New Polyhalogenated Monoterpenes from the Sea Hare *Aplysia punctata*

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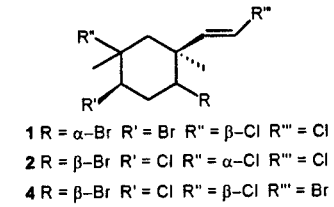
The sea hare *Aplysia punctata* from Sancti Petri (Cádiz, Spain) contains four new unusual acetates of linear polyhalogenated monoterpenes (**5**−**8**) together with four known cyclic derivatives (**1**−**4**). The structures were elucidated by interpretation of spectral data. It is suggested that the new constituents of *A. punctata* might be biotransformation products. Compounds **6**−**8** showed significant in vitro cytotoxicity against four tumor cell lines.

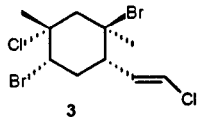
Sea hares have traditionally been a source of terpenoids of dietary origin.[1] In particular, the genus *Aplysia* has been extensively studied,[2] affording monoterpenes, sesquiterpenes, and diterpenes with varied degrees of halogenation, accumulated both from the algal diet and also modified by metabolic transformations.[3] In only a few instances is there no direct relationship between the metabolites isolated from *Aplysia* and the chemical constituents of the dietary source.[4,5]

In our project directed toward the discovery of bioactive compounds from marine organisms of the southern coast of Spain we obtained specimens of *Aplysia punctata* Cuvier (Aplysiidae) collected in Sancti Petri (Cádiz, Spain). *A. punctata* from Galicia, Spain, had been reported to contain seven cyclic polyhalogenated monoterpenes in an earlier report of cyclic monoterpenes from the genus *Aplysia*.[6] Our specimens of *A. punctata* contained four known cyclic polyhalogenated monoterpenes (**1**−**4**) together with four new acyclic derivatives (**5**−**8**).

Specimens of *A. punctata* were collected by hand using scuba and were immediately frozen. Chromatography of the Me_2CO-soluble material on Si gel followed by final purification using HPLC allowed isolation of the following compounds in order of increasing polarity: (1*S**,2*R**,4*R**,5*S**)-5-chloro-1(*E*)-(chlorovinyl)-2,4-dibromo-1,5-dimethylcyclohexane (**1**, 0.252% dry wt); (1*S**,2*S**,4*R**,5*R**)-2-bromo-1(*E*)-(chlorovinyl)-4,5-dibromo-1,5-dimethylcyclohexane (**2**, 0.315% dry wt); (1*R*,2*S*,-4*S*,5*R*)-5-chloro-2(*E*)-(chlorovinyl)-1,4-dibromo-1,5-dimethylcyclohexane (**3**, 1.735% dry wt); (1*S*,2*S*,4*R*,5*S*)-2-bromo-1(*E*)-(bromovinyl)-4,5-dichloro-1,5-dimethylcyclohexane (**4**, 1.609% dry wt); (3*Z*,5*E*)-1-acetoxy-4-chloro-3,7-dimethylocta-3,5,7-triene (**5**, 0.095% dry wt); (7*E*)-1-acetoxy-8-chloro-7-(dichloromethyl)-3-methyloct-7-en-4-one (**6**, 0.158% dry wt); (7*Z*)-1-acetoxy-8-chloro-7-(dichloromethyl)-3-methyloct-7-en-4-one (**7**, 0.140% dry wt), and (3*Z*,5*E*)-1-acetoxy-8-bromo-4,7-dichloro-3,7-dimethylocta-3,5-diene (**8**, 0.126% dry wt). Compounds **1**,[7] **2**,[8] **3**,[9] and **4**[9] were identified by comparison of spectral data with those reported in the literature.

The HRMS of compound **5** contained the correct cluster of peaks for the molecular formula $C_{12}H_{17}O_2Cl$, indicating four degrees of unsaturation. The IR absorption at 1740 cm^{-1}, together with the ^{13}C-NMR signals (Table 1) at δ 170.1 (s) and 20.3 (q), indicated that **5**





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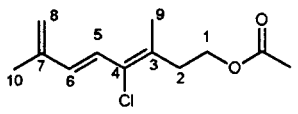

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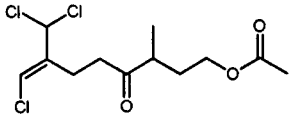

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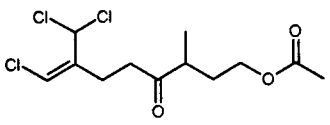

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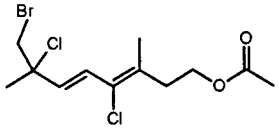

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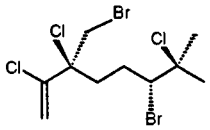

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was an acetate. A triplet at δ 4.84 (t, 2H, J = 7.0 Hz) in the ^1H-NMR spectrum (Table 1), which was mutually and exclusively coupled with the allylic methylene signal at δ 2.21 (t, 2H, J = 7.0 Hz), was assigned to a methylene attached to the acetoxyl group and defined a $CH_3CO_2CH_2CH_2-$ moiety attached to a fully substituted sp^2 carbon atom. Because the three degrees of unsaturation remaining were due to three double bonds, which gave rise in the ^{13}C-NMR spectrum to the signals at δ 141.8 (s), 134.9 (d), 132.1 (s), 129.6 (s), 122.8 (d), and 118.9 (t), it was concluded that the molecule was acyclic. The ^1H-NMR signals at δ 6.66 (d, 1H, J = 15.4 Hz) and 7.05 (d, 1H, J = 15.4 Hz) were assigned to a *trans* disubstituted double bond. and the signals 5.08 (br s, 1H), 4.95 (br s, 1H), and 1.84 (br s, 3H) were due to a terminal isopropylene group linked to the *trans* disubstituted double bond. The remaining double bond must join the $CH_3CO_2CH_2CH_2-$ and the 3-methylbuta-1,3-dienyl moieties. Its substitution pattern was proposed following the isoprene rule and upon observation of the ^{13}C-NMR signal at 21.2 (q) assigned to a methyl group on a *Z* double bond.[10] These spectral features could be accommodated by structure **5**.

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Table 1. Full Assignments for **5**, **6**, **7**, and **8**[a]

[a]	**5**[b]		6		7		8	
carbon no.	^{13}C	^{1}H, mult	^{13}C	^{1}H, mult	^{13}C	^{1}H, mult	^{13}C	^{1}H, mult
1	61.7	4.84, t, 7.0	62.3	4.10, dt, 11.1, 6.2 4.05, dt, 11.1, 6.2	62.2	4.08, ddd, 11.0, 6.9, 6.0 4.01, ddd, 11.0, 6.9, 6.0	61.8	4.20, t, 7.0
2	33.8	2.21, t, 7.0	31.4	2.09, m 1.67, m	31.3	2.04, m 1.65, dq, 12.8, 6.0	35.8	2.29, td, 7.0, 1.9
3	132.1		43.1	2.66, sextet, 7.1	43.1	2.65, sextet, 7.0	134.0	
4	129.6		211.9		211.6		132.8	
5	122.8	6.66, d, 15.4	39.1	2.92, ddd, 17.8, 8.6, 7.1 2.82, ddd, 17.8, 8.9, 6.1	39.8	2.80, ddd, 17.5, 7.5, 6.7 2.86, ddd, 17.5, 7.5, 6.7	123.7	6.10, d, 14.8
6	134.9	7.05, d, 15.4	20.7	2.70, m	23.6	2.73, m	135.6	6.78, d, 14.8
7	141.8		140.1		138.4		71.8	
8	118.9	4.95, 5.08, br s	121.4	6.30, br s	117.5	6.03, td, 1.2, 0.4	45.0	3.52, s
9	21.2	1.77, br s	16.7	1.14, d, 7.1	16.6	1.12, d, 7.0	19.4	2.00, s
10	18.8	1.84, br s	72.8	6.52, br s	68.1	6.92, d, 0.4	26.7	1.47, s
COCH$_3$	170.1		171.0		170.9		170.1	
CO*CH*$_3$	20.3	1.56, s	20.9	2.04, s	20.9	2.02, s	21.0	2.01, s

[a] 400 MHz, $CDCl_3$, 25 °C, δ, mult, J in Hz. [b] Measured in C_6D_6.

Compound **8** was isolated as a colorless oil of molecular formula $C_{12}H_{17}O_2Cl_2Br$ as ascertained by the cluster of peaks observed in the HRMS. The spectroscopic data of **8** (Table 1) were quite similar to those of compound **5** and indicated that the (3*Z*,5*E*)-1-acetoxy-4-chloro-3-methylhexa-3,5-dienyl fragment must be present in the structure of **8**. However, the absence of signals for the H-8 vinylic protons and the presence of a triplet at δ 45.0 in the ^{13}C-NMR spectrum assigned to the carbon of a bromomethylene group, a singlet at δ 71.8 due to a fully substituted carbon atom bearing chlorine, and a quartet at δ 26.7 assigned to a methyl group that must be located on the quaternary carbon indicated the presence of the terminal 7-(bromomethyl)-7-chloroethyl unit. Structure **8** was therefore proposed for this compound.

Compound **6** was isolated as a colorless oil. The molecular formula, $C_{12}H_{17}O_3Cl_3$, was deduced from the cluster of peaks observed in the HRMS measurement. The IR absorption at 1745 cm^{-1} together with the ^{13}C-NMR signals (Table 1) at δ 171.0 (s) and 20.9 (q), indicated that **6** was an acetate. The ^{1}H-NMR signals (Table 1) at δ 4.10 (dt, 1H, J = 11.1, 6.2 Hz) and 4.05 (dt, 1H, J = 11.1, 6.2 Hz) assigned to a methylene group attached to the acetoxyl group were correlated in the COSY spectrum with the methylene proton signals at δ 2.09 (m, 1H) and 1.67 (m, 1H) and defined the CH_3-$CO_2CH_2CH_2$− moiety. In contrast with compound **5** the methylene proton signals at δ 2.09 and 1.67 were additionally coupled to a signal at δ 2.66 (sextet, 1H, J = 7.1 Hz) assigned as the proton of a methine group attached to a carbonyl group. Because the signal at δ 2.66 showed a cross peak in the COSY spectrum with the methyl proton signal at δ 1.14 (d, 3H, J = 7.1 Hz) a 4-acetoxy-2-methylbutanoyl moiety must be present in the structure of **6**. The five remaining carbons were assigned to the second isoprene unit as follows. The methylene proton signals mutually and exclusively coupled at δ 2.92 (ddd, 1H, J = 17.8, 8.6, 7.1 Hz), 2.82 (ddd, 1H, J = 17.8, 8.9, 6.1 Hz), and 2.70 (m, 2H) were assigned to two methylene groups in the α and β positions to the ketone carbonyl group, respectively. The two remaining signals in the ^{1}H-NMR spectrum at δ 6.52 (br s, 1H) and 6.30 (br s, 1H) were due to a dichloromethine proton and to a vinylic proton, respectively. The dichloromethyl substituent and the third chloro atom must be located on the double bond, which gave rise to the ^{13}C-NMR singlets at δ 140.1 and 121.4. The substitution pattern and the *E* geometry of this double bond were defined following the isoprene rule and upon observation of the mutual NOE enhancements produced on irradiation of the vinylic and the dichloromethine proton signals. These spectral data were in agreement with the proposed structure for compound **6**.

The cluster of peaks observed in the HRMS of **7** indicated a molecular formula $C_{12}H_{17}O_3Cl_3$ and that compound **7** was an isomer of **6**. The ^{1}H- and ^{13}C-NMR data (Table 1) of both compounds were quite similar, suggesting that the 4-acetoxy-2-methylbutanoyl moiety was present in the structure of **7**. However some differences were observed in the H-8 vinylic proton signal at δ 6.03 (td, 1H, J = 1.24, 0.35) and H-10 dichloromethine proton signal at 6.92 (d, 1H, J = 0.35). These differences were explained assuming an opposite geometry at C-7,C-8 double bond whose stereochemistry was therefore proposed as *Z*. The absence of NOE enhancements upon irradiation of both H-8 and H-10 proton signals confirmed that compound **7** was the geometric isomer of compound **6**.

The close relationship existing between the constituents of sea hares and those of the algal dietary source is well known.[1] In fact, monoterpenes **1**−**4** from *A. punctata* are known metabolites of *Plocamium* algae.[7−9] The new compounds **5**−**8** are also related to the algal monoterpenes, but the study of different collections of *Plocamium cartilagineum* found near our specimens did not afford any of these metabolites. Although it might be suggested that compounds **5**−**8** could have been acquired by the mollusk from an undiscovered dietary source, inasmuch as the pattern of oxygenated functions is uncommon among algal monoterpenes, it seems plausible that the new components isolated from *A. punctata* may be biotransformation products arising from further metabolism of dietary compounds.

Most of the halogenated monoterpenes isolated from mollusks and algae were described 20 years ago in challenging studies made by marine natural product chemists.[11] However, it was not until the discovery that halomon (**9**) selectively inhibits brain-, renal-, and colon-tumor cell lines[12] that there has been renewed interest in the medical potential of polyhalogenated compounds. This reason prompted us to examine the cytotoxicity of

the new compounds[13] isolated from *A. punctata* against P-388 mice lymphoma, A-549 human lung carcinoma, HT-29 human colon carcinoma and MEL-28 human melanoma cell lines. Compounds **6**−**8** showed identical cytotoxic properties with ED_{50}= 2.5 μg/mL against P-388 and HT-29 cell lines and ED_{50}= 1.5 μg/mL against A-549 and MEL-28 cell lines.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Perkin-Elmer 881 spectrophotometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian 400 spectrometer at 400 MHz and 100 MHz, respectively, using $CDCl_3$ as solvent. The resonances of residual $CHCl_3$ at δ_H 7.26 and δ_C 77.0 were used as internal reference for ^{1}H-NMR and ^{13}C-NMR spectra, respectively. MS were measured on a VG 12250 or on a Kratos MS 80RFA spectrometer. In HPLC separations LiChrosorb Si-60 was used in normal-phase mode using a differential refractometer. All solvents were distilled from glass prior to use.

Collection, Extraction, and Isolation Procedures. Five specimens of *A. punctata* (3.17 g dry wt) were collected by hand using scuba in Sancti Petri (Cádiz, Spain) and immediately frozen. A voucher specimen is available at Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad de Cádiz. The material was cut in small pieces an extracted with Me_2CO at room temperature. The solution was filtered, and the solvent was evaporated under reduced pressure to obtain a residue that was partitioned between H_2O and Et_2O. The Et_2O solution was dried over anhydrous Na_2SO_4 and the solvent removed to afford a violet oil (1.0 g). The organic extract was subjected to SiO_2 column separation eluting with mixtures of increasing polarity from hexane to Et_2O and subsequently $CHCl_3$−MeOH (8:2). Selected nonpolar fractions contained a mixture of cyclic monoterpenes that was further separated by HPLC in normal-phase mode (LiChrosorb 10 μ, 10 mm × 25 cm; hexane) affording **1** (8 mg, 0.252% dry wt), **2** (10 mg, 0.315% dry wt), **3** (55 mg, 1.735% dry wt), and **4** (51 mg, 1.609% dry wt). Fractions eluted with hexane−Et_2O (8:2) afforded, after purification by HPLC (LiChrosorb 10 μ, 10 mm × 25 cm; hexane−EtOAc, 99:1), compound **5** (3 mg, 0.095% dry wt). Fractions eluted with hexane−Et_2O (7:3) contained a mixture of isomers that was subjected to HPLC separation (LiChrosorb 10 μ, 10 mm × 25 cm; hexane−EtOAc, 99:1) yielding **6** (5 mg, 0.158% dry wt) and **7** (13 mg, 0.410% dry wt). More polar fractions provided compound **8** (4 mg, 0.126% dry wt) after purification by HPLC (LiChrosorb 10 μ, 10 mm × 25 cm; hexane−EtOAc, 8:2).

(3*Z*,5*E*)-1-Acetoxy-4-chloro-3,7-dimethylocta-3,5,7-triene (5): colorless oil; IR (dry film) ν_{max} 2980 (CH, aliphatic), 1740 (C=O), 1635 (C=C), 1244 (CO), 726 (CCl) cm^{-1}; EIMS (70 eV) *m*/*z* (rel int) 228, 230 (0.8:0.2), 168, 170 (13.0:4.7), 133 (100); HREIMS *m*/*z* 228.0917, calcd for $C_{12}H_{17}O_2{}^{35}Cl$, 228.0901.

(7*E*)-1-Acetoxy-8-chloro-7-(dichloromethyl)-3-methyloct-7-en-4-one (6): colorless oil; $[\alpha]^{25}_D$ −3.7° (*c* 0.19, $CHCl_3$); IR (dry film) ν_{max} 2960, 2930 (CH, aliphatic), 1745 (C=O), 1700 (C=C), 1454, 1370 (CH_3−), 1236 (CO), 746 (CCl) cm^{-1}; EIMS (70 eV) *m*/*z* (rel int) 255, 257, 259, 261 (2.9:2.6:0.6:0.1), 199, 201, 203, 205 (100:98.1:47.0:4.60), 171, 173, 175, 177 (18.9:17.6:2.5:0.9); HREIMS *m*/*z* 271.0070, calcd for $C_{10}H_{14}O_2{}^{35}Cl_3$, 271.0094.

(7*Z*)-1-Acetoxy-8-chloro-7-(dichloromethyl)-3-methyloct-7-en-4-one (7): colorless oil; $[\alpha]^{25}_D$ −5.0° (*c* 1.3, $CHCl_3$); IR (dry film) ν_{max} 2963, 2927 (CH, aliphatic), 1747 (C=O), 1690 (C=C), 1454, 1380 (CH_3−), 1236, 1057 (CO), 746 (CCl) cm^{-1}; EIMS (70 eV) *m*/*z* (rel int) 314, 316, 318, 320 (3.2:3.2:1.1:0.1), 255, 257, 259, 261 (8.3:7.7:2.5:0.6), 219, 221, 223 (34.5:23.1:4.1), 199, 201, 203, 205 (51.0:51.5:18.5:4.8), 135, 137, 139 (100.0:57.2:15.0); HREIMS *m*/*z* 256.0002, 254.0032; calcd for $C_{10}H_{13}O^{35}Cl_2{}^{37}Cl$ 256.0001, for $C_{10}H_{13}O^{35}Cl_3$, 254.0026.

(3*Z*,5*E*)-1-Acetoxy-8-bromo-4,7-dichloro-3,7-dimethylocta-3,5-diene (8): colorless oil; $[\alpha]^{25}_D$ −4.8° (*c* 0.2, $CHCl_3$); UV λ_{max} (MeOH) 250 (ϵ 9300) nm; IR (dry film) ν_{max} 2975, 2933 (CH, aliphatic), 1639 (C=C), 1463, 1369 (CH_3−), 1238 (CO), 725 (CCl) cm^{-1}; EIMS (70 eV) *m*/*z* (rel int) 264, 266, 268 (7.3:6.0:1.1), 229, 231 (5.6:4.1), 171, 173 (30.3:16.3), 135 (100); HREIMS *m*/*z* 341.9792; calcd for $C_{12}H_{17}O_2{}^{35}Cl_2{}^{79}Br$, 341.9823.

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(13) Compound **5** was highly unstable and could not be tested in cytotoxicity assays.

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