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Pregnane-10, 2-Carbolactones from a Hawaiian Marine Sponge in the Genus *Myrmekioderma*

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Abstract

Four new pregnanes, 3 β ,4 β -dihydroxy-17-methyl-17 α -pregna-5,13-diene-10,2-carbolactone (**1**), 6 β -chloro-3 β -hydroxy-17-methyl-17 α -pregna-4,13-diene-10,2-carbolactone (**2**), 3 β -hydroxy-6 β -methoxy-17-methyl-17 α -pregna-4,13-diene-10,2-carbolactone (**3**) and 3 β ,6 β -dihydroxy-17-methyl-17 α -pregna-4,13-diene-10,2-carbolactone (**4**), were isolated from an undescribed species of *Myrmekioderma* Ehlers (Order Axinellida Lévi, Family Heteroxyidae Dendy) along with the known pregnane carbolactone (**5**). The structures of the new compounds were determined by spectroscopic methods and comparison with previously described compounds. Compound **5** showed almost four-fold activation of pregnane X receptor (PXR) while **2** inhibited BACE1 with an IC₅₀ value of 82 μ M.

Abstract

Pregnane X receptors (PXR) belong to the super family of nuclear receptors (NR) encoded in humans by *NR112*. These transcription factors are widely expressed in normal tissues

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: Copies of the spectroscopic data for **1-6**

Notes

The authors declare no competing financial interest.

such as the liver, colon, kidney, brain, prostate and immune cells,¹ triggered by a diverse set of ligands and are involved in detoxification of endogenous and exogenous toxins via activation of key genes that increase drug metabolism and efflux. Activators of PXR have attracted considerable attention primarily because of their role in activating phase I and II drug-metabolizing enzymes, and the possible resulting drug-drug interactions. For example, PXR contributes to the regulation of genes encoding the cytochrome P450s, CYP3A4, CYP2B6, CYP2C9, CYP2C19, that metabolize more than 80% of prescribed drugs. A significant amount of emerging evidence also suggests that modulation of PXR and the functionally overlapping CAR (Constitutive androstane receptor) would not only be useful for preventing undesirable drug-drug interactions but may be beneficial in the treatment of cancer and inflammatory diseases.²

Marine invertebrates are a rich source of biologically active chemicals, including a large number of triterpenes and steroids. Despite the diversity of terpenoids known from these sources, few pregnanes derivatives have been reported.³⁻⁵ An investigation of a Hawaiian sponge identified as an undescribed species of *Myrmekioderma* Ehlers (Order Axinellida Lévi, Family Heteroxyidae Dendy) has led to the isolation of four rare pregnane-10,2-carbolactones.³ The extract also contained two known compounds, 3 β ,4 β -dihydroxypregna-5,17-diene-10,2-carbolactone **5** and the sesquiterpene (+)-curcudiol. The isolation and structure determination of these new compounds are described herein, along with biological screening of **1-5** against β -secretase (BACE1), and PXR activation by **5**.

Compound **1** was obtained as a white powder with a molecular formula of C₂₁H₂₈O₄ as determined by LC-MS. The IR spectrum showed strong absorbances for alcohol and ester functional groups at 3409 and 1764 cm⁻¹, respectively, which accounted for one of the implied eight elements of unsaturation. The ¹H and ¹³C NMR spectroscopic data (Table 1 and Table 2), together with analysis of the HSQC spectrum, revealed several notable features: a methyl singlet (δ_H 0.99), a methyl triplet (δ_H 0.78, H₃-21), three oxygenated methines (δ_H 4.67, H-2; δ_H 3.85, H-3; δ_H 4.38, H-4), one trisubstituted olefin, and a carbonyl (δ_C 178.4). A four-carbon fragment containing C-1 through C-4 was assembled beginning with the C-1 methylene (δ_H 2.72, dd, H-1 β and 1.64, d, H-1 α) using a network of COSY correlations between vicinal protons. Based on carbon and proton chemical shift analyses C-2, C-3, and C-4 within this fragment were all oxygenated. Three other fragments consisting of an ethyl group (C-20/C-21), a six-carbon chain of methylenes and methines terminating in the vinylic proton H-6 (δ_H 6.03), and a two carbon methylene chain (C-15/C-16) were constructed in a similar manner (Figure 1).

Analysis of HMBC correlations assembled **1** from the subunits and the remaining unassigned atoms. In particular, HMBC correlations to the tetrasubstituted olefin ^{13,14} from H-20 of the ethyl group, from H-12 and H-7 of the six-carbon chain, from H-15, and from the methyl singlet H-18 established the majority of the classic steroidal ring system. The trioxxygenated unit spanning C-1 to C-4 was appended to this larger fragment based on HMBC correlations from H-1 $\alpha\beta$ and H-4 to C-5 (δ_C 138.4), while the only carbonyl (C-19) was attached to the quaternary carbon C-10. This left only one issue unresolved with regards to the planar structure; which of the three oxygens in the A-ring was the acyloxy oxygen of

the ester. This issue was solved by the observation of HMBC correlations between H-1 α and H-2 to C-19.

A review of the literature revealed **1** was similar to 3 β ,4 β -dihydroxypregna-5,17-diene-10,2-carbolactone (**5**) previously isolated from a sponge identified as a species of *Petrosia* (*Strongylophora*) Dendy (Order Haplosclerida Topsent, Family Petrosiidae van Soest), collected around Kauai, and also isolated from our sample.³ The proton and carbon chemical shifts between the two compounds were in good agreement, supporting the proposed structure of **1**, with the anticipated minor differences in the spectra attributable to the different location of the methyl and alkene groups in their D-rings.

The mass spectrum of the analog **2** showed a 3:1 cluster of adduct ion peaks indicative of a chlorinated compound which defined a molecular formula of C₂₁H₂₇ClO₃. Comparison of the ¹H and ¹³C NMR spectra of **2** (Table 1 and Table 2) with those of **1** indicated that **2** contained the same pregnane core with slight chemical shift differences for resonances within the A and B rings. Analysis of the 2D NMR data indicated **2** contained a double bond between C-4 and C-5, and that a chlorine atom was attached to C-6, based on HMBC correlations and chemical shift considerations (δ_C 61.3 and δ_H 5.07), which defined the planar structure of **2**.

With the basic carbon skeleton elucidated in **1** and **2**, the structures of two other analogs were easily identified at this point. Compound **3** was readily determined to be the 6-methoxy derivative of **2** by analysis of its HRMS spectrum, which lacked the characteristic chlorine isotope pattern, but contained an additional CH₃O. Detailed NMR analysis supported this assignment with a characteristic methoxy singlet at 3.16ppm (Table 1 and Table 2). Similarly, **4** was identified as the hydroxy analog of **3**, by the loss of the methoxy signal in the ¹H and ¹³C NMR spectra.

Compounds **1-4** have the same relative configuration at their conserved centers as determined by coupling constant and ROESY analysis. Figure 2 shows the key ROESY correlations used in this determination. Specifically, the multiplicity of H-8 (dd, J = 11.4, 11.4 Hz) indicates it and H-9 are axial given the proposed structure, while sequential ROESY correlations between H-9/ H-12 α (δ_H 1.79) and H-12 β / H-18 established three of the stereocenters. ROESY correlations between H-12 α and H-1 $\alpha\beta$, and then H-1 α to H-2 and H-3 established the configurations of the two last conserved stereocenters C-2 and -3. The α -orientation of the proton on the unique stereocenters in **1-4**, i.e. C-4 in **1** and C-6 in **2-4**, was then elucidated on the basis of the multiplicity of the corresponding proton resonance.

New compounds **1-4** were accompanied by the known pregnane carbolactone **5** as previously mentioned. Interestingly, pregnane 10,2-carbolactones are rare; only three examples have been reported, all of which are from Hawaii.³ The three known compounds were originally isolated from a specimen identified as *Petrosia* (*Strongylophora*), collected from Puako on the Big Island, while our *Myrmekioderma* sp. was collected off the coast of Poipu, Kauai; essentially opposite ends of the Hawaiian island chain. These genera are unrelated taxonomically, the former in Order Haplosclerida, the latter in Order Axinellida,

and are morphologically distinct, so taken together this may suggest either an erroneous identification of the former specimen or the involvement of a bacterial or fungal producer.

Biosynthetically, **1** could be derived from **5** directly via a cationic rearrangement that results in a 1,2-methyl shift and deprotonation, as proposed in Scheme 1, to generate the modified D-ring. Compounds **2**, **3** and **4** would then be formed by a similar cationic process involving an allylic cation at C-4 through C-6 and quenching with MeOH, H₂O or a chloride ion. Given the use CHCl₃, MeOH and formic acid during column chromatography, we considered the possibility that **2-4** were artifacts. Treatment of **1** with aqueous MeOH with trace amounts of formic acid at 60 °C for 4 h did not yield **3** or **4** though, and a reexamination of our original LC-MS chromatograms of the extract shows weak ions corresponding to these compounds supporting the conclusion that **2**, **3**, and **4** are natural products.

Compound **1-5** were screened against BACE1, which has a central role in the etiology of Alzheimer's disease. Interestingly, while in general these compounds were inactive at the initial 30 µg/mL test concentration (ca. 80 µM), the chlorinated analog **2** showed weak activity with an IC₅₀ value of 82 µM. Compound **5**, the most abundant compound isolated, was further screened at several concentrations for activation of pregnane X receptors (PXR) which are involved in the regulation of several CYP450s (Table 3). At 40 µM, **5** induced a 3.7-fold increase in PXR indicating weak activation at this test concentration. Using the classification of Luo *et al.*, a potent activator, such as rifampin, shows 10-fold or greater activation at 50 µM.⁶ Solubility issues in DMSO prevented testing **5** up to this concentration though, and unfortunately there were insufficient quantities left at this point to test the effects of compounds **1-4**.

EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotations were measured on a Jasco-DIP-700 polarimeter at the sodium line (589 nm). UV spectra were obtained on a Hewlett-Packard 8453 spectrophotometer, and IR spectra were measured as a thin film on a CaF₂ disk using a Perkin-Elmer 1600 series FTIR. NMR spectra were acquired on a Varian Unity INOVA 500 MHz spectrometer operating at 500 (¹H) or 125 (¹³C) MHz using the residual solvent signals as an internal reference (CD₃OD δ_H 3.30ppm, δ_C 49.0 ppm). Samples were in 3 mm Shigemi tubes during NMR analyses. High-resolution mass spectrometric data were obtained on an Agilent LC-MSTOF with ES ionization in the positive mode. Gradient separations used a Shimadzu system consisting of LC-20AT solvent delivery modules, a SPD-M20A VP diode photodiode array detector, and a SCL-20A VP system controller. TLC analyses were performed on Si₆₀F₂₅₄ plates and visualized under UV or by heating after spraying with a 1% anisaldehyde solution in acetic acid/H₂SO₄ (50:1). General reagents were purchased from Sigma-Aldrich or VWR.

Collection and Identification

The sponge was collected from the Sheraton dive site on Kauai, Hawai'i (21° 51.920N 159° 27.097W; 60 fsw), and formed a thick undulating encrustation with a fine polygonal

structure on the otherwise smooth surface. The color in life was white, with orange-brown patches, and the texture crumbly. The skeleton is composed of a tangential/paratangential ectosomal layer of relatively large (450–550 μm) oxeas/strongyles and small (320–450 μm), lightly acanthose, oxeas/strongyles, below which are ascending tracts of oxeas forming large broad irregular meshes. These are interspersed with raphides in two size categories: 1) 75–100 μm ; 2) 20–50 μm . The sponge is an undescribed species of *Myrmekioderma* (Order Axinellida, Family Heteroxyidae) that differs from the common Indo-Pacific species *M. granulatum* (Esper, 1794) in the possession of smaller oxeas (*M. granulatum*: over 1000 μm), a second category of small raphides in trichodragmata (*M. granulatum* has a single category about 140–160 μm) and in lacking the thick mucus typical of *M. granulatum* and other species. A voucher specimen has been deposited at the Natural Museum, London (NHMUK2012.3.27.5).

Extraction and Isolation

The freeze-dried sponge (10g) was chopped into small pieces and then exhaustively extracted with MeOH (3 $\times$ 0.5 L) at room temperature to afford 2.68 g of lipophilic extract. The extract was subjected to Si flash column chromatography eluting with a gradient of EtOAc in hexane to afford 20 fractions. The active residue from fraction 16 (20 mg) was further purified by RP-HPLC (Luna C₈, 250 $\times$ 10 mm, a linear gradient of 40–100% MeOH in H₂O over 20 min, then 100% MeOH for an additional 10 min, flow rate 3 mL/min, PDA and ELSD detection) to afford compound **1** (t_R 17.7 min, 1.0 mg, 0.037% yield, 100% purity at 203 nm), compound **2** (t_R 19.5 min, 0.6 mg, 0.022% yield, 94% purity at 203 nm), compound **3** (t_R 19.0 min, 0.5 mg, 0.019% yield, 98% purity at 203 nm) and compound **4** (t_R 15.8 min, 0.9 mg, 0.034% yield, 96% purity at 203 nm). Fraction 12 (10 mg) was separated by RP-HPLC (Luna C₈, 250 $\times$ 10 mm, a linear gradient of 10–100% MeCN in H₂O over 40 min, flow rate 3 mL/min, PDA and ELSD detection) to yield compound **5** (t_R 15.3 min, 1.0 mg, 0.037%, 98% purity at 203 nm) and (+)-curcudiol (t_R 17.1 min, 0.5 mg, 0.019%, 99% purity at 203 nm).

3 β ,4 β -Dihydroxy-17-methyl-17 α -pregna-5,13-diene-10,2-carbolactone (1)—white powder; $[\alpha]_D^{22}$ -44 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (0.87), 236 (0.27) nm; IR ν_{max} (CaF₂) 3409, 1764 cm^{-1} ; ¹H and ¹³C NMR data, Table 1 and 2; HRESI-TOFMS m/z 367.1877 [M + Na]⁺ (calcd for C₂₁H₂₈O₄Na, 367.1883; error -2.3 ppm).

6 β -Chloro-3 β -hydroxy-17-methyl-17 α -pregna-4,13-diene-10,2-carbolactone (2)—white powder; $[\alpha]_D^{22}$ -8 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (0.86), 237 (0.44) nm; IR ν_{max} (CaF₂) 3400, 1763 cm^{-1} ; ¹H and ¹³C NMR data, Table 1 and 2; HRESI-TOFMS m/z 363.1723 [M + H]⁺ (calcd for C₂₁H₂₈O₃³⁵Cl, 363.1727; error -1.1 ppm).

3 β -Hydroxy-6 β -methoxy-17-methyl-17 α -pregna-4,13-diene-10,2-carbolactone (3)—white powder; $[\alpha]_D^{22}$ -20 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (0.5), 237 (0.13) nm; IR ν_{max} (CaF₂) 3391, 1740 cm^{-1} ; ¹H and ¹³C NMR data, Table 1 and 2; HRESI-TOFMS m/z 381.2043 [M + Na]⁺ (calcd for C₂₂H₃₀O₄Na, 381.2041; error 0.3 ppm).

3 β ,6 β -Dihydroxy-17-methyl-17 α -pregna-4,13-diene-10,2-carbolactone(4)—white powder; $[\alpha]_D^{22}$ -40 (*c* 0.2, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 206 (0.22), 237 (0.05) nm; IR (CaF₂) $\nu_{\max}$ 3391, 1744 cm⁻¹; ¹H and ¹³C NMR data, Table 1 and Table 2; HRESI-TOFMS *m/z* 367.1875 [M + Na]⁺ (calcd for C₂₁H₂₈O₄Na, 367.1885; error -2.8 ppm).

3 β ,4 β -Dihydroxypregna-5,17-diene-10,2-carbolactone (5)—white powder; $[\alpha]_D^{22}$ -75 (*c* 0.2, CHCl₃); lit. $[\alpha]_D^{22}$ -82 (*c* 1.2, CHCl₃); IR $\nu_{\max}$ (CaF₂) 3300 and 1775 cm⁻¹; NMR data consistent with literature values.³ HRESI-TOFMS *m/z* 367.1871 [M + Na]⁺ (calcd for C₂₁H₂₈O₄Na, 367.1885; error -3.9 ppm).

(+)-Curcudiol—colorless oil; $[\alpha]_D^{22}$ +4.2 (*c* 0.2, CHCl₃); lit. $[\alpha]_D^{22}$ +9.2 (*c* 10.8, CHCl₃); NMR data consistent with literature values.⁷ HRESI-TOFMS *m/z* 259.1658 [M + Na]⁺ (calcd for C₁₅H₂₄O₂Na, 259.1669; error -4.4 ppm).

BACE1 assay

β -Secretase mediated cleavage of amyloid precursor protein was determined as described by Naqvi⁸ using an enzyme fragment complementation assay. Compounds were dissolved in DMSO and tested in triplicate (n=3) in parallel to the controls (DMSO and β -secretase inhibitor IV from Calbiochem, Purity >95% by HPLC), and analyzed as previous described (same instrument and statistical analysis).⁹ The experimentally determined IC₅₀ value (9 $\pm$ 2 nM) for the positive control was consistent with reported data.¹⁰

PXR Activation

Tests were performed at Puracyp, Inc. The human assay utilizes DPX2 cells that harbor the human PXR gene (NR1I2) and a luciferase reporter gene linked to two promoters identified in the human CYP3A4 gene. Stable cells transfected with the nuclear receptor and the corresponding response elements were seeded in a 96-well plate. Twenty-four hours after seeding, the cells were treated with six concentrations of compound (in duplicate wells) and cells then returned to the incubator for an additional 24 h. At the end of this incubation period, the number of viable cells/well was determined using Promega's Cell Titer Fluor cytotoxicity assay. Following this assay, Promega's ONE-Glo was added to the same wells and reporter gene activity assessed, and by comparing the results to vehicle-treated cells (DMSO). Positive controls consist of cells treated with seven different concentrations of rifampicin.

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Figure 1.
Selected COSY (bold solid bars) and HMBC (H→C) correlations of **1**.

Figure 2.
Key ROESY correlations used to establish the relative configuration of **1**. Lactone removed for clarity. Remaining centers established by $^3J_{\text{H,H}}$ analysis

Scheme 1.

Proposed biosynthesis of **1** from **5** via 1,2-methyl shifts.

Table 1¹HNMR Spectroscopic Data (500 MHz, MeOH-*d*₄) for **1-4**

H#	1	2	3	4
	δ_{H} (J in Hz)	δ_{H} (J in Hz)	δ_{H} (J in Hz)	δ_{H} (J in Hz)
1 α	1.64, d (12.0)	1.91, d (11.8)	1.92, d (11.7)	1.88, d (11.7)
1 β	2.72, dd (12.0, 6.6)	2.77, dd (11.8, 6.7)	2.76, dd (11.7, 6.7)	2.75, dd (11.7, 6.7)
2	4.67, dt (6.6, 1.4)	4.75, ddd (6.7, 2.7, 2.7)	4.76, ddd (6.7, 2.6, 2.6)	4.74, ddd (6.7, 2.7, 2.7)
3	3.85, dd (5.7, 1.4)	4.40, dd (2.7, 2.7)	4.42, dd (2.6, 2.6)	4.39, dd (2.7, 2.7)
4	4.38, d (5.7)	5.90, dd (2.7, 2.7)	5.69, dd (2.6, 2.6)	5.72, dd (2.7, 2.7)
6	6.03, dd (6.0, 1.5)	5.07, dd (2.9, 2.9)	3.77, dd (2.8, 2.8)	4.36, dd (2.6, 2.6)
7 α	1.69, ddd (17.7, 11.4, 1.5)	1.57, ddd (14.5, 11.0, 2.9)	1.26, ddd (13.5, 11.0, 2.8)	1.23, ddd (13.8, 11.7, 2.6)
7 β	2.37, ddd (17.7, 6.0, 4.6)	2.32, ddd (14.5, 2.9, 2.9)	2.24, ddd (13.5, 2.8, 2.8)	2.15, ddd (13.8, 2.6, 2.6)
8	2.52, dd (11.4, 11.4)	3.12, dd (11.0, 11.0)	2.89, dd (11.0, 11.0)	3.03, dd (11.7, 11.7)
9	1.40, ddd (11.4, 11.4, 2.4)	1.38, ddd (11.0, 11.0, 2.0)	1.35, ddd (11.0, 11.0, 2.0)	1.34, ddd (11.7, 11.7, 2.0)
11 α	1.80, m	1.77, m	1.79, m	1.78, m
11 β	1.66, m	1.70, m	1.64, m	1.66, m
12 α	1.79, m	1.82, m	1.80, m	1.81, m
12 β	2.02, m	2.05, m	2.02, m	2.03, m
15 α	2.26, m	2.25, m	2.25, m	2.26, m
15 β	2.14, m	2.15, m	2.15, m	2.17, m
16 α	1.81, m	1.87, m	1.78, m	1.78, m
16 β	1.57, m	1.56, m	1.55, m	1.56, m
18	0.99, s	0.99, s	0.97, s	0.98, s
20	1.32, m	1.32, m	1.31, m	1.31, m
21	0.78, t (7.4)	0.77, t (7.5)	0.77, t (7.4)	0.77, t (7.4)
OCH ₃			3.16, s	

Table 2¹³C NMR Spectroscopic Data (125 MHz, MeOH-*d*₄) for **1-3**

Position	1	2	3 ^a
	δ _C , type	δ _C , type	δ _C , type
1	39.3, CH ₂	δ _C , type 40.7, CH ₂	40.8, CH ₂
2	81.4, CH	78.9, CH	78.9, CH
3	70.7, CH	68.7, CH	68.5, CH
4	72.4, CH	133.5, CH	132.8, CH
5	138.4, C	142.0, C	138.2, C
6	131.8, CH	61.3, CH	80.0, CH
7	32.0, CH ₂	39.0, CH ₂	36.2, CH ₂
8	35.3, CH	32.4, CH	32.3, CH
9	42.7, CH	43.0, CH	45.5, CH
10	48.1, C	47.3, C	46.5, C
11	23.4, CH ₂	23.8, CH ₂	23.8, CH ₂
12	23.1, CH ₂	23.2, CH ₂	23.2, CH ₂
13	141.7, C	142.3, C	141.7, C
14	137.0, C	136.5, C	136.5, C
15	31.4, CH ₂	31.4, CH ₂	31.4, CH ₂
16	36.4, CH ₂	36.3, CH ₂	36.5, CH ₂
17	50.5, C	50.6, C	49.9, C
18	25.8, CH ₃	25.8, CH ₃	26.0, CH ₃
19	178.4, C	178.2, C	178.0, C
20	33.0, CH ₂	32.9, CH ₂	31.0, CH ₂
21	9.4, CH ₃	9.4, CH ₃	9.5, CH ₃
OCH ₃			55.5, CH ₃

^aDetermined from HMBC experiment.

Table 3Activation of PXR by **5**

Conc. μM	Fold Activation	% of Rifampicin ^b Activation
0.1	1.3	3
0.4	1.4	4
1.0	1.2	2
4.0	1.2	2
10.0	1.6	6
40.0	3.7	28

^b
10 μM