Tetrahedron Vol. 48, No. 43, pp. 9561-9566, 1992
Printed in Great Britain

0040-4020/92 $5.00+.00

α- and γ-Pyrone-Polypropionates from the Mediterranean Ascoglossan Mollusc *Ercolania funerea*

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(*Received in UK* 4 *August* 1992)

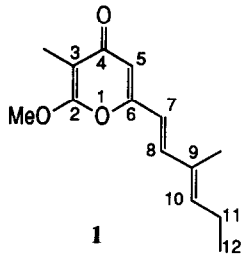
Key words : Ascoglossa, Stiligeridae, Polybranchiidae, pyrones, polypropionates.

Abstract: *Six polypropionic α- and γ-pyrones were isolated from the Stiligeridae ascoglossan mollusc Ercolania funerea. Their specific structures, elucidated by means of spectroscopic methods, are related to those of α- and γ-pyrones recently found in Polybranchioidea ascoglossans. Preliminary in vivo biosynthesis experiments conducted with (^{14}C)-sodium propionate suggest that α- and γ-pyrones derive from an endogenous secondary metabolic pathway common to ascoglossan species belonging to the Polybranchioidea super-family.*

Polypropionates are quite uncommon in nature: typical examples are some metabolites with antibiotic activity from Streptomycetes.[1] However, polypropionic metabolites were found in many marine molluscs: ascoglossans, pulmonates, cephalaspidcans.[2] While continuing our studies on the chemical ecology of marine opisthobranchs,[3] our interest has been focussed on species belonging to the order Ascoglossa, which are extraordinary from an evolutive point of view since they include both conchoid and aconchoid genera. The aconchoids are classified in two main super-families: Elysioidea and Polybranchioidea.[4] The former are characterized by the presence of two large parapodia, where the chloroplasts, sequestered from the algal prey, remain active for many days producing molecules useful both for nutrition and for defense. The main morphological characteristic of the Polybranchioidea molluscs, on the other hand, is a series of dorsal appendages (cerata), which in some species are easily detached as part of a typical defensive mechanism (autotomy). Recent work on Polybranchioidea led to the characterization of some α- and γ-pyrones, formally constructed from five propionic units: from *Cyerce cristallina*,[5] belonging to the family Polybranchiidae, and from *Placida dendritica*[6], belonging to the family Stiligeridae. Related metabolites had previously been found in nature only in the Pacific *Cyerce nigricans*.[7,8] In this paper, we describe a chemical study on *Ercolania funerea* (Costa 1867), a Stiligeridae

ascoglossan which lives closely associated with the green alga *Chaetomorpha capillaris*. *E. funerea* is a very small mollusc, barely 0.5 cm long, which is completely camouflaged in its natural habitat. Samples of *Chaetomorpha* were kept in an aquarium and specimens of the mollusc could be collected only after careful observation.

E. funerea (600 specimens) was extracted with acetone; the chloroform-soluble fraction from the acetone extract was analyzed by TLC (SiO_2, diethyl ether/petroleum ether, 1:1) showing at least four UV sensitive spots (in order of decreasing polarity: A, Rf= 0.10; B, Rf= 0.15; C, Rf= 0.55 and D, Rf= 0.60). The four fractions were recovered by semipreparative TLC and purified by reverse phase HPLC, which yielded two components from both A (**1-2**) and B (**3-4**) whereas D and C resulted pure compounds, **5** and **6** respectively. Spectral analysis of the components of the first pair rapidly led to identify cyercene-B (**1**), previously found in *C.*



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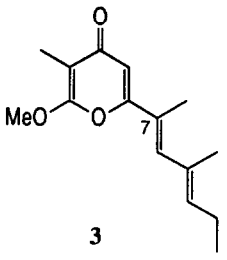

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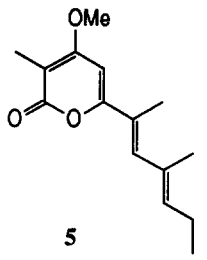

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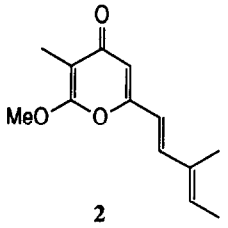

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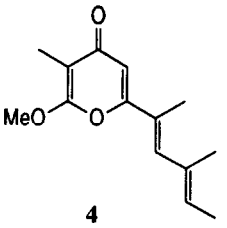

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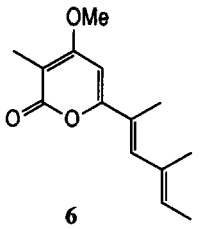

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cristallina,[5] along with a closely related new metabolite, named 12-norcyercene-B (**2**). Analogously to cyercene-B (**1**), the γ-pyrone nature of 12-norcyercene-B (**2**) was supported by IR absorption at 1654 cm^{-1}. The ^{1}H-NMR spectra of **1** and **2** display (**Table 1**) almost identical resonances; the only difference is the absence in **2** of the terminal methyl group. In fact, H-10 resonated at δ 5.90 as a quartet due to coupling with a methyl (H-11, 3H, d, δ 1.84). The geometry of both double bonds of **2** was *E* on the basis of $J_{7\text{-}8}$=15.8 *Hz* and the ^{1}H-NMR chemical shift of CH_3-9 (δ 1.83), identical to that observed in the ^{1}H-NMR spectrum of **1** for the corresponding protons. ^{1}H-NMR assignments of H-7 and H-8 were supported by 2D ^{1}H-^{13}C-heterocorrelation experiments conducted on **1**, which connected the ^{1}H-NMR signals at δ 6.03 and 6.90 with the ^{13}C-NMR resonances at δ 116.5 (C-7) and 139.5 (C-8) respectively. This suggested the inversion of the reported[5] chemical shifts previously assigned only on the basis of a supposed downfield effect of the pyrone ring on H-7. An analogous inversion must be suggested for the same two protons in *C. cristallina* cyercenes 1-3.[5]

Spectral analysis of the second pair of pyrones, **3** and **4**, led to stuctures closely related to **1** and **2**, but displaying another methyl at C-7, 7-methyl-cyercene-B (**3**) and 7-methyl-12-norcyercene-B (**4**). Elemental composition, based on HREIMS, suggested that **3** (C_{15} H_{20} O_3) is the higher homologue of **4** (C_{14} H_{18} O_3). Presence of a γ-pyrone ring in **3** and **4** was suggested by an IR band at 1655 cm^{-1}. Very similar ^{1}H-NMR spectra ($CDCl_3$, **Table 1**) of **3** and **4** displayed signals attributable to a 2-methoxy-3-methyl-6-substituted-γ-pyrone

(δ 6.27, H-5; δ ~ 4.04, OCH_3-2; δ ~ 1.86, CH_3-3). The alkyl chain at C-6 was identified as a tetrasubstituted butadiene system with methyls at C-7 (broad singlet at δ 2.02) and C-9 (broad singlet at δ 1.83) allylically coupled to two vinyl protons (δ 6.71, H-8; δ ~ 5.58, H-10).

TABLE 1. ^{1}H-NMR Chemical Shifts [a], Multiplicities and Coupling Constants (in *Hz*, in parentheses).

H	1[b]	2	3[c]	4	5	6
5	6.12 *s*	6.12 *s*	6.27 *s*	6.27 *s*	6.15 *s*	6.15 *s*
7	6.03 *d* (16.0)	6.02 *d* (15.8)				
8	6.90 *d* (16.0)	6.91 *d* (15.8)	6.71 *bs*	6.71 *bs*	7.00 *bs*	7.00 *bs*
10	5.80 *bt* (7.3)	5.90 *bq* (7.3)	5.53 *bt* (7.1)	5.62 *bq* (6.8)	5.55 *bt* (7.5)	5.64 *bq* (6.8)
11	2.24 *m* (7.5,7.3)	1.84 *d* (7.3)	2.17 *m* (7.3,7.1)	1.77 *d* (6.8)	2.16 *m* (7.5,7.5)	1.76 *d* (6.8)
12	1.05 *t* (7.5)		1.03 *t* (7.3)		1.02 *t* (7.5)	-
Me-9	1.83 *bs*	1.83 *bs*	1.83 *bs*	1.83 *bs*	1.82 *bs*	1.83 *bs*
Me-7			2.02 *bs*	2.02 *bs*	2.04 *bs*	2.03 *bs*
Me-3	1.86 *s*	1.86 *s*	1.86 *s*	1.87 *s*	1.94 *s*	1.94 *s*
O-Me	4.06 *s*	4.05 *s*	4.04 *s*	4.03 *s*	3.91 *s*	3.91 *s*

[a] 500.13 MHz, $CDCl_3$, chemical shifts referred to $C\underline{H}Cl_3$ at 7.26 ppm.
[b] Already reported in ref. 5.
[c] The data in C_6D_6 identical to those reported in ref. 8 are listed in the Experimental section.

TABLE 2. ^{13}C-NMR Chemical Shifts[a] and Multiplicities[b] of Cyercenes.

C	3[c]	4[d]	5	6	C	3[c]	4[d]	5	6
2	162.2 *s*	162.2 *s*	166.0 *s*	165.8 *s*	10	134.6 *d*	128.9 *d*	136.8 *d*	129.0 d
3	101.4 *s*	100.5 *s*	n. d.	101.9 *s*	11	21.8 *t*	13.8 *q*	21.7 *t*	14.0 q*
4	180.3 *s*	181.0 *s*	n. d.	164.9 *s*	12	13.8 *q*		14.0 *q**	
5	110.5 *d*	109.5 *d*	92.3 *d*	92.2 *d*	Me-9	16.4 *q*	16.2 *q*	16.5 *q*	16.4 q
6	159.0 *s*	158.5 *s*	160.8 *s*	160.8 *s*	Me-7	13.8 *q*	13.9 *q*	13.8 *q**	13.9 q*
7	125.5 *s*	124.4 *s*	124.0 *s*	123.7 *s*	Me-3	7.0 *q*	6.4 *q*	8.5 *q*	8.5 q
8	135.9 *d*	135.2 *d*	136.8 *d*	136.7 *d*	O-Me	54.7 *q*	55.4 *q*	56.1 *q*	56.0 q
9	131.3 *s*	132.3 *s*	131.9 *s*	132.9 *s*					

[a] 125.75 MHz, $CDCl_3$, chemical shifts referred to $C\underline{D}Cl_3$ at 77.0 ppm; some quaternary carbons were not detected (n. d.).
[b] Determined by DEPT sequence experiments.
[c] C_6D_6, TMS=0, partially assigned in ref. 8.
[d] Assignments made by ^{1}H-^{13}C heteronuclear experiments.
* Starred values in the same column can be interchanged.

The coupling pattern of H-10 (triplet in **3** and quartet in **4**) established that **3** and **4**, analogously to **1** and **2**, differ only by the presence in **3** of an additional methyl at C-11. The suggested structures were confirmed by the ^{13}C-NMR values (**Table 2**) and by ^{1}H-^{1}H spin decoupling. *E* -stereochemistry of the two double bonds was suggested by the values of the ^{13}C-NMR resonances assigned to CH_3-7 and CH_3-9, δ ~ 13.8 and δ ~ 16.3, respectively. ^{13}C-NMR multiplicities were assigned by DEPT sequence experiments. The NMR data of **3** (in $CDCl_3$) were very similar to those of the γ-pyrone-polypropionate isolated from *C. nigricans*[8] (in C_6D_6).

Structures of the two metabolites proved identical by recording both ^{1}H- and ^{13}C-NMR spectra of **3** in C_6D_6, which suggested *E*-stereochemistry of the terminal double bond of the *C. nigricans* polypropionate.

The two less polar TLC fractions (D, C) yielded two single products in the corresponding HPLC analyses (**5** and **6**). Both compounds displayed an IR band at 1682 cm^{-1} which, along with the absence of ^{13}C-NMR signals at δ ~180 and the ^{1}H-NMR singlets at δ 3.91 (3H), 1.94 (3H) and 6.15 (1H), suggested the presence of a 3-methyl-4-methoxy-6-substituted-α-pyrone system. HREIMS of the molecular peaks furnished the composition $C_{15}H_{20}O_3$ for the less polar compound (**5**, 7-methylcyercene-2) and $C_{14}H_{18}O_3$ for the second metabolite (**6**, 7-methylcyercene-1). Analogously to the above described pairs, the alkyl chain at C-6 of **5** and **6** was characterized by a tetrasubstituted *E, E* -butadiene system, where the terminal methyl of **6** is replaced in **5** by an ethyl group. All ^{1}H-NMR data were confirmed by ^{1}H monodimensional spin-decoupling experiments. The two new metabolites are closely related to similar α-pyrones found in *C. cristallina*.[5]

This study further supports the ability of molluscs belonging to the Polybranchioidea superfamily to biosynthetize unusual degraded polypropionates. It is extraordinary that *E. funerea* possesses some of the rare metabolites (**3** and **1**) found only in two species (*C. nigricans* and *C. cristallina*) of the genus *Cyerce* living in very distinct geographical areas. Preliminary biosynthetic experiments on *E. funerea* with (^{14}C)-sodium propionate led to a moderate incorporation into cyercenes. The small size of the molluscs prevented direct injection of the precursor and, therefore, the experiments were performed by absorption as described by Manker *et al.*.[9] *De novo* synthesis of the pyrones in the presence of ^{14}C-propionate was induced by using 90 "naked" (without cerata) specimens of *E. funerea* and by conducting the absorption experiment during their rapid (8 days) regeneration period. After incorporation, the specimens, possessing newly regenerated cerata, were extracted as usual, and the extract fractionated either by semipreparative TLC or by HPLC. Both purifications revealed that the major radioactive fractions coeluted or comigrated with cyercenes. Because of the small amounts of the metabolites, it was impossible to define further, by additional purification and chemical derivatization, the effective incorporation.

E. funerea, when molested and in analogy with *C. cristallina*, loses its dorsal appendages and secretes a supposedly toxic mucus. Both properties are likely to protect the animals against predation. HPLC profiles of the extracts from the mucus and from the cerata revealed different metabolite-patterns. Briefly, a) **2** and **4** were the major components in the mucous secretion, b) **2** is only a minor component in cerata extracts, where the main products are **4** and the other cyercenes (**3**, **5**, **6**).[13] Ichthyotoxicity tests on *Gambusia affinis* were carried out only for the most abundant 7-methyl-12-norcyercene-B (**4**) and 7-methylcyercene-B (**3**), which were toxic to *Gambusia affinis* at concentrations of 20 and 16 ppm; ichthyotoxicity of **1**, also present in *C. cristallina* (10 ppm), had been already reported,[10] thus suggesting their potential role as defense allomones.

Studies are now in progress to ascertain the possible involvement of some *E. funerea* metabolites in the extraordinary regeneration of the cerata , as had previously been suggested for cyercene-A from *C. cristallina*.

Experimental

Extraction and purification of cyercenes

E. funerea (600 specimens, wet weight 23.4 g), was collected in the Gulf of Naples. The animals were immediately extracted with acetone and sand (Merck, 1:1 w/w); the chloroform-soluble fraction (100 mg) from the acetone extract was loaded onto semipreparative TLC plates (Merck) and developed with petroleum ether/diethyl ether (1:1) and yielding four UV-active bands at Rf= 0.1 (band A, 4.0 mg), 0.15 (band B, 4.5 mg),

0.55 (band C, 1.4 mg) and 0.60 (band D, 1.9 mg). These were further purified by reverse phase HPLC (Spherisorb ODS2 column, 4.5 x 250 mm, 5 μm, 40 min gradient from 60 to 75 % methanol in water; flow rate 1.0 mL/min). TLC bands at Rf= 0.1 and Rf= 0.15 yielded two HPLC peaks each, corresponding to cyercene-B (**1**, 1.7 mg) and 12-norcyercene-B (**2**, 2 mg), and 7-methylcyercene-B (**3**, 0.8 mg), 7-methyl-12-norcyercene-B (**4**, 3.3 mg), respectively. The two less polar TLC fractions (Rf= 0.60, 0.55) yielded two single products in the corresponding HPLC analysis: 7-methylcyercene-2 (**5**, 1.6 mg) and 7-methylcyercene-1 (**6**, 1.0 mg).

Acquisition of IR, UV, MS and NMR spectra.

IR spectra were recorded as thin films on KBr on a Biorad FTS-7 FTIR instrument. UV spectra were obtained in methanol using a DMS-90 Varian spectrophotometer. Electron impact mass spectra were run on Kratos MS30 and MS50 machines. ^{1}H-(500.13 MHz) NMR and ^{13}C-(125.78 MHz) spectra were obtained in $CDCl_3$ for all compounds; **3** was also analyzed in C_6D_6. The instrument used was a Bruker AMX-500 Spectrometer equipped with X32 data system. HPLC analyses were performed using a Waters liquid chromatograph equipped with M510 pumps and a 490E multiwavelength UV detector, monitoring UV absorbance at 248, 300 and 350 nm.

Further spectral data of cyercene-B (**1**)

UV, IR, EIMS, ^{1}H and ^{13}C-NMR spectral data of **1** were identical to those of cyercene-B extracted from *C. cristallina*.[5] Therefore, because of the small amounts of cyercene-B (**1**) extracted from *E. funerea*, it was possible to conduct heterocorrelation experiments on **1** by purifying it also from *C. cristallina* .

Further spectral data of 12-norcyercene-B (**2**)

IR ν_{max} at 1654 cm^{-1}. UV and ^{13}C-NMR spectral data of **2** are lacking because the product was subject to rapid degradation. EIMS fragments at *m/z* 220 (molecular ion, 100%), 205 (-CH_3, 33%), 177 (-CH_3,-CO,44%). HREIMS Calcd. for $C_{13}H_{16}O_3$: M, 220.1099. Found: *m/z* 220.1090.

Further spectral data of 7-methylcyercene-B (**3**)

IR ν_{max} at 1655 cm^{-1}. UV λ_{max} at 227 nm (ε= 13000) and 288 nm (ε= 10700). ^{1}H-NMR (C_6D_6, 500 MHz, δ): 6.48 (H-8, 1H, bs), 6.36 (H-5, 1H, s), 5.37 (H-10, 1H, bt, *J*= 7.1 *Hz*), 3.13 (O-CH_3, s), 2.13 (CH_3-3, s), 1.96 (H-11, 2H, m, *J*= 7.3, 7.1 *Hz*), 1.64 (CH_3-7, bs), 1.59 (CH_3-9, bs), 0.90 (H-12, 3H, t, *J*= 7.3 *Hz*). EIMS fragments at *m/z* 248 (molecular ion, 100%). HREIMS Calcd. for $C_{15}H_{20}O_3$: M, 248.1412. Found: *m/z* 248.1418.

Further spectral data of 7-methyl-12-norcyercene-B (**4**)

IR ν_{max} at 1655 cm^{-1}. UV λ_{max} at 229 nm (ε= 10320) and 276 nm (ε= 6785). EIMS fragments at *m/z* 234 (molecular ion, 86%), 206 (-CO,100%). HREIMS Calcd. for $C_{14}H_{18}O_3$: M, 234.1255. Found: *m/z* 234.1246.

Further spectral data of 7-methyl-cyercene-2 (**5**)

IR ν_{max} at 1682 cm^{-1}. UV spectral data of **5** are lacking because the product was subject to degradation. EIMS fragments at *m/z* 248 (molecular ion, 100%). HREIMS Calcd. for $C_{15}H_{20}O_3$: M, 248.1412. Found: *m/z* 248.1418.

Further spectral data of 7-methylcyercene-1 (**6**)

IR ν_{max} at 1682 cm^{-1}. UV λ_{max} at 227 nm (ε= 7142), 252 nm (ε= 5978) and 345 nm (ε= 6428). EIMS fragments at *m/z* 234 (molecular ion, 100%), 219 (- CH_3, 70%). HREIMS Calcd. for C_{14} H_{18} O_3: M, 234.1255. Found: *m/z* 234.1266.

In vivo incorporation experiments

All 90 specimens were molested in order to induce spontaneous detachment of cerata. The resulting "naked" molluscs were then placed in an aerated tank with 5 L of sea water containing 25 μCi of ^{14}C-sodium propionate labelled at C-1 (57.0 mCi/mmol, Amersham). After 4 days the water was changed and additional 25 μCi of labelled precursor added. After 8 days, cerata were almost completely regenerated and the molluscs were extracted as usual and the extract analyzed by HPLC and TLC carried out as described above. Scintillation liquid 10 mL (INSTA Fluor-II, Packard) was added to 1 mL HPLC fractions and to TLC silica scraped off the plate at Rf 0.05 intervals, and β-emission was counted. Specific incorporation of the purified pyrones was: **1**, 2813 cpm/mg, 0.1 mg; **2**, 4842 cpm/mg, 0.1 mg; **3**, 2521 cpm/mg, 0.05 mg; **4**, 3617 cpm/mg, 0.2 mg; **5**, 1495 cpm/mg, 0.09 mg; **6**, 7087 cpm/mg, 0.06 mg.

Ichthyotoxicity assay

The *Gambusia affinis* ichthyotoxicity test was carried out following the procedure described by Gunthorpe and Cameron[11] and using the toxicity scale defined by Coll *et al.*.[12]

Acknowledgments

The authors are deeply grateful to Prof. P. Scheuer for the critical reading of manuscript and to Prof. W. Fenical for the ^1H and ^{13}C-NMR data of compounds from *C. nigricans*. Special acknowledgment goes to Drs. G. Villani and E. Mollo for their help during collection of materials and biological observations, and to A. Crispino, A. Trabucco and R. Turco for their valuable technical assistance. NMR and mass Spectra were performed at the ICMIB-NMR Service and at the Servizio di Spettrometria di Massa CNR-University of Naples, respectively. This work is part of a bilateral Italian-Spanish project and was partly funded by the "Progetto Finalizzato Chimica Fine" (CNR) and the CEE contract MAS 2-CT 91-0004 (MAST-II programm 1991-1994).

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