4-Acetylaplykurodin B and Aplykurodinone B, Two Ichthyotoxic Degraded Sterols from the Mediterranean Mollusk Aplysia fasciata

A. Spinella, M. Gavagnin, A. Crispino, G. Cimino, E. Martinez, J. Ortea, and G. Sodano

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4-ACETYLAPLYKURODIN B AND APLYKURODINONE B, TWO ICHTHYOTOXIC DEGRADED STEROLS FROM THE MEDITERRANEAN MOLLUSK *APLYSIA FASCIATA*

A. SPINELLA,* M. GAVAGNIN, A. CRISPINO, G. CIMINO,

Istituto per la Chimica di Molecole di Interesse Biologico, C.N.R., Via Toiano, 6, 80072 Arco Felice (NA), Italy

E. MARTINEZ, J. ORTEA,

Departamento de Biologia de Organismos y Sistemas, Universidad de Oviedo, c/J. Arias de Velasco, Oviedo, Spain

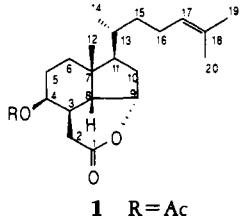
and G. SODANO

Dipartimento di Fisica, Università di Salerno, 84081 Baronissi (SA), Italy

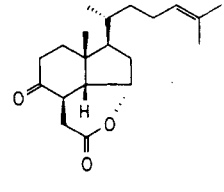
ABSTRACT.—Two ichthyotoxic lactones, 4-acetylaplykurodin B [**1**] and aplykurodinone B [**2**], were isolated from the external parts of the body of the mollusk *Aplysia fasciata*. Their structures, determined by spectroscopic and chemical means, are closely related to aplykurodin B [**3**] previously isolated from *Aplysia kurodai*. The interconversion of δ- and γ-lactones in the aplykurodin derivatives has been also investigated.

Anaspidean mollusks of the family Aplysiidae are known to have few predators. Several studies have been conducted on various *Aplysia* species, and it has been postulated that toxic compounds, ingested and stored in the digestive glands, are mainly responsible for their defense (1).

In the course of our study of chemical aspects of the ecology of the Mediterranean opisthobranchs (2), we have investigated the chemical defense of *Aplysia fasciata* Poiret. We report here the isolation and structure elucidation of 4-acetylaplykurodin B [**1**] and aplykurodinone B [**2**], two ichthyotoxic metabolites contained in those parts of the animal ex-



1 R=Ac
3 R=H



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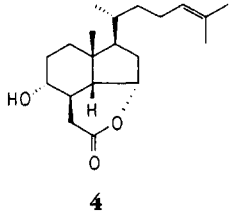

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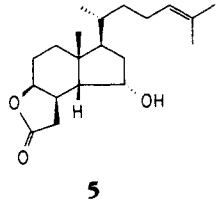

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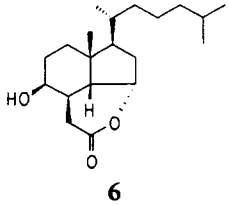

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posed to predators. These compounds were named as aplykurodin derivatives because of their structural similarity with **3** and **6** isolated from *Aplysia kurodai* (3).

Specimens of *A. fasciata* collected in the Bay of Naples were carefully dissected, and different parts of the animals were extracted separately with Me_2CO. In this way it was possible to compare metabolite content of the exposed and internal parts of the mollusk. Et_2O-soluble fractions of Me_2CO extracts obtained from the parapodial lobes and mantles (external parts) contained two metabolites, **1** and **2**, which were completely absent in the extracts from the other parts of the animals. Si gel chromatography afforded 15 mg of **1** and 39 mg of **2**.

The molecular formula, $C_{22}H_{34}O_4$, of **1** was determined on the basis of the hreims spectrum. The analysis of both ^{1}H- and ^{13}C-nmr data (Table 1), obtained from 1D and 2D experiments, allowed the assignment of structure **1**. The presence of a δ-lactone, instead of the alternative γ-lactone, was supported

TABLE 1. ^{1}H- and ^{13}C-nmr Data[a] (in $CDCl_3$) of Compounds **1** and **2**.

Position	Compound			
	1		**2**	
	δ_H[b]	δ_C[c]	δ_H[b]	δ_C[c]
1	—	171.0[d] (s)	—	171.2 (s)
2	2.12 (dd, 17.4, 10.8) 2.47 (dd, 17.4, 3.0)	33.3 (t)	2.06 (dd, 17.3, 12.9) 2.90 (dd), 17.3, 2.8)	30.0 (t)
3	1.95 (m)	32.0 (d)	2.67 (ddd, 13.3, 12.9, 2.8)	41.9 (d)
4	4.98 (m)	69.6 (d)	—	209.8 (s)
5	0.93 (m) 1.10 (m)	24.8 (t)	2.34 (m) 2.44 (m)	35.5 (t)
6	1.55 (m) 1.85 (m)	29.6 (t)	2.02 (m) 1.92 (m)	35.1 (t)
7	—	43.1 (s)	—	44.5 (s)
8	2.05 (m)	43.8 (d)	1.95 (m)	51.7[d] (d)
9	4.98 (m)	80.4 (d)	4.85 (bt)	79.3 (d)
10	2.12 (m)	37.6 (t)	2.00 (m) 2.27 (m)	38.5 (t)
11	1.87 (m)	48.4 (d)	1.95 (m)	50.9[d] (d)
12	0.97 (s)	23.0 (q)	1.01 (s)	21.3 (q)
13	1.40 (m)	35.2 (d)	1.50 (m)	33.9 (d)
14	0.94 (d, 6.5)	18.6 (q)	0.98 (d, 6.5)	18.4 (q)
15	1.08 (m) 1.40 (m)	36.3 (t)	1.16 (m) 1.45 (m)	36.1 (t)
16	1.86 (m) 2.03 (m)	25.7 (t)	1.90 (m) 2.03 (m)	24.7 (t)
17	5.06 (bt)	124.4 (d)	5.06 (bt)	124.3 (d)
18	—	131.5 (s)	—	131.5 (s)
19	1.68 (bs)	25.6 (q)	1.68 (s)	25.6 (q)
20	1.60 (bs)	17.7 (q)	1.60 (s)	17.7 (q)
Me-CO-	2.08 (s)	21.0 (q)		
MeCO-	—	170.3[d] (s)		

[a]Table entries are chemical shifts (multiplicity, *J* in Hz), in ppm referred to $CHCl_3$ as internal standard (δ 7.26 for ^{1}H and δ 77.0 for ^{13}C).

[b]Assignments made by 1D decoupling and 2D COSY experiments.

[c]Assignments were aided by 1D DEPT and 2D ^{1}H-^{13}C COSY experiments.

[d]Assignments may be interchanged.

by the band at 1730 cm^{-1} in the ir spectrum. Furthermore, alkaline hydrolysis of **1** gave a compound with spectroscopic properties completely in agreement with those of aplykurodin B [**3**], isolated from *A. kurodai* (3). The structure of **3** was determined by X-ray crystallography and cd (3).

The more polar compound, **2**, $[\alpha]^{20}D$ $-198.0°$ ($c=0.73$, $CHCl_3$), was obtained as a white solid (mp 58–60°). Hreims indicated a molecular formula $C_{20}H_{30}O_3$, while analysis of 1D and 2D nmr data (Table 1) indicated that **2** was related to **1** but with a ketone rather than alcohol function at C-4. This was confirmed by reduction of **2** with $NaBH_4$, which afforded a mixture of products including aplykurodin B [**3**]. The absolute stereochemistry was confirmed by the cd spectrum of **2**, which showed a negative Cotton effect ($[\theta]_{290}$ -8026) (3). It is interesting to note that the $NaBH_4$ reduction of **2** gave the expected mixture of **3** and its 4α epimer **4** (ca. 1:1) together with the γ-lactone **5** which was the most abundant product. The structures of **4** and **5** were established from ir and nmr data (Experimental). In particular, the C-4 epimeric nature of **4** was inferred from the width at half height ($W_{1/2}$) of the H-4 signal in the ^{1}H-nmr spectrum, compared with H-4 of **3**, while the structure of **5** was deduced by comparison of its spectral data with those reported for the analogous γ-lactone obtained from aplykurodin A [**6**] (3).

The formation of **5** was not unexpected, since it has been reported that aplykurodin A [**6**] "upon treatment with alkali or acid gave the γ-lactone isomer" (3); the same isomerization occurred when **3** was hydrogenated (3). However, when **1** was hydrolyzed as reported above, only the δ-lactone **3** was obtained. These apparently conflicting findings stimulated us to investigate the conditions that favor the production and interconversion of the two lactones. When the γ-lactone **5** was dissolved in alkali [5% KOH in MeOH-H_2O (1:1)], neutralization of the solution with dilute acid caused the immediate formation of a precipitate. Extraction of the suspension with Et_2O yielded the δ-lactone **3**. On the other hand, when the δ-lactone **3** was dissolved in slightly acidic conditions, the γ-lactone **5** was slowly formed, reaching more than 90% conversion after 24 h.

From these results it is apparent that neutralization of the carboxylate form leads to the formation of the δ-lactone (kinetic product), while when this latter equilibrated in acidic solution the γ-lactone was obtained (thermodynamic product).

Aplykurodinone B [**2**] was found to be very toxic to the mosquito fish *Gambusia affinis* (4) at 10 ppm, while **1** was toxic in the same test at the same concentration. Antifeedant assays conducted with the fish *Carassius auratus* (6) showed feeding deterrence for both **1** and **2** at a concentration of 60 $\mu g/cm^2$ of food pellets. The distribution of the biological activities (ichthyotoxicity and feeding deterrence) on the mollusk's body suggests that compounds **1** and **2** could act as defense allomones against predators in *A. fasciata*. Compound **1** was toxic (LC_{50} 29.1 ppm) in the brine shrimp assay (7,8).

It is possible that 4-acetylaplykurodin B [**1**] and aplykurodinone B [**2**] are biogenetically derivable by oxidative pathways involving the loss of A-ring carbon atoms from a parent sterol or triterpenic alcohol. Incidentally, 9,10-secosterols (9,10) and 5,6-secosterols (11), which could be considered likely intermediates in the biosynthesis of aplykurodins, have been isolated from other marine invertebrates. Structurally related lactones have also been isolated from a sponge (12). However, *Aplysia* species are known to be generally herbivorous (1). In particular, *A. fasciata* (=*limacina*) has been reported to contain typical algal halogenated monoterpenoids (13).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ir spectra were measured with a Nicolet FT 5DXB spectrophotometer. Optical rotations were measured on a Perkin-Elmer 141 polarimeter, and the cd curve was recorded on a Jasco 710 dicograph. Hplc purifications were performed on a Waters chromatograph equipped with two pumps and a uv-vis detector. ^{1}H- and ^{13}C-nmr spectra were recorded on Bruker WM 500 and Bruker WM 250 spectrometers; chemical shifts are reported in parts per million referred to $CHCl_3$ as internal standard (δ 7.26 for proton and δ 77.0 for carbon). Mass spectra were obtained on AEI MS 30 and MS 50 Kratos instruments.

BIOLOGICAL MATERIAL.—*A. fasciata* was collected in the Bay of Naples and authenticated by E. Martinez. Voucher specimens are available at the Istituto per la Chimica di Molecole di Interesse Biologico.

EXTRACTION AND ISOLATION OF THE METABOLITES.—Twenty-four freshly collected specimens of *A. fasciata* were carefully dissected into exposed external parts such as parapodial lobes, mantle, metapodium, gills, and head, and the internal viscera. All these sections were separately extracted with Me_2CO at room temperature. The filtered Me_2CO solutions were concentrated and then, after dilution with H_2O, extracted with Et_2O. The composition of the Et_2O extracts was compared by tlc on SiO_2 using light petroleum ether-Et_2O (2:8). Compounds **1** (15 mg) and **2** (39 mg), only present in extracts from parapodial lobes and mantles, were isolated by cc on SiO_2 using light petroleum ether-Et_2O (1:1).

4-Acetylaplykurodin B [**1**].—$[\alpha]^{20}D$ −26.1° (c = 0.74, $CHCl_3$); ir ($CHCl_3$) 1730 cm^{-1}; ^{1}H and ^{13}C nmr see Table 1; eims *m/z* $[M]^+$ 362.2411 ($C_{22}H_{34}O_4$ requires 362.2457), 302, 287, 191.

Aplykurodinone B [**2**].—White solid: mp 58–60° (*n*-hexane); $[\alpha]^{20}D$ −198.0° (c = 0.73, $CHCl_3$); ir ($CHCl_3$) 1736, 1726 cm^{-1}; ^{1}H and ^{13}C nmr see Table 1; eims *m/z* $[M]^+$ 318.2173 ($C_{20}H_{30}O_3$ requires 318.2195), 300, 259, 205.

HYDROLYSIS OF 4-ACETYLAPLYKURODIN B [**1**].—Compound **1** (6 mg) was dissolved in MeOH (1 ml) and added to a 10% aqueous solution of KOH (1 ml). After 1 h of stirring at room temperature, MeOH was removed by flushing the solution with N_2. Neutralization with 2 N HCl caused the formation of a white precipitate which was dissolved by addition of Et_2O. Washing the organic phase with H_2O and removal of the solvent afforded 4 mg of **3**; eims *m/z* (%) 320 (9), 302 (6), 235 (6), 207 (100). ^{1}H nmr ($CDCl_3$) δ 5.07 (1H, bt, H-17), 4.97 (1H, m, H-9), 3.91 (1H, bs, $W_{1/2}$ = 11 Hz, H-4), 2.45 (1H, dd, J = 17.0 and 3.0, H_a-2), 2.36 (1H, dd, J = 17.0 and 12.5, H_b-2), 1.69 (3H, bs, Me-19), 1.60 (3H, bs, Me-20), 0.95 (3H, s, Me-12), 0.94 (3H, d, J = 6.5, Me-14); ^{13}C nmr ($CDCl_3$) δ 172.4 (s), 131.4 (s), 124.5 (d), 80.6 (d), 66.8 (d), 47.5 (d), 43.6 (d), 43.2 (s), 37.7 (t), 36.4 (t), 35.2 (d), 33.6 (t), 33.2 (d), 29.0 (t), 28.8 (t), 25.7 (q), 24.8 (t), 23.0 (q), 18.6 (q), 17.6 (q).

$NaBH_4$ REDUCTION OF APLYKURODINONE B [**2**].—To a solution containing **2** (7 mg) dissolved in EtOH (1 ml), $NaBH_4$ (1 mg) was added and the reaction mixture was stirred at room temperature for 3 h. Excess $NaBH_4$ was eliminated adding a drop of HOAc, and usual workup gave 7 mg of crude product. This product was chromatographed on a Si gel column using light petroleum ether-Et_2O (2:8) as eluant, affording 4 mg of **5** and 2.5 mg of a mixture containing **3** and **4** in a 1:1 ratio. The mixture of **3** and **4** was separated by hplc on a μ-Porasil column using *n*-hexane-iPrOH (94:6) as eluant, affording 1.0 mg of **3** and 1.1 mg of **4**.

Compound **4**.—Ir ($CHCl_3$) 1733 cm^{-1}; ms *m/z* (%) $[M]^+$ 320 (34), 302 (38), 287 (13), 191 (71), 93 (64), 69 (100); ^{1}H nmr ($CDCl_3$) δ 5.07 (1H, bt, J = 6.8, H-17), 4.95 (1H, m, H-9), 3.33 (1H, m, $W_{1/2}$ = 20 Hz, H-4), 2.99 (1H, dd, J = 17.4 and 3.0, H_a-2), 1.92 (1H, dd, J = 17.4 and 13.1, H_b-2), 1.69 (3H, bs, Me-19), 1.60 (3H, bs, Me-20), 0.94 (3H, d, J = 6.5, Me-14), 0.92 (3H, s, Me-12); ^{13}C nmr ($CDCl_3$) δ 172.1, 131.4, 124.5, 80.2, 73.0, 52.8, 43.7, 43.2, 37.5, 36.9, 36.3, 35.3, 33.7, 33.5, 31.0, 25.7, 24.8, 22.9, 18.6, 17.7.

Compound **5**.—Ir ($CHCl_3$) 1767 cm^{-1}; ms *m/z* (%) $[M]^+$ 320 (8), 302 (7), 235 (7), 207 (100); ^{1}H nmr ($CDCl_3$, assignments made by ^{1}H-^{1}H COSY 2D experiment) δ 5.07 (1H, bt, J = 6.8, H-17), 4.66 (1H, dt, J = 5.8 and 5.2, H-4), 4.44 (1H, bs, H-9), 2.75 (1H, dd, J = 17.5 and 8.0, H_a-2), 2.58 (1H, m, H-3), 2.46 (1H, dd, J = 17.5 and 3.2, H_b-2), 2.02 (1H, m, H_a-16), 1.97 (1H, m, H_a-5), 1.88 (1H, m, H_b-16), 1.85 (1H, m, H_b-5), 1.81 (1H, m, H-11), 1.81 (2H, m, H-10), 1.68 (3H, s, Me-19), 1.61 (3H, s, Me-12 or Me-20), 1.60 (3H, s, Me-20 or Me-12), 1.60 (1H, m, H-8), 1.55 (2H, m, H-6), 1.43 (1H, m, H-13), 1.40 (1H, m, H_a-15), 1.05 (1H, m, H_b-15), 0.94 (3H, d, J = 6.0, Me-14); ^{13}C nmr ($CDCl_3$) δ 177.5, 131.5, 124.7, 79.6, 71.9, 53.2, 47.3, 42.5, 39.4, 37.8, 36.2, 34.1, 32.7, 30.6, 25.7, 25.0, 24.1, 23.7, 18.9, 17.7.

CONVERSION OF **3** INTO **5**.—To (1.0 mg) **3** dissolved in Et_2O (0.1 ml), HCl 2 N (0.01 ml) and MeOH (0.1 ml) were added. The resulting solution was stirred at room temperature and monitored by tlc. After 24 h, more than 90% of

the starting product was transformed into **5**, which was identified by tlc and ^{1}H nmr.

CONVERSION OF **5** INTO **3**.—To a solution of **5** (1.0 mg) in MeOH (0.2 ml), 10% aqueous solution of KOH (0.2 ml) was added. The solution was stirred at room temperature for 1 h. At the end of this period, the MeOH was evaporated by N_2 flushing, and the resulting solution was neutralized with 2 N HCl; the white precipitate was extracted with Et_2O. Usual workup afforded 0.5 mg of aplykurodin B [**3**] and trace amounts of **5**.

BIOLOGICAL ASSAYS.—Brine shrimp assays (*Artemia salina*) (7,8) ichthyotoxicity tests (*Gambusia affinis*) (4,5), and antifeedant assays (*Carassius auratus*) (6) were conducted as described in the literature.

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