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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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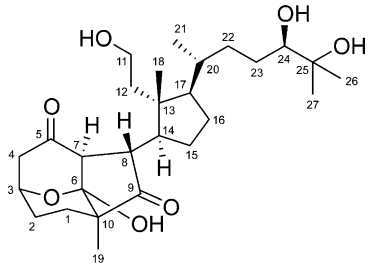
Abstract: *A new 9,11-secosteroid having an unprecedented tricyclic γ-diketone structure, aplysiasecosterol A (**1**), was isolated from the sea hare Aplysia kurodai. The structure was determined by one- and two-dimensional NMR spectroscopic analysis, molecular modeling studies, a comparison of experimental 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 known cholesterol was proposed and includes twice α-ketol rearrangements and an intramolecular acetalization.*



Aplysiasecosterol A (**1**)

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 which 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 interactions 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 herein the isolation, structure determination, and bioactivity of **1**.

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 which 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}%, $[\alpha]_D^{24} = +19.3$ (*c* 0.228, MeOH)}. Aplysiasecosterol A (**1**) did not show significant cytotoxicity against the human cervical carcinoma cell line HeLa S3 at 200 μM, but 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 one- and two-dimensional NMR analysis (Figure 1). The ^1H, ^{13}C NMR, DEPT135, and HSQC spectra in $CDCl_3$ showed that **1** had the following signals: four singlets representing methyl groups ($\delta_H = 0.89$, 1.11, 1.21, 1.15 ppm), one doublet

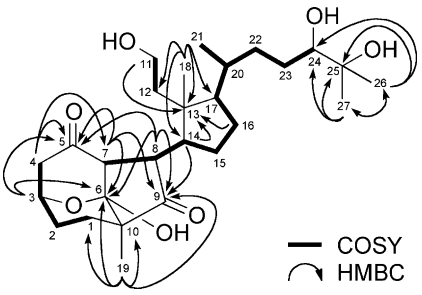


Figure 1. Planar structure of aplysiasecosterol A (**1**) determined by two-dimensional NMR analysis (bold line, COSY; arrows, selected HMBC correlations).

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representing a methyl group ($\delta_H = 0.92$ ppm), two carbonyl carbon atoms ($\delta_C = 208.28$, 216.50 ppm), and one acetal carbon atom ($\delta_C = 101.31$ ppm). The IR ($CHCl_3$) spectrum of **1** showed absorption bands for hydroxy groups (3568 cm^{-1}) and two carbonyl groups (1736, 1708 cm^{-1}).

A detailed analysis of the COSY spectrum of **1** allowed us to construct three partial structures: C1–C4, C7–C24, and C11–C12 (Figure 1, Table S1). HMBC correlations between Me-18/C12, C13, C14, and C17 revealed that the C7–C24 unit was linked to the C11–C12 unit via the sp^3 quaternary carbon C13. Similarly, HMBC correlations between Me-19/C1, C6, C9, and C10 indicated that C1, C6, C9, and C19 were each connected to the sp^3 quaternary carbon C10. The C8–C9 connectivity was established based on HMBC correlations between H7, H8, and H14/C9. Further HMBC correlations between H7 and H8/C5 and C6 indicated that the carbonyl carbon C5 and the acetal carbon C6 were connected to the methine carbon C7. Thus, the results showed that **1** possesses a cyclopentanone moiety (C9, 1736 cm^{-1}) with an acetal carbon atom. Moreover, the presence of both a tetrahydropyran ring and a 4-oxacyclohexanone moiety (C5, 1708 cm^{-1}) in **1** was established by HMBC correlations between H3/C5 and C6 and H4/C5 and C7. Furthermore, the connectivity between two single methyl groups (C26, 27) and an 1,2-diol moiety was clarified based on HMBC correlations between Me-26/C24, C25, and C27, and Me-27/C24, C25, and C26. Based on the molecular formula and the degree of unsaturation, **1** was shown to contain four hydroxy groups. Therefore, the planar structure of **1** was determined to be as shown in Figure 1.

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 $J_{2b,3} = 0$ Hz suggested that H1b and H2a were oriented in an *anti*-arrangement in the tetrahydropyran ring (Figure 2a). Key NOEs were observed for H1b/H4b and H1b/H8, and indicated that these three protons faced each other on the concave face of the rigid tricyclic structure. Similarly, NOE correlations for H4a/H7 and H2a/Me-19 suggested that all of the protons H2a, H4a, H7, and Me-19 were located in the convex face. Thus, H1b, H2a, and Me-19 were thought to be oriented in an axial position with respect to the tetrahydropyran ring in 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 C8 was replaced with an isopropyl group, had only one conformer within 7 kcal mol^{-1} 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 H1b/H4b, H1b/H8, H2a/Me-19, and H4a/H7 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 H2b/H3 and H7/H8 in **2** were −67.8° and −105.8°, respectively. Based on the Karplus equation,[10] the coupling constants of H2b/H3 and H7/H8 in **2** were estimated to be 0–2 Hz, which coincided with those observed in **1** (ca. 0 and 1.7 Hz, respectively). For

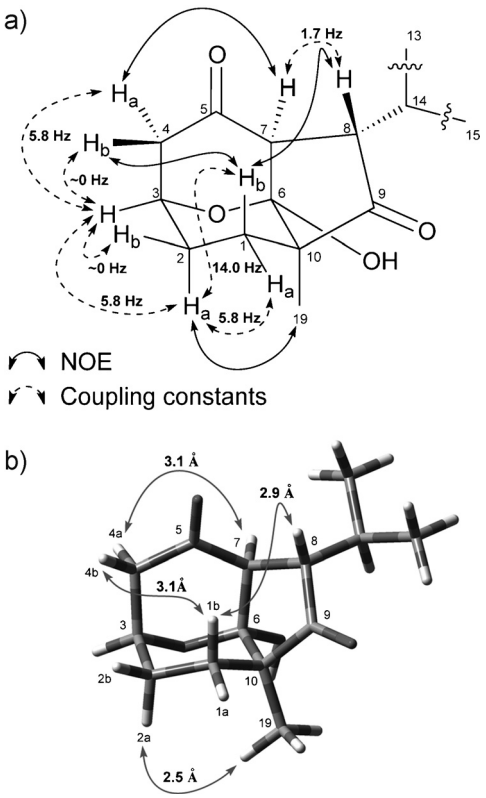


Figure 2. a) Relative stereochemistry of the tricyclic γ-diketone structure of **1** determined by one- and two-dimensional NMR analysis (solid arrows, selected NOE correlations; dashed arrows, coupling constants). b) Optimized structure of the tricyclic model compound **2** at the B3LYP/6-31G+ level of theory in the gas phase. The substituent on C8 in **2** was replaced with an isopropyl group. Solid arrows are the calculated distances between two selected protons.

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 H11a/H14, H14/H17, H17/Me-21, H12a/Me-21, H16a/H22a, and Me-18/H20. These data strongly indicated that H12, H14, H17, and Me-21 are oriented in the one face, and Me-18 and H20 are oriented in the other face of the

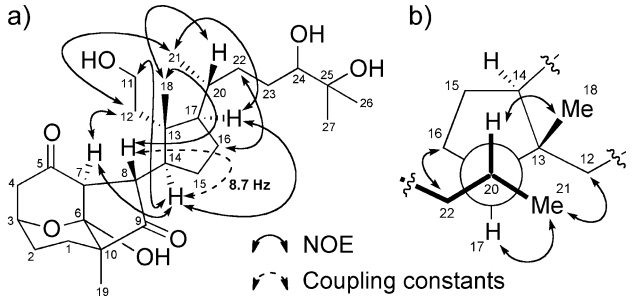


Figure 3. a) Relative stereochemistry around the cyclopentane ring of **1** determined by one- and two-dimensional NMR analysis (solid arrows, selected NOE correlations; dashed arrows, coupling constants). b) A Newman projection with a view along the C20–C17 bond.

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]

The relative stereochemistry between the tricyclic structure and cyclopentane ring part in **1** was also determined based on a detailed NMR analysis. While the C8(sp^3)–C14(sp^3) bond is able to freely rotate, the large magnitude of $J_{8,14} = 8.7$ Hz indicated that H8 is positioned antiperiplanar to H14. NOEs were observed for H7/H14, H7/H12, and H8/Me-18. These results suggested that H7, H12, and H14 are oriented in the α-face, and H8 and Me-18 are oriented on β-face of the cyclopentane ring. Thus, the relative stereochemistry of **1** except for the oxymethine carbon C24 was established to be as shown in Figure 3.

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

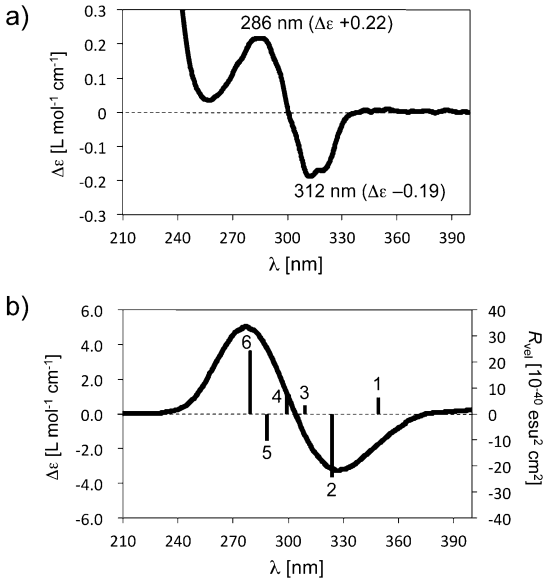


Figure 4. a) Experimental ECD spectrum of **1** measured in MeOH at 0.73 mM. b) Calculated ECD data for the model compound **2** at the PBEPBE/6-311G++(d,p) level of theory in implicit solvent model (CPCM, MeOH) (solid line, left scale). Six first excited states for **2** were shown in solid bars (right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

1 showed a negative first Cotton effect at $\lambda = 312$ nm ($\Delta\varepsilon$ −0.19) and a positive second Cotton effect at $\lambda = 286$ nm ($\Delta\varepsilon$ +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 $\lambda =$ 327 nm ($\Delta\varepsilon$ −3.3) and a positive ECD peak at $\lambda = 277$ nm ($\Delta\varepsilon$ +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 $n\rightarrow\pi^*$ transition of the C9 ketone (HOMO→LUMO+1; Figure 5). Meanwhile, slightly positive ECD bands at $\lambda =$ 309 nm (band 3, R_{vel} +3.3) and $\lambda = 299$ nm (band 4, R_{vel} +7.4) were mainly ascribed to the $n\rightarrow\pi^*$ transition of the C5 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**.

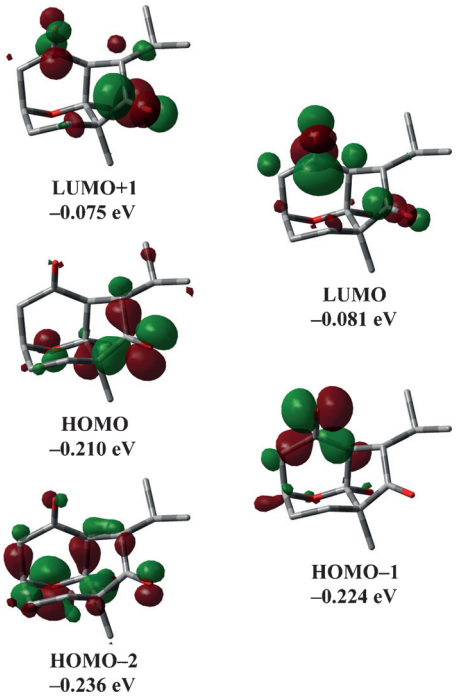
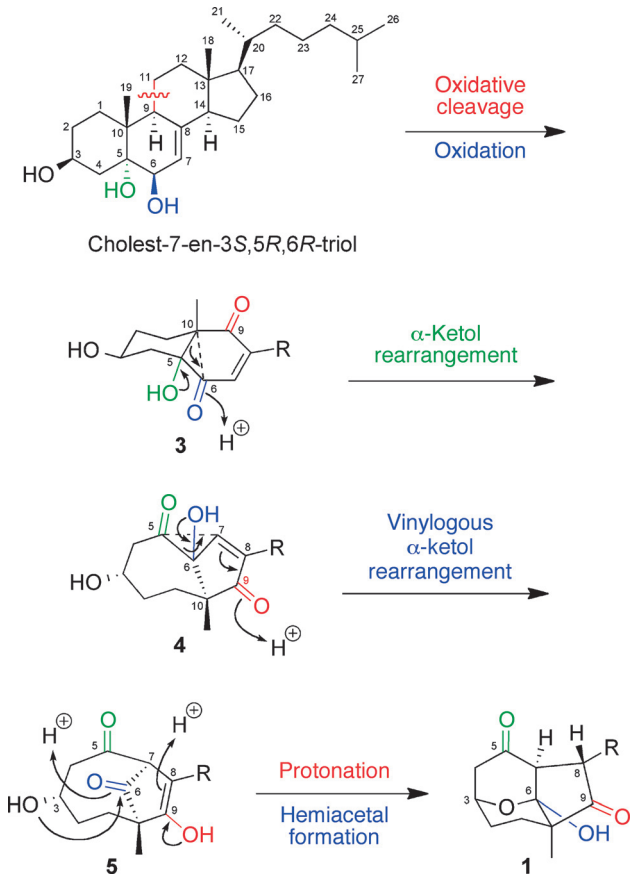


Figure 5. Molecular orbitals involved in the $n\rightarrow\pi^*$ transition of the C5 and C9 ketones in the model compound **2**.

Finally, **1** was converted into an 11,24-(MTPA) ester (see Figure S2 in the Supporting Information). Through the use of a modified Mosher's method,[15] the stereochemistry of C24 was determined to be *R*. For the above results, the absolute stereochemistry of **1** was completely established.

A biosynthetic pathway for the tricyclic γ-diketone structure of **1** was proposed, as shown in Scheme 1. Because of 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 C9–C11 bond and oxidation of the C6 hydroxy 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 the α,β-unsaturated ketone **4**. The vinylogous α-ketol rearrangement in **4** might form the C5–C7 bond, and subsequent enolization at the C9 ketone would afford the enol **5**. Finally, protonation at C8 and intramolecular acetalization of **5** would afford the tricyclic γ-diketone structure of **1**. The stereochemistry of the methine carbon at C8 in general steroids is opposite to that in **1**. However, protonation at C8 in **5** might occur to give priority to the thermodynamically stable *exo*-substituent 8*S* isomer.

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-,



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

9,10-, 9,11-, and 13,17-secosteroids.[12] To the best of our knowledge, however, there are no examples having tricyclic ring systems similar to that of **1**. Further biological and biosynthetic studies on **1** are in progress.

Keywords: biosynthesis · circular dichroism · natural products · rearrangements · structure elucidation

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