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Graphical Abstract

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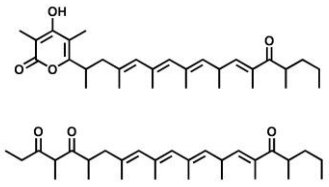

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Exiguapyrone and exiguaone, new polypropionates from the Mediterranean cephalaspidean mollusc *Haminoea exigua*

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ABSTRACT

Two new polypropionates, named exiguapyrone (**2**) and exiguaone (**3**) along with the known haminol-1 (**4**) and -2 (**5**), have been isolated from the lipidic extract of the Mediterranean cephalaspidean mollusc *Haminoea exigua*. The regular propionate skeletons, structurally related to other polypropionates from the congener *Haminoea fusari*, have been elucidated by means of NMR techniques as natural (**2** and **3**) and α-/γ-pyrone methyl derivatives (**2a** and **2b**). This is a further report showing the co-occurrence of alkyl-pyridines and polypropionates in *Haminoea* molluscs strenghtening the role of polypropionates as chemical markers among cephalaspideans.

1. Introduction

Polypropionates are polyketides biosynthetically derived by condensation of C_3-units in regular or mixed acetate/propionate polyketide chain. They are typically encountered in marine organisms,[1] mainly in Pulmonata[2], Sacoglossa[3] and Cephalaspidea molluscs.[4] In a previous study we investigated the organic extract of *Haminoea fusari*, reporting the new finding of two polypropionates (i.e. fusaripyrone A, **1**, Figure 1) featured by an α-pyrone ring and an unusually long chain, along with known haminols, alkyl-pyridine pheromones biosynthesized *de novo* and to be considered a chemical marker of the genus *Haminoea*.[5-9] In the course of our chemical research on marine invertebrates, we studied the secondary metabolites of another *Haminoea* species, namely *Haminoea exigua* Schaefer 1992, a very small Mediterranean species (average 10-15 mm) collected in the Etang de Thau Lagoon (France) and never chemically investigated before.

According to our classical analytical procedure, frozen animals (80 specimens) were extracted with acetone by gentle sonication (mantle extract) and then by grinding of the tissues (viscera extract). Both organic extracts were concentrated under vacuum and the aqueous residue partitioned with diethyl ether. The raw ethereal mantle extract, despite the tiny amount (2.4 mg) gave a clear and diagnostic ^{1}H NMR spectrum (600 MHz, C_6D_6): along with a pattern of signals at δ 8.51, 8.47, and 7.05 typical of a pyridine ring and suggesting the occurrence of haminol derivatives, several methyl groups indicated the presence of polypropionate metabolites. Silica gel purification of this extract by Pasteur pipette with an EP/EE gradient elution led to the isolation of two UV-absorbing polyketides, **2** and **3**, as pure compounds, along with the known haminol-1 (**4**, 0.13 mg) and -2 (**5**, 0.25 mg) (Figure 1).

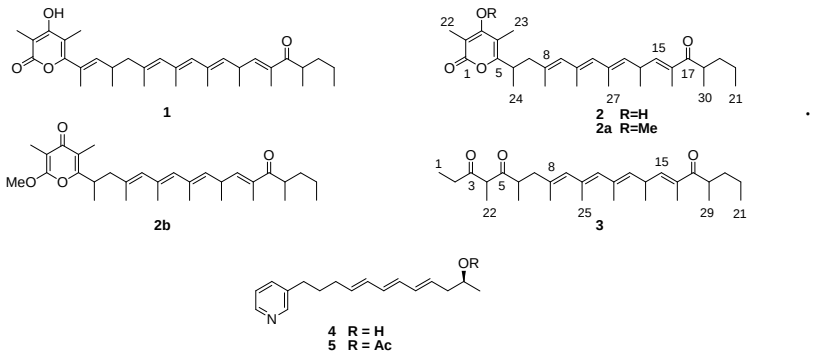

Figure 1. Structures of natural polyketides **1**-**5** and methyl derivatives **2a** and **2b.**

2. Result and discussion

HRESIMS of compound **2** (0.25 mg) gave a sodium adduct ion $[M+Na]^+$ at *m/z* 491.3132 in agreement with the molecular formula $C_{30}H_{44}O_4$. The ^{1}H-NMR spectrum (600 MHz, C_6D_6) of **2** displayed ten methyl resonances in the region δ 0.77-1.92 that indicated a regular polypropionate skeleton formed by ten units. An α-pyrone substructure was inferred by diagnostic HMBC correlations of the two vinyl methyl groups at δ 1.52 and 1.63 with the quaternary carbons at 163.5 (C-1), 97.7 (C-2), 162.4 (C-3), 104.3 (C-4) and 161.5 (C-5) ppm. The assemblage of the entire propionate backbone was straightforward by inspection of homo and heteronuclear bidimensional NMR spectra. Thus, ^{1}H-^{1}H COSY and TOCSY data revealed only three spin systems. The first one was a short C_3-fragment including a methyl doublet signal at δ 1.04 (H-24), a methine proton at δ 2.78 (H-6), and an allylic methylene at δ 2.07/2.38 (H_2-7). Diagnostic long range cross peaks observed for the above resonances joined the propyl unit to the pyrone ring through the oxygenated quaternary carbon C-5 (161.5 ppm). In turn, the allylic methylene H_2-7 was connected by proton-carbon long range couplings to a substituted conjugated triene system (C-8/C-13), exhibiting two olefinic protons resonating as two singlets at δ 5.72 (C-9, 132.0 ppm) and δ 5.76 (C-11, 132.5 ppm) and a doublet at δ 5.21 (C-13, 132.0 ppm). Furthermore, key HMBC correlations allowed to unambiguously locate the three vinyl methyls at δ 1.67 (C-25), 1.80 (C-26) and 1.63 (C-27) on the corresponding quaternary olefinic carbons at 132.7 (C-8), 133.0 (C-10) and 132.4 (C-12) ppm, respectively. The second spin system was associated to a bis-allylic methine at δ 3.39 (C-14, 32.9 ppm) coupled to a methyl doublet at δ 0.97 (C-28, 20.5 ppm), and spaced the triene system from a trisubstituted α,β-unsaturated keto moiety at 204.9 ppm (C-17; H-15, δ 6.34; C-15, 143.9 ppm; C-16, 134.5 ppm; H_3-29, δ 1.92; C-29, 11.5 ppm). Finally, the last spin system reconstructed by homonuclear bidimensional experiments (COSY and TOCSY) allowed the identification of a terminal 2-pentenyl residue (Table 1) which was linked to the carbonyl group at C-17, thus completing the carbon skeleton framework.

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Table 1. NMR data (C_6D_6, 600 MHz) of exiguapyrone (**2),** its α- and γ-pyrone methyl derivatives (**2a** and **2b**, respectively**)** and exiguaone (**3**).

	2		**2a**		**2b**		**3**	
	δ_H, *J* (Hz)	**δ_C**	**δ_H, *J* (Hz)**	**δ_C**	**δ_H, *J* (Hz)**	**δ_C**	**δ_H, *J* (Hz)**	**δ_C**
1		163.5		165.7		161.7	0.99, t (6.3)	8.0
2		97.7		109.6		99.4	2.04, m; 2.12, m	33.6
3		162.4		167.5		180.0		209.4
4		104.3		107.8		118.2	3.38, q (m)	54.5
5		161.5		161.0		159.8		206.5
6	2.78, m	33.1	2.80, m	33.6	2.87, m	33.2	2.78, m	43.4
7	2.07, dd (13.0, 6.5); 2.38, dd (13.0, 8.0)	44.9	2.09, dd (13.0, 6.5); 2.39, dd (13.0, 8.0)	45.6	2.0, dd (13.0, 7.0); 2.16, dd (13.0, 8.0)	45.1	2.35, dd (14.0, 7.5); 2.52, dd (14.0, 6.5)	44.0
8		132.7		133.2		133.0		133.5
9	5.72, s	132.0	5.73, s	132.2	5.66, s	132.3	5.71, s	132.5
10		133.0		132.6		132.5		133.2
11	5.76, s	132.5	5.75, s	132.5	5.79, s	132.9	5.76, s	132.1
12		132.4		132.7		133.0		133.1
13	5.21, d (9.4)	132.0	5.23, d (9.2)	132.7	5.26, d (9.0)	132.5	5.27, d (8.5)	132.4
14	3.39, m	32.9	3.39, m	33.0	3.40, m	32.9	3.41, m	33.0
15	6.34, d (9.4)	143.9	6.35, d (9.5)	143.9	6.35, d (9.4)	143.7	6.37, d (8.5)	143.9
16		134.5		135.2		135.2		135.4
17		204.9		204.7		204.9		204.9
18	3.07, m	38.4	3.07, m	38.8	3.07, m	38.4	3.08, m	38.3
19	1.26, m; 1.72, m	36.1	1.26, m; 1.73, m	36.5	1.26, m; 1.72, m	36.5	1.27, m;1.74, m	36.6
20	1.16, m	20.4	1.17, m	20.9	1.17, m	20.5	1.17, m	20.2
21	0.77, t (7.4)	13.8	0.77, t (7.0)	14.9	0.78, t (7.3)	13.6	0.78, t (6.5)	13.9
22	1.52, s	7.2	1.96, s	10.1	2.12, s	6.8	1.11, d (6.5)	16.7
23	1.63, s	8.6	1.62, s	9.1	1.08, s	17.5	1.10, d (6.5)	12.2
24	1.04, d (7.0)	17.0	1.08, d (7.0)	17.5	0.93, d (7.0)	16.8	1.71, s	17.5
25	1.67, s	17.4	1.67, s	17.7	1.64, s	17.3	1.85, s	18.2
26	1.80, s	18.5	1.80, s	18.8	1.81, s	18.3	1.67, s	17.5
27	1.63, s	16.8	1.63, s	16.9	1.65, s	17.4	1.00, d (6.5)	20.2
28	0.97, d (7.0)	20.5	0.98, d (6.6)	20.8	0.99, d (7.0)	20.2	1.93, s	11.4
29	1.92, s	11.5	1.92, s	11.8	1.92, s	11.8	1.08, d (6.5)	17.5
30	1.06, d (7.0)	17.7	1.06, d (6.6)	17.8	1.07, d (6.7)	17.6		
OMe			3.10, s	59.0	3.23, s	53.8		

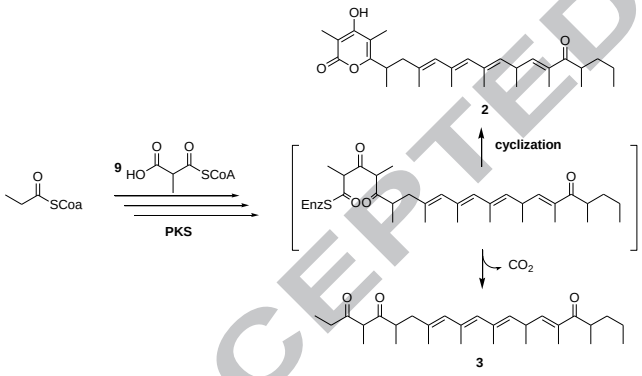
The geometry of all the double bonds was assigned as *E* according to the chemical shift values of the vinyl methyl groups, all below 20 ppm.[10] On the whole, NMR and MS data accounted for a new polypropionate skeleton, named exyguapyrone, and depicted in **2** (Figure 1). Structurally, the new polypropionate from *H. exygua* exhibited a high resemblance with the known fusaripyrone A (**1**) which we have previously isolated from the congener *H. fusari*. However, fusaripyrone A revealed a high chemical lability and was only partially characterized in the natural form, requiring its conversion into corresponding methyl derivatives to complete the structural assignment.[5] Hence, in order to compare the spectroscopic data of the two skeletons of **1** and **2**, we attempted the conversion of this latter metabolite into the α- and γ-pyrone methyl derivatives **2a** and **2b**, respectively (Figure 1). Part of the ethereal extract (70 mg) of viscera which contained the natural α-pyrone polypropionate, was methylated with diazomethane and successively purified by silica gel radial chromatography with a petroleum ether/diethyl ether gradient affording the α- and γ-pyrone methyl derivatives, **2a** (2.9 mg) and **2b** (1.4 mg) respectively, as pure compounds. NMR data of methyl pyrones (table 1), confirmed the structure proposed for **2** and evidenced the strict similarity of the two natural skeletons of compounds **1** and **2,** thus suggesting also the same configuration at C-14 and C-18 for both metabolites.

HRESIMS of compound **3** gave a molecular ion $[M+Na]^+$ at *m/z* 465.3337 that was indicative of the molecular formula $C_{29}H_{46}O_3$, accounting for seven formal double bonds. ^{13}C NMR spectrum contained 11 sp^2 signals (table 1), three of which at 204.9, 206.5 and 209.4 ppm were attributable to keto groups. The eight remaining signals were all ascribable to the olefinic carbons of four double bonds thus satisfying the unsaturation level

required and indicating a linear molecular scaffold. A complete NMR assignment of protons and carbons was obtained by homo- and heteronuclear bidimensional experiments (table 1). Most of the aliphatic carbons were assigned to methyl groups indicative also for this molecule of a regular polypropionate skeleton. Indeed, signals in the region C-8/C-21 were superimposable to those corresponding to the acyclic part of **2**. However, in the structure of polypropionate **3**, the pyrone ring was replaced by a linear terminus containing the 1,3-diketone functionality at 206.5 and 209.4 ppm. Key HMBC correlations were observed from the terminal ethyl residue (H-1 δ 0.99, C-1 8.0 ppm; H_2-2 δ 2.04/2.12, C-2 33.6 ppm), to the carbonyl group at δ 209.4 ppm and from the methine proton at δ 3.38 ppm to both the keto groups at C-3 and C-5 (δ 209.4 and 206.5 ppm, respectively). Finally, the proton at δ 2.78 (C-6, 43.4 ppm) was coupled to the carbon at 206.5 ppm, connecting the above 1,3-diketo functionality with the remaining aliphatic chain. Thus, the molecular structure of a new linear propionate, named exiguaone, was elucidated as depicted in **3** (Figure 1).

From a biogenetical point of view, the two polypropionates **2** and **3** clearly arise from the same PKS process as depicted in scheme 1. The chain assembly likely follows a regular condensation process starting with propionyl-CoA and continuing with elongation of C-3 units up to the linear C_{30}-polypropionate thioester precursor. The fate of the thioester can implicate either a cyclization with release from PKS enzyme of the pyrone derivative **2** or a decarboxylation step of the terminal unit affording the linear polypropionate **3**.

Scheme 1. Biogenetical proposal for a common origin of **2** and **3** in *H. exigua* by a PKS-like assembly based on ten propionate units.



The structure of exiguaone (**3**) resembles that of the known linear marine polypropionates such as aglajne-1,[11] niuhinone-B[12] and nalodionol[13] identified as chemical markers for Bullidae species.[4] Compounds **2** and **3** from *H. exigua* represents the second report of polypropionates chemically characterized in the genus *Haminoea*, which follows our previous chemical description of fusaripyrones from *H. fusari*.[5] However, the occurrence of these polyketides seems to be anything but a casual finding in *Haminoea* congeners, as attested by former undetermined polypropionates put forwarded by Marin and coworkers in *H. hydatis*[15] as well as by our own observation in *H. navicula* extracts (unpublished results). Considering that the ability to produce polyketides has been proven in both cephalaspidean *Haminoea* and *Bulla* species,[8,9,14] the recurrent co-occurrence of both haminols and polypropionates suggests the *de novo* polyketide origin in *H. exigua*.[5] However, the lack of molecular studies on PKS gene clusters in Mollusca prevents any further speculation and doesn't rule out a different origin for these polypropionates.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Jasco P2000 digital polarimeter. UV spectra were acquired on a Jasco V-650 Spectrophotometer. IR spectra were registered on a Jasco FT-IR 4100 spectrometer. NMR spectra were recorded on a Bruker Avance DRX 600 equipped with a cryoprobe operating at 600 MHz for proton. Chemical shifts values are reported in ppm (δ) and referenced to internal signals of residual protons (C_6D_6 ^{1}H δ 7.15, ^{13}C 128.0 ppm). High resolution mass spectra were acquired on a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific). Silica gel chromatography was performed using precoated Merck F254 plates and Merck Kieselgel 60 powder. Radial chromatography was carried out on a pre-coated silica plate by using a Chromatotron® apparatus.

3.2. Extraction and isolation of metabolites

The mollusc *H. exigua* (80 specimens) was collected in Etang De Thau (France) in October 2014 and kept frozen until analyses. The frozen material was extracted with acetone by gentle sonication (mantle extract) and then by grinding of the tissues (viscera extract). The organic solvent was removed under reduced pressure and the water residue partitioned with diethyl ether (4 x 15 ml) to give 2.4 mg and 150 mg of mantle and viscera extract, respectively.

The raw ethereal extract of the mantle (2.4 mg) was chromatographed by SiO_2-gel column on a Pasteur pipette with a light petroleum ether (PE)/diethyl ether (EE) gradient. The fractionation afforded, in order of increasing polarity, exiguaone **3** (0.1 mg, eluted with PE/EE 80:20), haminol-2 (**5**, 0.25 mg, PE/EE 60:40), exiguapyrone **2** (0.25 mg, eluted with PE/EE 40:60), and haminol-1 (**4**, 0.13 mg, PE/EE 20:80).

Methylation of exiguapyrone (**2**). Part of the extract from viscera (70 mg) was methylated with ethereal diazomethane for 1h at r.t. After solvent removal, the reaction product was subjected to radial chromatography on silica plate with a petroleum ether /diethyl ether gradient. The α- and γ-pyrone methyl derivatives **2a** (2.9 mg) and **2b** (1.4 mg) were eluted as pure compounds with PE/EE 70:30 and 60:40, respectively.

3.3. Chemical Data

3.3.1. Exiguapyrone (2). Colorless amorphous oil (0.25 mg). HRESIMS *m/z* 491.3132 $[M+Na]^+$ (calcd for $C_{30}H_{44}O_4Na$, 491.3132). $[\alpha]_D^{25}$ -10 (*c* 0.15, CH_2Cl_2); IR (film KBr) ν_{max} 1715, 1670 cm^{-1}. UV (MeOH) λ_{max} (ε) 217 (9884), 236 (11484), 275 (10048) nm; NMR data: see Table 1.

*3.3.2. Compound **2a**.* Colorless amorphous oil (2.9 mg). HRESIMS *m/z* 505.3285 $[M+Na]^+$ (calcd for $C_{31}H_{46}O_4Na$, 505.3288). $[\alpha]_D^{25}$ -48 (*c* 0.3, CH_2Cl_2); IR (film KBr) ν_{max} 1725, 1680 cm^{-1}. UV (MeOH) λ_{max} (ε) 217 (28136), 236 (36380), 275 (35396) nm; NMR data: see Table 1.

*3.3.3. Compound **2b**.* Colorless amorphous oil (1.3 mg). HRESIMS *m/z* 505.3289 $[M+Na]^+$ (calcd for $C_{31}H_{46}O_4Na$, 505.3288). $[\alpha]_D^{25}$ +14 (*c* 0.3, CH_2Cl_2); IR (film KBr) ν_{max} 1650 cm^{-1}. UV (MeOH) λ_{max} (ε) 256 (30010), 278 (14730) nm; NMR data: see Table 1.

3.3.4. Exiguaone (3). Colorless amorphous oil (0.1 mg). HRESIMS *m/z* 465.3337 $[M+Na]^+$ (calcd for $C_{29}H_{46}O_3Na$, 465.3339); IR (film KBr) ν_{max} 1710, 1660 cm^{-1}. UV (MeOH) λ_{max} (ε) 236 (2433), 270 (2051) nm. NMR data: see Table 1.

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Highlights

This is the second report of polypropionates isolated from *Haminoea* species.
Polypropionates co-occurr with haminols in *Haminoea exigua*
The two polypropionates likely arise from the same PKS-like assembly based on ten C-3 units