Aplysiasecosterol?A: A 9,11-Secosteroid with an Unprecedented Tricyclic γ-Diketone Structure from the Sea Hare Aplysia kurodai

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Aplysiasecosterol A: A 9,11-Secosteroid with an Unprecedented Tricyclic γ-Diketone Structure from the Sea Hare *Aplysia kurodai* **

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Abstract: A new 9,11-secosteroid having an unprecedented tricyclic γ-diketone structure, aplysiasecosterol A (**1**), was isolated from the sea hare *Aplysia kurodai*. The structure was determined by 1D and 2D NMR spectroscopic analysis, molecular modeling studies, a comparison of experimental and calculated ECD spectra, and a modified Mosher's method. Aplysiasecosterol A (**1**) exhibited cytotoxicity against human myelocytic leukemia HL-60 cells. A biosynthetic pathway for **1** from a cholesterol was proposed, which includes twice α-ketol rearrangement and an intramolecular acetalization.

Sea hares (family Aplysiidae) belong to the opisthobranch group of mollusks (clade Aplysiomorpha).[1] They are shell-less and benthic marine invertebrates, and have been postulated to contain chemical defense substances. Among them, the genera *Aplysia* and *Dolabella* are known to be rich sources of bioactive molecules.[2] For example, dolastatin 10 is an antineoplastic peptide that was obtained from the Indian Ocean sea hare *Dolabella* sp.[3] In 2011, an mAb-targeted dolastatin 10 conjugate was approved for the treatment of Hodgkin's lymphoma. Aplyronine A, a highly potent antitumor and actin-depolymerizing macrolide, was isolated from the Japanese sea hare *Aplysia kurodai*.[4] Recently, aplyronine A was shown to induce protein–protein interaction between actin and tubulin and to prevent spindle formation and mitosis.[5] In addition, various secondary metabolites have been isolated from *A. kurodai*, including the shikimate derivatives pericosines,[6] the sterol derivatives aplykurodins,[7] and the alkaloids gliocladins [8] and aplaminal.[9] These molecules are expected to give new insights for the discovery and development of a new class of pharmacological tools and therapeutic agents.

In our continuing search for new bioactive compounds from *A. kurodai*, aplysiasecosterol A (**1**), a 9,11-secosteroid having an unprecedented tricyclic γ-diketone structure, was isolated. We report here the isolation, structure determination, and bioactivity of **1**.

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The sea hare *A. kurodai* (54.8 kg, wet wt.) was extracted with aqueous ethanol. The concentrated extract was partitioned between ethyl acetate and water. The ethyl acetate layer was further partitioned with *n*-hexane, dichloromethane, and 60% aqueous methanol. Apart from the highly cytotoxic fractions that contain aplyronines, we investigated the other constituents in the dichloromethane layer. Repeated SiO_2, Al_2O_3, and ODS column chromatography and final reverse-phase HPLC purification afforded aplysiasecosterol A (**1**) as a colorless oil {6.7 mg, 1.2×10^{-7} %, $[\alpha]^{24}_D$ +19.3 (*c* 0.228, MeOH)}. Aplysiasecosterol A (**1**) did not show significant cytotoxicity against the human cervical carcinoma cell line HeLa S3 at 200 μM, while it exhibited moderate cytotoxicity against the human myelomonocytic leukemia cell line HL-60 (IC_{50} = 16 μM).

The molecular formula of **1** was established to be $C_{27}H_{44}O_7$ by HR-ESIMS ($[M+Na]^+$, *m/z* 503.2979, Δ –0.6 mmu). The planar structure of **1** was determined by 1D and 2D NMR analysis (Figure 1). The ^1H, ^{13}C NMR, DEPT135 and HSQC spectra in $CDCl_3$ showed that **1** had four singlet methyl groups (δ_H 0.89, 1.11, 1.21, 1.15), one doublet methyl group (δ_H 0.92), two carbonyl carbons (δ_C 208.28, 216.50), and one acetal carbon (δ_C 101.31). The IR ($CHCl_3$) spectrum of **1** showed absorption bands for hydroxyl groups (3568 cm^{-1}) and two carbonyl groups (1736, 1708 cm^{-1}).

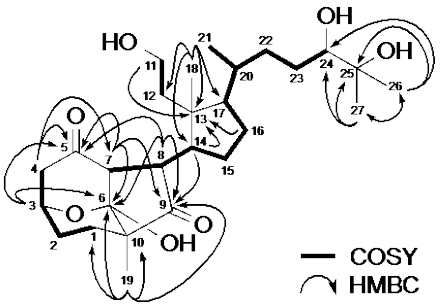


Figure 1. Planar structure of aplysiasecosterol A (**1**) determined by 2D-NMR analysis (bold line, COSY; arrows, selected HMBC correlations).

A detailed analysis of the COSY spectrum of **1** allowed us to construct three partial structures: C-1–C-4, C-7–C-24, and C-11–C-12 (Figure 1, Table S1). HMBC correlations between Me-18/C-12, C-13, C-14 and C17 revealed that the C-7–C-24 unit was linked to the C-11–C-12 unit via the sp^3 quaternary carbon C-13. Similarly, HMBC correlations between Me-19/C-1, C-6, C-9, and C-10 indicated that C-1, C-6, C-9, and C-19 were each connected to the sp^3 quaternary carbon C-10. The C-8–C-9 connectivity was established based on HMBC correlations between H-7, H-8, and H-14/C-9. Further HMBC correlations between H-7 and H-8/C-5 and C-6 indicated that the carbonyl

carbon C-5 and the acetal carbon C-6 were connected to the methine carbon C-7. Thus, the results showed that **1** possesses a cyclopentanone moiety (C-9, 1736 cm^{-1}) with an acetal carbon. Moreover, the presence of both a tetrahydropyran ring and a 4-oxacyclohexanone moiety (C-5, 1708 cm^{-1}) in **1** was established by HMBC correlations between H-3/C-5 and C-6 and H-4/C-5 and C-7. Furthermore, the connectivity of two singlet methyl groups (C-26, 27) and an 1,2-diol moiety was clarified based on HMBC correlations between Me-26/C-24, C-25, and C-27, and Me-27/C-24, C-25, and C-26. Based on the molecular formula and the degree of unsaturation, **1** was shown to contain four hydroxyl groups. Therefore, the planar structure of aplysiasecosterol A (**1**) was determined to be as shown in Figure 1.

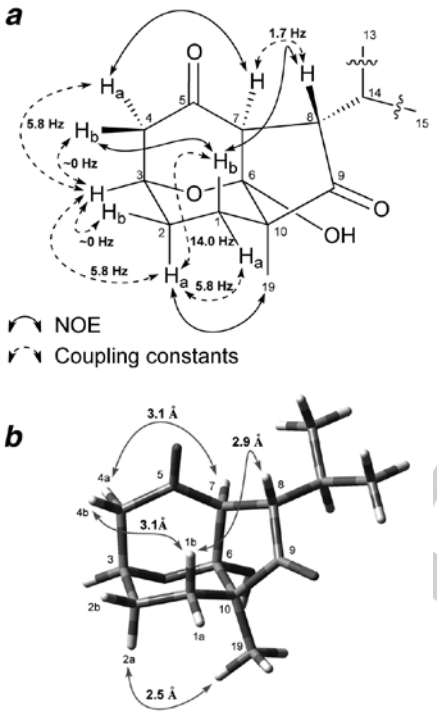


Figure 2. (a) Relative stereochemistry of the tricyclic γ-diketone structure of **1** determined by 1D and 2D-NMR analysis (solid arrows, selected NOE correlations; dashed arrows, coupling constants). (b) Optimized structure of the tricyclic model compound **2** at the B3LYP/6-31G+ level of theory in the gas phase. The substituent on C-8 in **2** was replaced with an isopropyl group. Solid arrows are the calculated distances between two selected protons.

Next, the relative stereochemistry of the tricyclic γ-diketone structure of **1** was established. The large magnitude of $J_{1b,2a}$ = 14.0 Hz and the relatively small magnitudes of $J_{1a,2a}$ = 5.8 Hz, $J_{2a,3}$ = 5.8 Hz, and $J_{2b,3}$ = ca. 0 Hz suggested that H-1b and H-2a were oriented in an *anti* arrangement in the tetrahydropyran ring (Figure 2a). Key NOEs were observed for H-1b/H-4b and H-1b/H-8, which indicated that these three protons faced each other on the concave face of the rigid tricyclic structure. Similarly, NOE correlations for H-4a/H-7 and H-2a/Me-19 suggested that all of the protons H-2a, H-4a, H-7, and Me-19 were located in the convex face. Thus, H-1b, H-2a and Me-19 were thought to be oriented in an axial position with respect to the tetrahydropyran ring with a chair conformation.

A molecular modeling study using a Merck molecular force field 94x (MMFF94x) showed that the tricyclic γ-diketone model compound **2**, in which the substituent on C-8 was replaced with an isopropyl group, had only one conformer within 7 kcal/mol of the lowest energy conformation. Geometry optimization with the density functional theory (DFT) method for **2** was conducted using the B3LYP/6-31G+ level of theory (Figure 2b). The calculated distances of H-1b/H-4b, H-1b/H-8, H-2a/Me-19, and H-4a/H-7 for **2** were 3.1, 2.9, 2.5, and 3.1 Å, respectively. Thus, this model satisfied all of the key NOEs observed for **1**. The dihedral angles for H-2b/H-3 and H-7/H-8 in **2** were –67.8° and –105.8°, respectively. Based on the Karplus equation,[10] the coupling constants of H-2b/H-3 and H-7/H-8 in **2** were estimated to be 0–2 Hz, which coincided with those observed in **1** (~0 and 1.7 Hz, respectively). For the above reason, the relative stereochemistry of the tricyclic γ-diketone portion of **1** was determined to be as shown for the model compound **2** in Figure 2.

The relative stereochemistry around the cyclopentane ring in **1** was also determined by NOE experiments and coupling constant analysis (Figure 3). NOEs were observed for H-11a/H-14, H-14/H-17, H-17/Me-21, H-12a/Me-21, H-16a/H-22a, and Me-18/H-20. These data strongly indicated that H-12, H-14, H-17, and Me-21 are oriented in the one face, and Me-18 and H-20 are oriented in the other face of the cyclopentane ring. As a result, the relative stereochemistry of the cyclopentane ring part in **1** was identical to those of typical 9,11-secosteroids.[11,12]

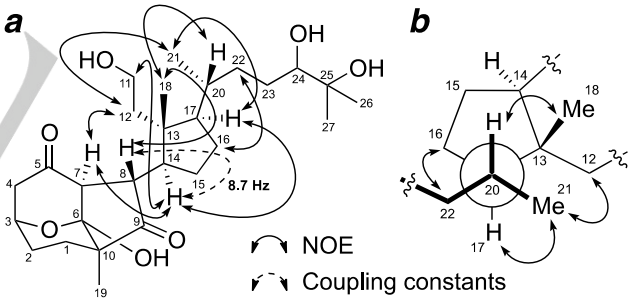


Figure 3. (a) Relative stereochemistry around the cyclopentane ring of **1** determined by 1D and 2D-NMR analysis (solid arrows, selected NOE correlations; dashed arrows, coupling constants). (b) A Newman projection with a view along the C20-C17-bond.

The relative stereochemistry between the tricyclic structure and cyclopentane ring part in **1** was also determined based on a detailed NMR analysis. While the C-8–C-14 sp^3–sp^3 bond is able to freely rotate, the large magnitude of $J_{8,14}$ = 8.7 Hz indicated that H-8 is positioned anti-periplanar to H-14. NOEs were observed for H-7/H-14, H-7/H-12, and H-8/Me-18. These results suggested that H-7, H-12, and H-14 are oriented in the α-face, and H-8 and Me-18 are oriented in β-face of the cyclopentane ring. Thus, the relative stereochemistry of **1** except for the oxymethine carbon C-24 was established to be as shown in Figure 3.

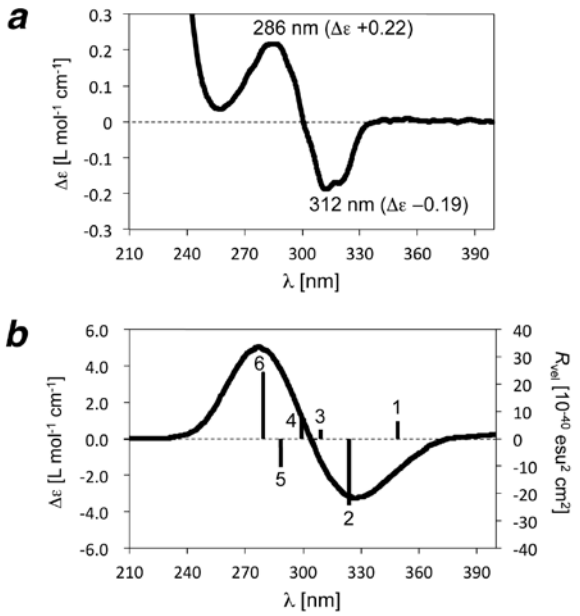


Figure 4. (a) Experimental ECD spectrum of **1** measured in MeOH at 0.73 mM. (b) Calculated ECD data for the model compound **2** at the PBEPBE/6-311G++(d,p) level of theory in implicit solvent model (CPCM, MeOH) (solid line, left scale). Six first excited states for **2** were shown in solid bars (right scale).

To determine the absolute stereochemistry of **1**, the experimental electronic circular dichroism (ECD) spectrum of **1** was compared with the calculated ECD data for the model compound **2** (Figure 4).[13] The ECD spectrum of **1** showed a negative first Cotton effect at 312 nm (Δε –0.19) and a positive second Cotton effect at 286 nm (Δε +0.22). The calculated ECD spectrum for **2** at the PBEPBE/6-311G++(d,p) level of theory in implicit solvation model (CPCM[14], MeOH) also had a negative ECD peak at 327 nm (Δε –3.3) and a positive ECD peak at 277 nm (Δε +5.0), which reproduced the signs of the experimental Cotton effects. As inferred from the molecular orbital analysis, the negative ECD band (band 2, R_{vel} –24.3) was mainly ascribed to the n → π* transition of the C-9 ketone (HOMO → LUMO+1) (Figure 5). Meanwhile, slightly positive ECD bands at 309 nm (band 3, R_{vel} +3.3) and 299 nm (band 4, R_{vel} +7.4) were mainly ascribed to the n → π* transition of the C-5 ketone (HOMO–1 → LUMO and HOMO–1 → LUMO+1, respectively). In addition, the positive ECD band (band 6, R_{vel} +23.8) corresponded to the transition from HOMO–2 to LUMO+1. These results suggested that the absolute configuration of the tricyclic γ-diketone part of **1** was identical to that of the model compound **2**.

Finally, compound **1** was converted to an 11,24-bis-MTPA ester (Figure S2). Through the use of a modified Mosher's method,[15] the stereochemistry of C-24 was determined to be *R*.

For the above results, the absolute stereochemistry of **1** was completely established.

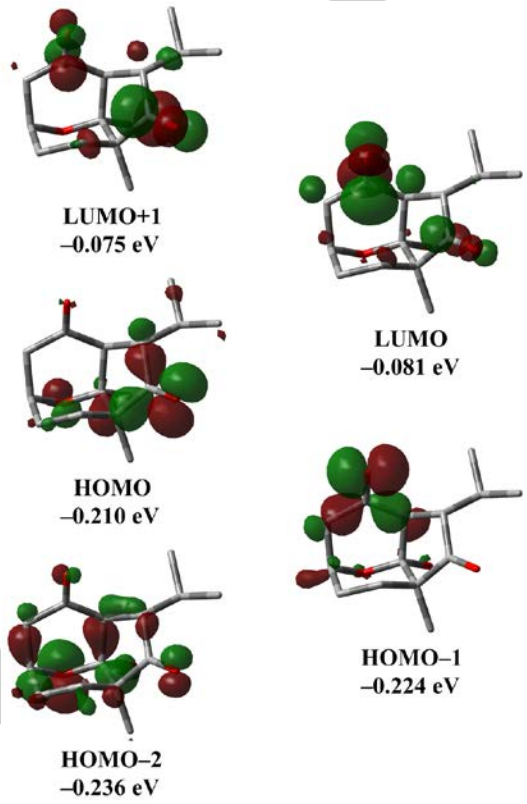
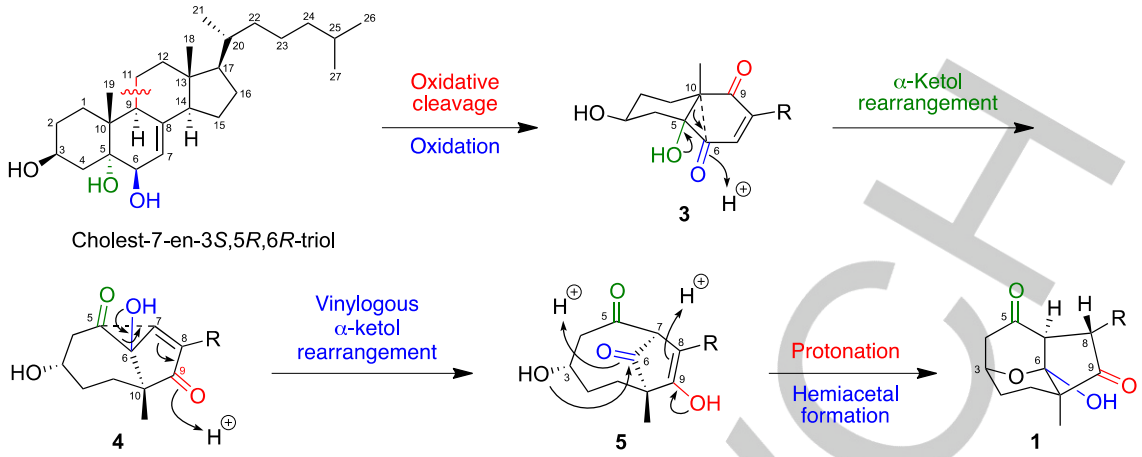


Figure 5. Molecular orbitals involved in the n → π* transition of the C-5 and C-9 ketones in the model compound **2**.

A biosynthetic pathway for the tricyclic γ-diketone structure of **1** was proposed, as shown in Scheme 1. Due to the structural similarity of the cyclopentane ring and the side-chain part of **1** with those of known 9,11-secosteroids, cholest-7-en-3*S*,5*R*,6*R*-triol [16] was assumed to be a biosynthetic precursor of **1**. Oxidative cleavage of the C-9–C-11 bond and oxidation of the C-6 hydroxyl group would give 1,4-diketone **3**. The α-ketol rearrangement [17] in **3** would lead to the formation of the C6–C10 bond to give α,β-unsaturated ketone **4**. The vinylogous α-ketol rearrangement in **4** might form the C-5–C-7 bond, and subsequent enolization at the C-9 ketone would afford enol **5**. Finally, protonation at C-8 and intramolecular acetalization of **5** would afford the tricyclic γ-diketone structure of **1**. The stereochemistry of the methine carbon at C-8 in general steroids is opposite to that in **1**. However, protonation at C-8 in **5** might occur to give priority to the thermodynamically stable *exo*-substituent 8*S* isomer.



Scheme 1. Proposed biosynthetic pathway for the tricyclic γ-diketone structure of **1**.

In summary, the structure and bioactivity of aplysiasecosterol A (**1**), a 9,11-secosteroid with a novel tricyclic γ-diketone structure, was established. Structurally and functionally diverse secosteroids have been discovered from both terrestrial and marine origin, which include 5,6-. 8,9-, 8,14-, 9,10-, 9,11-, and 13,17-secosteroids.[12] To the best of our knowledge, however, there are no examples that have similar tricyclic ring systems like **1**. Further biological and biosynthetic studies on aplysiasecosterol A (**1**) are in progress.

Keywords: marine natural products • isolation and structure determination • electronic circular dichroism (ECD) • α-ketol rearrangement • biosynthesis

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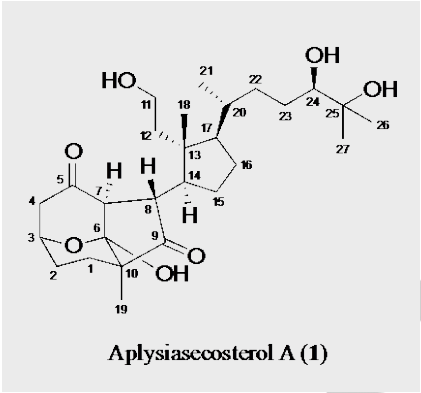
COMMUNICATION

A new cytotoxic 9,11-secosteroid having an unprecedented tricyclic γ-diketone structure, aplysiasecosterol A (**1**), was isolated from the sea hare *Aplysia kurodai*. A biosynthetic pathway for **1** from a cholesterol was proposed, which includes twice α-ketol rearrangement and an intramolecular acetalization.



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