

Article

Isolation of Smenopyrone, a Bis- γ -Pyrone Polypropionate from the Caribbean Sponge *Smenospongia Aurea*.

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Abstract: The organic extract of the Caribbean sponge *Smenospongia aurea* has been shown to contain an array of novel chlorinated secondary metabolites derived from a mixed PKS-NRPS biogenetic route, such as the smenamides. Here we report the presence of a biogenetically different compound, smenopyrone (**1**), a polypropionate containing two γ -pyrone rings. The structure of smenopyrone, including its relative and absolute stereochemistry, was determined by spectroscopic analysis (NMR, MS, ECD) supported by comparison with model compounds from the literature. Pyrone polypropionates are unprecedented in marine sponges, but are commonly found in marine mollusks, where their biosynthesis by symbiotic bacteria has been hypothesized and at least in one case demonstrated. As pyrones have recently been recognized as bacterial signaling molecules, we speculate that smenopyrone could mediate inter-kingdom chemical communication between *S. aurea* and its symbiotic bacteria.

Keywords: *Smenospongia aurea*, γ -pyrone, polypropionate, marine sponges, secondary metabolites, signaling molecules.

1. Introduction

γ -Pyrone are a quite large class of biologically active compounds that are biosynthesized via the polyketide pathway. They are mainly found in marine organisms [1], where they are thought to play a role as allomones or to be biosynthesized for defense purposes [2]. An important class of polyketides that often contain one or more γ -pyrone rings are polypropionates, polyketides that are mostly composed of propionate rather than acetate building blocks, and in the marine environment are typically found in mollusks [3]. In 2013, Gavagnin *et al.* [4] reported a comprehensive study of polypropionates (e.g. onchnidionol, Figure 1) from marine pulmonate mollusks belonging to the family of Onchidiidae. This family is known to produce a large array of C₃₂ bis- γ -pyrones having cytotoxic properties at micromolar concentrations. Earlier in 1996, auripyrones A and B were isolated from the Japanese sea hare *Dolabella auricularia* [5], a species known for being the first source of dolastatin-10 [6]. A synthetic analogue of dolastatin-10 is one of the marketed drugs derived from a marine lead compound, the antibody drug-conjugate brentuximab vedotin, commercialized under the name of Adcetris.

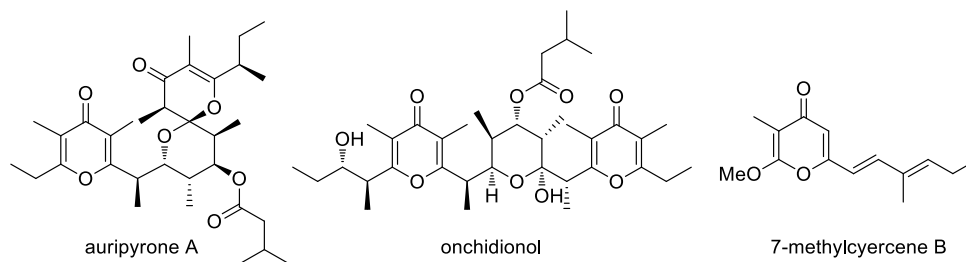


Figure 1. Some γ -pyrones from marine source.

Among marine organisms, sponges represent a magnitude of biodiversity. Their holobiome is one of the richest marine sources of secondary metabolites [7,8,9,10], often showing skeletons with no counterpart in the terrestrial environment. However, no pyrone polypropionates have been isolated so far from a marine sponge. In this paper we report on the isolation and structure elucidation of smenopyrone (**1**) (Figure 2), a bis- γ -pyrone compound, from a sample of *Smenospongia aurea*. The Caribbean sponges of the genus *Smenospongia* have been previously shown to contain a wide array of chlorinated metabolites deriving from the PKS-NRPS pathway, such as smenamides, conulothiazoles, and smenothiazoles [11]. They have been used as guiding structures in our anticancer drug discovery programs [12,13].

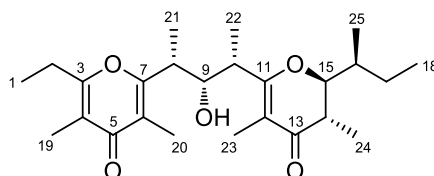


Figure 2. The structure of smenopyrone (**1**).

2. Results

A sample of the sponge *Smenospongia aurea* was collected along the coast of the Florida Keys Little Inagua (Bahamas Islands) in June 2013 at -15 m by experts of our group [14]. It was identified onboard of the vessel after the collection, immediately frozen, and kept frozen until extraction. The sample (712 g wet weight) was extracted using our standard procedure involving extraction with MeOH/ CHCl_3 mixtures and partitioning between H_2O and BuOH [15]. The total organic extract was chromatographed on a column packed with RP-18 silica gel. The fraction eluted with MeOH/ H_2O (9:1) was partitioned in a two-phase system composed of H_2O (160 mL), MeOH (260 mL), CHCl_3 (140 mL), and AcOH (5 mL). The organic layer was shown to contain known smenothiazoles and smenamides, and one unknown compound. This fraction was subjected to repeated reversed-phase HPLC separations, affording a fraction containing pure smenopyrone (**1**).

The $[\text{M}+\text{H}]^+$ ion peak at m/z 419.2780 in the high-resolution ESI MS spectrum of **1** was indicative of the molecular formula $\text{C}_{25}\text{H}_{38}\text{O}_5$, corresponding to seven unsaturations. No olefinic proton was present in the ^1H NMR spectrum, while the ^{13}C NMR spectrum showed the presence of eight non-protonated sp^2 carbon atoms ranging from δ 109.2 to 197.9. Combined information from the DEPT spectrum and the molecular formula showed only one OH group to be present in the molecule, the remaining protons being linked to carbon atoms.

The proton NMR spectrum revealed the presence of nine methyl groups (two triplets, four doublets, and three singlets) out of 25 carbon atoms, suggesting a polypropionate structure. It has been shown that for methyl rich compounds like **1** the analysis of 2D NMR spectra can conveniently start from the HMBC spectrum [16]. Indeed, as a result of the presence in **1** of nine methyl groups on alternated carbon atoms, the HMBC correlations of methyl protons provided enough information to build the whole carbon skeleton of **1** (Figure 3).

Table 1. NMR Data of smenopyrone (**1**) (^1H 700 MHz, ^{13}C 175 MHz, CD_3OD).

Position	δ_{C} , type	δ_{H} , mult (J in Hz)	HMBC ^a
1	11.7 (CH_3)	1.25 (t, 7.5)	2, 3
2	25.7 (CH_2)	a,b 2.71 (m)	1, 3
3	167.5 (C)	-	
4	119.0 (C)	-	
5	182.0 (C)	-	
6	120.3 (C)	-	
7	167.7 (C)	-	
8	41.7 (CH)	3.15 (quintet, 7.1)	9, 21
9	75.6 (CH)	4.02 (t, 7.1)	7, 8, 21, 22
10	41.5 (CH)	2.93 (quintet, 7.0)	9, 11, 22
11	175.6 (C)	-	
12	109.2 (C)	-	
13	197.9 (C)	-	
14	41.5 (CH)	2.53 (dq, 12.8, 6.9)	13, 15, 24
15	88.0 (CH)	3.84 (dd, 12.8, 3.0)	
16	36.6 (CH)	1.78 (m)	
17	23.0 (CH_2)	a 1.64 (m) b 1.29 (m)	
18	12.1 (CH_3)	0.98 (t, 7.5)	16, 17
19	9.6 (CH_3)	1.93 (s)	3, 4, 5
20	10.1 (CH_3)	1.91 (s)	5, 6, 7
21	15.5 (CH_3)	1.28 (d, 7.1)	7, 8, 9
22	14.1 (CH_3)	1.26 (d, 6.9)	9, 10, 11
23	9.4 (CH_3)	1.63 (s)	11, 12, 13
24	10.7 (CH_3)	1.06 (d, 6.9)	13, 14, 15
25	16.6 (CH_3)	1.11 (d, 6.9)	15, 16, 17

^a HMBC correlations from proton stated to the indicated carbon.

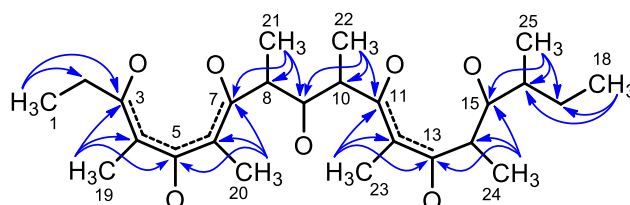


Figure 3. HMBC correlations (blue arrows) of methyl protons unequivocally defined the whole carbon skeleton of **1**. The π systems are depicted as dashed lines.

The chemical shifts of the carbon atoms allowed the identification of two groups of contiguous sp^2 carbon atoms, composed of five (C3-C7) and three (C11-C13) carbon atoms respectively; among them, C-3, C-5, C-7, C-11 and C-13 were bonded to an oxygen atom, as shown by their respective ^{13}C chemical shift. Two oxygen-bound sp^3 carbon atoms (C-9 and C-15) were also present. The ^{13}C chemical shift also suggested the C3-C7 system to be part of a pyrone, because they fit well with the values reported for the similar tetrasubstituted pyrone system present in auripyrone A and B [5]. Likewise, the C11-C13 system (together with C14 and C15) was part of a tetrasubstituted dihydropyrone, as suggested by comparison of the chemical shift of **1** with those of the corresponding atoms of maurenone [17,18]. Therefore, the sole hydroxyl group in the molecule was linked to C-9. This completed the planar structure of **1**, which was further confirmed by the analysis of the fragment ions present in the tandem mass spectrum (Figure 4).

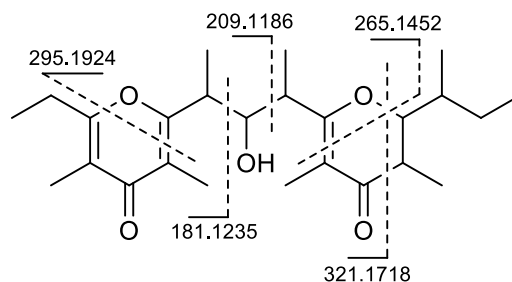


Figure 4. The main fragment peaks detected in the ESI tandem mass spectrum of smenopyrone (1)

Determination of the stereochemistry of smenopyrone (1) was based on the following evidence. The large coupling constant between H-14 and H-15 showed them to be *trans*-axial and so demonstrated the *trans* orientation of the groups at C-14 and C-15 (in the following discussion, we will assume the 14*S*,15*S* absolute configuration for these carbon atoms, which will be eventually proven to be correct). Configuration at C-9, C-10 and C-16 was based on a comparison of ^{13}C NMR chemical shifts of smenopyrone (1) with those of the corresponding atoms of synthetic models, i.e. eight diastereomers of maurenone [18] (4-11, Figure 5). The chemical shift of C-25, very similar to that of compounds 4-7 but very far from that of compounds, 8-11, clearly demonstrated the *S* configuration at C-16. Configurations at C-9 and C-10 were based on an overall comparison with ^{13}C NMR chemical shifts of model compounds, expressed as the sum of the absolute values of chemical shift differences, $\sum|\Delta\delta|$ (Table S1 and Figures 6 and S1). The best fit ($\sum|\Delta\delta|=3.1$) was shown by model compound 6, and therefore the configuration was assigned as 9*S*,10*S*. Configuration at C-8 was assigned based on the observation that in the isomers of the model compound 2,4-diphenyl-3-pentanol (2) (Figure 5), the ^{13}C NMR chemical shift of the methyl groups is remarkably different whether they are *syn* ($\delta_{\text{C}} \approx 15$) or *anti* ($\delta_{\text{C}} \approx 19$) to the OH group (Figure 5) [19]. This implied C-21 and C-22 (both resonating at about δ 15) to be both *syn* to the OH group at C-9, and, therefore, confirmed the relative configuration between C-9 and C-10 and determined the *R* configuration at C-8.

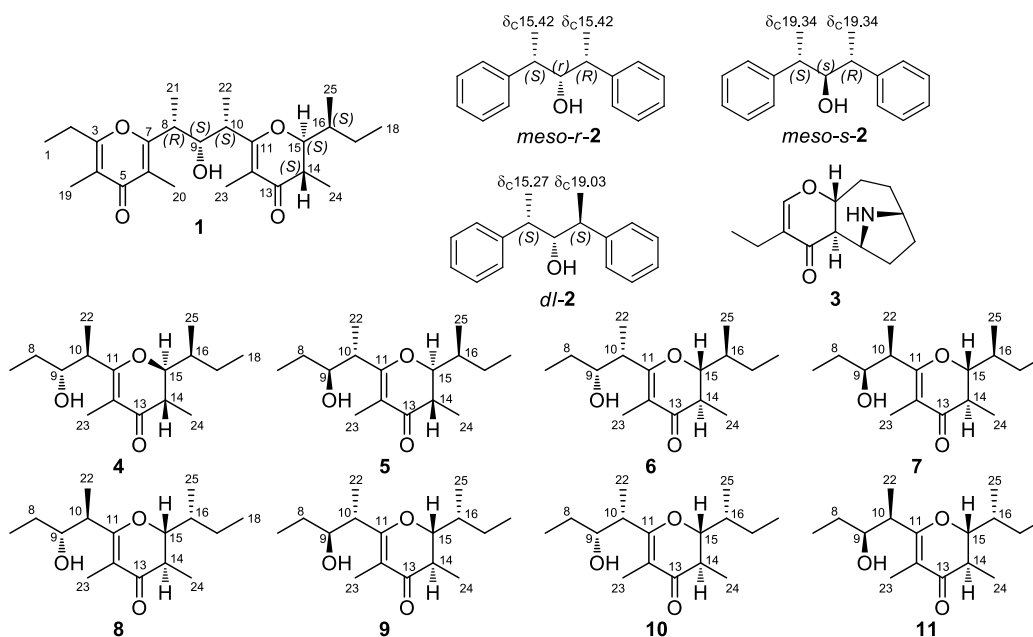


Figure 5. The three possible diastereomers of 2,4-diphenyl-3-pentanol (2), pinnamine (3), and the eight diastereomers of maurenone (4-11, the relative configuration of natural maurenone is as in 6). These compounds were used as model compounds to elucidate the stereochemistry of smenopyrone (1). ^{13}C NMR chemical shifts shown for 2 are from ref. 19.

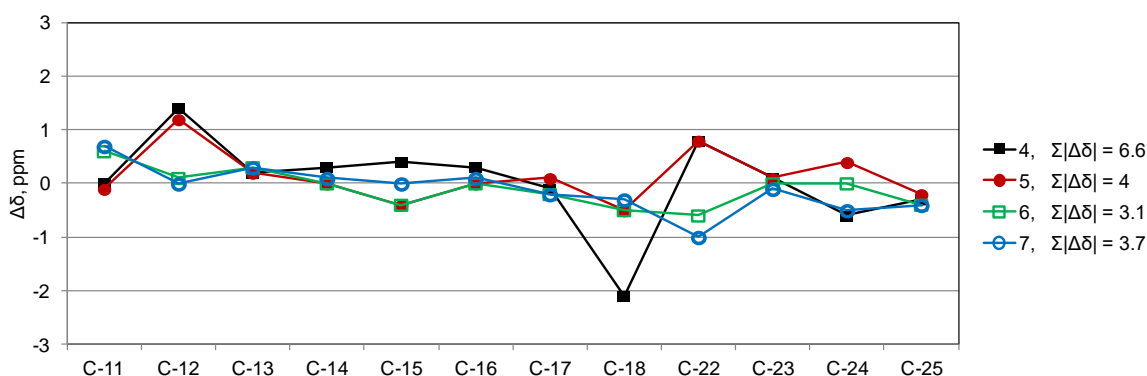


Figure 6. Difference in ^{13}C NMR chemical shift ($\delta\Delta$) between corresponding atoms of smenopyrone (**1**) and the four stereoisomers of the model compound maurenone (**4-7**). The sum of absolute values of $\delta\Delta$ ($\Sigma|\delta\Delta|$) was used to evaluate the overall fit between smenopyrone and **4-7**. Complete data can be found in Table S1 and Figure S1.

The absolute configuration of smenopyrone was based on its ECD spectrum. Smenopyrone (**1**) contains two chromophores, a γ -dihydropyrone and a γ -pyrone. A *trans*-disubstituted γ -dihydropyrone as in **1** exists predominantly in the half-chair conformation with *trans*-diequatorial substituents (this is supported by the 12.8 Hz coupling constant between H-14 and H-15). In a half-chair, the enone system is slightly skewed and becomes an inherently chiral chromophore, which is expected to dominate the ECD spectrum. In contrast, a γ -pyrone is a planar, inherently non-chiral chromophore, and is expected to give a minor contribution to the overall ECD spectrum. Indeed, the ECD curve recorded for smenopyrone (**1**) (Figure S2) showed a profile very similar to the ECD spectrum of the γ -dihydropyrone alkaloid pinnamine (**3**) [20], but an opposite sign. This suggests that the γ -dihydropyrone moiety of **1** has the opposite configuration compared to pinnamine (**3**), thus defining the (8*R*,9*S*,10*S*,13*S*,14*S*,15*S*) absolute configuration for smenopyrone (**1**).

3. Discussion

Smenopyrone (**1**) is the first pyrone polypropionate isolated from a marine sponge. It contains a bis- γ -pyrone structure similar to that of auriopyrones A and B, previously isolated from the Japanese sea hare *Dolabella auricularia*. These compounds are typical of some classes of marine mollusks, but are also commonly found in terrestrial and marine fungi. Interestingly, it has been shown that polypropionates are biosynthesized through two entirely different pathways in mollusks and fungi, even when the chemical structure of the synthesized compound is the same [21]. In mollusks, intact propionate units are introduced in the growing polyketide chain through incorporation of methylmalonyl-CoA; in fungi, methyl branches derive from methylation by *S*-adenosyl methionine (SAM).

Recently, a series of closely related γ -pyrone polypropionates, namely nocapyrones A-C and H-Q, was shown to be produced by the bacterial symbiont *Nocardioopsis alba* CR167 isolated from the mollusk *Conus rolani* [22]. An iterative type-I polyketide synthase gene cluster has been identified as responsible for the biosynthesis of these compounds, whose dipropionate backbone derives from condensation of two methylmalonyl CoA units. Likewise, it can be predicted that smenopyrone is assembled by an iterative type-I polyketide synthase, through condensation of one acetate unit and eight methylmalonyl-CoA units. The definite bacterial origin of nocapyrones suggests that also other polypropionates from mollusks can be actually produced by bacteria, and the same may hold true for sponge-derived smenopyrone. However, if nocapyrones are only occasionally found in *C. rolani* because *N. alba* is a casual symbiont of this mollusk, it is important to mention that the presence of smenopyrone (**1**) is by no means occasional in *S. aurea*, because the compound has been consistently found in all specimens of Caribbean *S. aurea* we studied, and is also present in the closely related species *S. conulosa*. Therefore, smenopyrone (**1**) is likely to be produced by obligate rather than casual sponge-symbionts.

These ideas also suggest an intriguing speculation on the role of smenopyrone in sponges. Sponges are filter sessile animals that harbor communities of microorganisms, either digested as a nutrient source, or in a complex symbiotic relationship with the sponge. They can be up to 30-40% of sponge mass [23,24] and are often the real producer of the secondary metabolites isolated from the sponge extract. This is, for example, the case of the group of manzamine alkaloids, originally isolated from the sponge *Acanthostrongylophora ingens*, and subsequently shown to be produced by the associated actinomycete *Micromonospora* [25,26]. The idea of a possible inter-kingdom communication [27] between bacteria and sponges mediated by small molecules (e.g. lactones, which are regularly produced by many bacteria) is fascinating. It has been recently explored and demonstrated for the sponge *Suberites domuncula* [28,29], where *N*-3-oxodecanoyl-L-homoserine lactone can affect the expression of immune and apoptotic genes of the host, possibly enabling the sponge to monitor bacterial community. Moreover, molecules affecting the quorum-sensing bacterial system were found in sponge extracts [30], suggesting the possibility of complex communications and interactions between the two kingdoms.

Endogenous pyrone-containing polyketides have been recently identified as the signaling molecules of a previously orphan signal system of proteobacteria [31]. It is therefore exceedingly tempting to speculate that smenopyrone can be a signaling molecule as well, mediating the communication between obligate symbiotic bacteria of *S. aurea* and the host sponge. Although at present this is just a fascinating speculation, it can be a guide for our and others' future work to prove that smenopyrone or related pyrone polypropionates act as inter-kingdom signaling molecules for bacterial symbionts of sponges or mollusks.

4. Materials and Methods

4.1 General Experimental Procedure

ECD spectra were recorded using a Jasco-715 spectropolarimeter. NMR spectra were determined on Varian Unity Inova spectrometers at 700 MHz and 500 MHz; chemical shifts were referenced to the residual solvent signal (CD₃OD: δ_{H} 3.31, δ_{C} 49.0; CDCl₃: δ_{H} 7.26, δ_{C} 77.0). For an accurate measurement of the coupling constants, the one-dimensional ¹H NMR spectra were transformed at 128K points (digital resolution < 0.1 Hz). The HSQC spectra were optimized for ¹J_{CH} = 142 Hz, and the ¹³C HMBC experiments for ^{2,3}J_{CH} = 8.3 Hz. High-resolution ESI-MS and MS/MS experiments were performed on a Thermo LTQ Orbitrap XL mass spectrometer coupled to a Thermo U3000 HPLC system. High performance liquid chromatography (HPLC) separations were achieved on an Agilent 1260 Infinity Quaternary LC apparatus equipped with a Diode-Array Detector (DAD).

4.2 Collections, Extraction and Isolation

A specimen of *Smenospongia aurea* (712 g wet weight) was collected on the 9th July 2013 at depths of 15 m by scuba diving along the coast of Great Inagua (Bahamas Islands, 21°03'24.55" N -73°25'27.76" W). The collected sample was a relatively small portion of a much larger sponge, and was excised with a sharp scalpel to minimally affect the remaining sponge tissue and allow recovery and regrowth. After collection, the sample was unambiguously identified onboard using a web-based photographic and taxonomic key, The Sponge Guide (www.spongeguide.org), with subsequent confirmation by sponge taxonomist Dr. Sven Zea. The sample was frozen immediately after collection and stored at -20 °C until extraction. A voucher specimen of the organism is stored at Dipartimento di Farmacia, "TheBlueChemistryLab" laboratory, Università degli Studi di Napoli "Federico II" with the reference number 06/07/13.

To perform the present study, the sample was allowed to get to room temperature, then cut in small pieces, homogenized, and extracted following our standard procedure [32]. The total organic extract (16.31 g) was chromatographed on a column packed with RP-18 silica gel. The fraction eluted with MeOH/H₂O (9:1, 363.7 mg) was partitioned in a two-phase system composed of H₂O (160 mL), MeOH (260 mL), CHCl₃ (140 mL), and AcOH (5 mL); the organic layer, containing smenothiazoles

[33], smenamides [34], and smenopyrone (**1**), was subjected to reversed-phase HPLC separation [column 250 × 10 mm, 10 μm, Luna (Phenomenex) C18; eluent A: H₂O; eluent B: MeOH; gradient: 55→100% B, over 60 min, flow rate 5 mLmin⁻¹], thus affording a fraction (*t_R* = 29min) containing compound **1**. The former fraction was subjected to new separations on reversed-phase HPLC [column 250 × 4.6 mm, 5 μm, Luna (Phenomenex) C18; eluent A: H₂O; eluent B: ACN; gradient: 50→100% B, over 35 min, flow rate 1 mL min⁻¹], which gave 12 μg of pure compound **1** (*t_R* = 16 min).

Smenopyrone (1): Colorless glass; UV (MeOH): λ_{max} (ε) 260 (21,600); ECD (MeOH): λ_{max} (Δε) 306 (+1.2), 277 (−5.5); HR-ESI-MS (High Resolution-ElectroSpray Ionization-Mass Spectrometry) (positive ion mode, MeOH) *m/z* 419.2780, [M+H]⁺ (C₂₅H₃₉O₅⁺ gives 419.2792, Δ −2.8 ppm); ¹H NMR (CDCl₃): 3.99 (dd, *J* = 8.0 and 4.6 Hz, H-9), 3.84 (m, H-15), 3.07 (dq, *J* = 8.0 and 7.0 Hz, H-8), 2.92 (dq, *J* = 4.6 and 7.0 Hz, H-10), 2.71 (q, *J* = 7.6 Hz, H₂-2), 2.51 (dq, *J* = 12.4 and 3.2 Hz, H-14), 1.94 (s, H₃-20), 1.94 (s, H₃-19), 1.76 (m, H-16), 1.67 (s, H₃-23), 1.62 (m, H-17a), 1.26 (m, H-17b), 1.24 (d, *J* = 7.0 Hz, H₃-22), 1.24 (d, *J* = 7.0 Hz, H₃-21), 1.22 (t, *J* = 7.6 Hz, H₃-1), 1.09 (d, *J* = 6.9 Hz, H₃-24), 1.05 (d, *J* = 6.9 Hz, H₃-25), 0.94 (t, *J* = 7.4, H₃-18); ¹³C NMR (CDCl₃): 195.4 (C, C-13), 179.5 (C, C-5), 172.4 (C, C-11), 164.0 (C, C-3), 164.7 (C, C-7), 118.2 (C, C-4), 119.4 (C, C-6), 108.5 (C, C-12), 87.4 (CH, C-15), 75.1 (CH, C-9), 40.6 (CH, C-14), 38.6 (CH, C-10), 38.3 (CH, C-8), 35.1 (CH, C-16), 26.2 (CH₂, C-2), 22.1 (CH₂, C-17), 16.5 (CH₃, C-25), 15.3 (CH₃, C-21), 13.9 (CH₃, C-22), 12.2 (CH₃, C-18), 11.6 (CH₃, C-1), 10.6 (CH₃, C-24), 9.8 (CH₃, C-20), 9.4 (CH₃, C-19), 9.3 (CH₃, C-23); ¹H and ¹³C NMR (CD₃OD): Table 1.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: ¹³C NMR chemical shift of smenopyrone **1** compared with those of the eight diastereomers of maurenone **4-11**, Figure S1: Difference in ¹³C NMR chemical shift between corresponding atoms of **1** and **4-11**, Figure S2: UV and ECD spectra of **1**, Figure S3: High-resolution ESI mass spectrum of **1**, Figure S4: High-resolution ESI MS/MS spectrum (parent ion at *m/z* 419.28) and fragmentation of **1**, Figures S5-S10: 1H-NMR, COSY, NOESY, HSQC, and HMBC spectra of **1** (700 MHz, CD₃OD).

Author Contributions: Conceptualization, Alfonso Mangoni and Valeria Costantino; Data curation, Germana Esposito, Roberta Teta and Gerardo Della Sala; Funding acquisition, Alfonso Mangoni and Valeria Costantino; Investigation, Germana Esposito, Roberta Teta and Gerardo Della Sala; Supervision, Joseph R. Pawlik and Valeria Costantino; Writing – review & editing, Alfonso Mangoni and Valeria Costantino.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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