# New Bioactive Steroids from the Gorgonian *Acalycigorgia inermis*

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*Received February 7, 2000*

During the course of our search for bioactive compounds from marine organisms, we have shown that gorgonians of Korean water are very prolific sources of bioactive metabolites.[1] Recently, we reported acalycixeniolides C-F, novel cytotoxic diterpenoids of the xenicane class from the gorgonian *Acalycigorgia inermis* collected from Keomun Island.[2] In addition to the diterpenoids, however, $^1$H NMR analysis and cytotoxicity tests of silica flash chromatographic fractions indicated the presence of another class of metabolites in polar fractions. Subsequently activity-guided separation has yielded several steroids possessing unusual functionalities. Herein we describe the structures and bioactivity of two novel metabolites and an epimeric mixture of polyoxygenated steroids of the cholestane class. All of the compounds contain a 3$\beta$,5$\alpha$-dihydroxy-6-one group as a common structural feature.

Compound **1** was analyzed for $C_{29}H_{46}O_5$ by a combination of high-resolution mass and $^{13}$C NMR spectrometry. The $^{13}$C NMR data for this compound contained twenty-nine signals. The presence of a ketone and an ester was readily recognized by the signals at $\delta$ 212.3 (C) and 171.2 (C) in the $^{13}$C NMR spectrum and strong absorption bands at 1740 and 1710 cm$^{-1}$ in the IR spectrum. The ester carbon was assumed to form an acetoxyl group by an occurrence of characteristic signals in NMR data; $^1$H $\delta$ 2.07 (3H, s), $^{13}$C $\delta$ 21.0 ($CH_3$). Therefore, compound **1** possessed a framework consisting of twenty-seven carbons that appeared to be a $C_{27}$ steroid of the cholestane class. Supporting evidence for this interpretation was the occurrence of a characteristic proton signal at $\delta$ 3.98 (1H, m) and upfield shift of a proton at $\delta$ 0.64 (3H, s) in the $^1$H NMR spectrum which corresponded to the H-3 and H-18 of a cholesterol-type steroid, respectively. Of the five oxygen atoms in the molecule, the remaining one appeared to form a hydroxyl group on the basis of the molecular formula and $^{13}$C NMR analysis.

With the aid of this information, the structure of **1** was determined by a combination of $^1$H COSY, TOCSY, gradient HSQC and gradient HMBC experiments. All of the protons and their respective carbons were precisely matched by the gradient HSQC data. The $^1$H COSY and TOCSY data revealed the presence of a spin system containing proton signals of a methine at $\delta$ 3.98 and three upfield methylenes. Of the two terminal methylene carbons of this partial structure at $\delta$ 29.8 and 36.4 in the $^{13}$C NMR spectrum, the former showed a long-range coupling with a methyl proton at $\delta$ 0.80 (3H, s) which was also coupled with a methine at $\delta$ 44.5 and two quaternary carbons at $\delta$ 80.8 and 42.4, respectively. The upfield shift of the terminal methylene carbon revealed its direct connection with the quaternary carbon at $\delta$ 42.4. On the other hand, a proton of the other terminal methylene at $\delta$ 1.79 (1H, dd, $J$ = 14.2, 11.2 Hz) showed a gradient HMBC correlation with the quaternary carbon at $\delta$ 80.8. Thus, all of the carbons and protons at the A ring of a steroid were confidently assigned and hydroxyl groups were placed at C-3 and C-5. The carbons at $\delta$ 44.5 and 42.4 in the $^{13}$C NMR spectrum were assigned at C-9 and C-10 of a steroidal framework, respectively.

The structure of B ring was also determined by combined NMR analysis. The gradient HSQC data assigned the signals of protons at $\delta$ 2.71 (1H, dd, $J$ = 13.2, 12.2 Hz) and 2.11 (1H, dd, $J$ = 13.2, 4.4 Hz) to a methylene carbon at $\delta$ 41.8. Initiated at these protons, a combination of the $^1$H COSY and TOCSY experiments traced a spin system containing several upfield proton resonances. The gradient HMBC data showed correlations between these protons and carbons at $\delta$ 212.3, 80.8, 56.3, 44.5, and 37.3. Since the carbons at $\delta$ 80.8 and 44.5 were already assigned at C-5 and C-9 by their long-range couplings with the H-19 protons, the remaining three were placed, in the order of chemical shifts, at C-6, C-14, and C-8, respectively. The structural elucidation of C and D rings was also accomplished by extensive NMR experiments and comparison of the $^{13}$C NMR data with those of known analogs.[1b,1f] Long-range couplings of H-12, H-15, H-17, H-18, and H-20 with neighboring carbons were crucial to assign all of the NMR signals. Hence compound **1** was determined to possess the same C and D rings as common steroids.

The $^1$H NMR data of **1** had downfield signals at $\delta$ 5.37 (1H, br dd, $J$ = 7.3, 6.8 Hz) and 4.57 (2H, s) which showed a weak but unnegligible cross-peak between each other in the $^1$H COSY experiment. A gradient HMBC experiment showed long-range correlations of these and a methyl proton at $\delta$ 1.74 (3H, s) with several carbon signals including the olefinic ones at $\delta$ 131.4 (CH) and 129.4 (C). An additional correlation was found between the acetoxyl carbon at $\delta$ 171.2 and the proton at $\delta$ 4.57. Accordingly the tri-substituted double bond was attached by an acetoxyl group at its allylic position. A combination of 2D NMR data accomplished the connection of this group to the C-20 methine *via* an ethylene group.

Compound **1** possessed an asymmetric double bond at C-24 and two new asymmetric carbon centers at C-3 and C-5. The geometry at the double bond was assigned as *Z* on the

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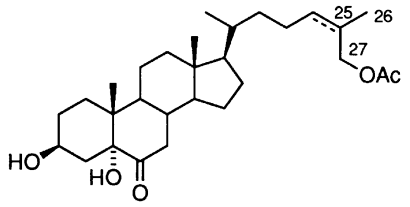
basis of downfield shift of the C-26 methyl carbon ($\delta$ 21.5). The characteristic splitting pattern of H-3 revealed an axial arrangement for this proton. ROESY data showed a strong 1,3-diaxial correlation between H-19 and H-4$\alpha x$ ($\delta$ 1.79) that was assigned on the basis of its large coupling ($J$ = 11.2 Hz) with H-3. Molecular modeling study showed that, to exhibit this spatial proximity, the C-5 hydroxyl group must be $\alpha$-oriented to the A/B ring junction. This interpretation was also ascertained by comparison of the $^{13}$C data with those of a synthetic analog.[3] Thus the structure of **1** was determined as 27-acetoxy-3$\beta$,5$\alpha$-dihydroxycholest-24-en-6-one.

Compound **2** was isolated as a white amorphous solid. The high-resolution mass data suggested the molecular formula for this compound to be $C_{29}H_{48}O_5$ ($\Delta$ -0.6 mmu). However, the $^{13}$C NMR data contained thirty-seven signals. Similar phenomenon was also observed in the $^1$H NMR spectrum in which integration of peak area revealed significant discrepancy among upfield signals. Careful examination of the $^{13}$C NMR data showed that **2** possessed the same ring system as **1**. Signals of carbons at the rings A-D also existed as single peaks while those at the side-chain were present as pairs. Therefore, **2** must be a mixture of analogs possessing structural differences only at the side-chain.

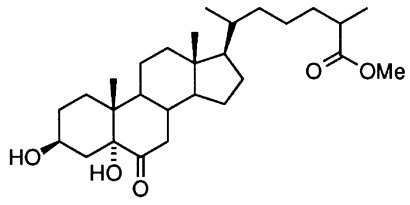
The most noticeable differences in the NMR data of **1** and **2** were those attributed to the presence of hydrogens at the C-24 double bond of **1** that was evident from detailed interpretation of 2D NMR data. Thus, **2** was defined as a mixture of the 24,25-dihydro derivatives of **1**. A combination of $^1$H COSY and TOCSY experiments revealed a long spin system initiating at the protons at $\delta$ 3.96 (dd, $J$ = 10.7, 7.3 Hz, H-27) and reaching at the methyl protons at $\delta$ 0.91 (3H, d, $J$ = 6.8 Hz, H-21). The same experiments also revealed the presence of an analogous spin system containing protons at $\delta$ 3.95 (dd, $J$ = 10.7, 7.3 Hz) and 0.91 (H-21). Since there was only one asymmetric center at C-25 at the side-chain, **2** was indeed a mixture of epimers at this center. Based on the integration of paired signals in the $^1$H NMR spectrum, the relative concentration between two isomers was calculated as 62:38. Due to the similar polarity between these, attempts to separate individual compound were not successful.

The molecular formula for **3** was deduced as $C_{28}H_{46}O_5$ by HREIMS. Apart from the loss of one carbon in the $^{13}$C NMR data, the NMR data for this compound were highly compatible with those obtained for **1** and **2**. In the $^{13}$C NMR data, however, the signals of the C-27 oxymethylene and acetoxyl methyl carbons were replaced by a methyl carbon at $\delta$ 51.5. Corresponding change was also observed in the $^1$H NMR spectrum in which the signal of the acetoxyl methyl proton was replaced by that of a new methyl proton at $\delta$ 3.67 (3H, s). In addition, the signal of the H-26 methyl proton was shifted upfield at $\delta$ 1.14 (3H, d, $J$ = 7.3 Hz). The $^1$H COSY experiment showed direct connectivity between H-26 and a methine proton at $\delta$ 2.43 (1H, tq, $J$ = 6.8, 7.3 Hz). The HMBC correlations of these and the methoxy proton with the carbon at $\delta$ 177.4(C) indicated the presence of a methoxycarbonyl functionality at the side-chain. The stereochemistry of a new asymmetric center at C-25 was not assigned due to the isolation of only one isomer.[1b, 4]




**1** $\Delta^{24,25}$

**2** (epimers at C-25)




**3**

A literature survey revealed that compounds **1-3** are structurally novel metabolites. Although the 3$\beta$,5$\alpha$-dihydroxy-6-one group and oxidation at C-27 at the side-chain have been previously observed in both natural and synthetic steroids, a combination of these in a molecule is unprecedented to the best of our knowledge.[3,5]

Polyoxygenated steroids derived from marine organisms are widely recognized to exhibit potent and diverse bioactivities.[1,5] For recent examples, polyhydroxysteroids from a Korean water gorgonian exhibited significant cytotoxicity and $PLA_2$ inhibitory activity.[1c] In our measurements, these steroids displayed moderate cytotoxicity against the human leukemia cell-line K-562 ($LC_{50}$ 1.1, 0.9, and 9.7 $\mu$g/mL, for **1-3**, respectively).

## Experimental Section

**Animal materials**. *Acalycigorgia inermis* (sample number 91K-4) was collected by hand using SCUBA at 20-25 m depth in November, 1991 along the offshore of Keomun Island, Korea. The collected samples were briefly dried under shade, frozen with dry ice, and kept at -25 °C until chemically investigated. The same animals (sample number 94K-11) were obtained again at the same area in August, 1994. Vouchers from both collections had identical morphological characters including the color and branching pattern of colonies, and length of calyces.

**Extraction and isolation**. The animals (10 kg, wet weight) were defrosted, macerated, and repeatedly extracted with $CH_2Cl_2$ (10 L × 3) and MeOH (10 L × 2). The combined crude extracts (41.2 g) were separated by silica vacuum flash chromatography by using stepped gradient mixtures of *n*-hexane and EtOAc as eluents. Fractions eluted with polar solvents (70-80% EtOAc in hexane, 290 mg) were combined and subjected to semipreparative $C_{18}$ reversed-phase HPLC (YMC ODS-AQ column, 1 cm × 25 cm, 5% aqueous

MeOH) to afford **3** and a mixture of **1** and **2**. Separation and purification of **1** and **2** were accomplished by reversed-phase HPLC (YMC ODS-A column, 1 cm × 25 cm, 10% aqueous MeOH) to afford 81.0 and 9.3 mg of **1** and **2**, respectively. Purification of **3** was made by reversed-phase HPLC (YMC ODS-A column, 20% MeCN in MeOH) to yield 13.9 mg of **3** as a white amorphous solid.

**Compound 1** (**1**): white amorphous solid; mp 183-185 °C; $[\alpha]^{25}_{D}$ -28.3° (*c* 0.1, MeOH); IR (KBr) $\nu_{max}$ 3400, 2940, 2870, 1740, 1710, 1440, 1375, 1240, 980 cm$^{-1}$; $^1$H NMR ($CDCl_3$) $\delta$ 5.37 (1H, br dd, $J$ = 7.3, 6.8 Hz, H-24), 4.57 (2H, s, H-27), 3.98 (1H, dddd, $J$ = 11.2, 11.2, 5.4, 5.4 Hz, H-3), 2.71 (1H, dd, $J$ = 13.2, 12.2 Hz, H-7), 2.11 (1H, dd, $J$ = 13.2, 4.4 Hz, H-7), 2.10 (1H, m, H-23), 2.07 (3H, s, OAc), 2.01 (1H, ddd, $J$ = 12.2, 3.4, 2.4 Hz, H-12), 1.94 (1H, m, H-23), 1.88 (1H, m, H-2), 1.86-1.82 (3H, m, H-4, H-9, H-16), 1.79 (1H, dd, $J$ = 14.2, 11.2 Hz, H-4), 1.74 (3H, br s, H-26), 1.70 (2H, m, H-1, H-8), 1.53 (2H, m, H-1, H-15), 1.45 (2H, m, H-2, H-11), 1.41 (1H, m, H-22), 1.38 (1H, m, H-20), 1.31 (1H, m, H-14), 1.28 (1H, m, H-11), 1.25 (1H, m, H-16), 1.22 (1H, m, H-12), 1.12 (1H, m, H-17), 1.06 (1H, m, H-22), 1.04 (1H, m, H-15), 0.92 (3H, d, $J$ = 6.5 Hz, H-21), 0.80 (3H, s, H-19), 0.64 (3H, s, H-18); $^{13}$C NMR ($CDCl_3$) $\delta$ 212.3 (C, C-6), 171.2 (C, OAc), 131.4 (CH, C-24), 129.4 (C, C-25), 80.8 (C, C-5), 67.3 (CH, C-3), 63.2 ($CH_2$, C-27), 56.3 (CH, C-14), 55.9 (CH, C-17), 44.5 (CH, C-9), 43.1 (C, C-13), 42.4 (C, C-10), 41.8 ($CH_2$, C-7), 39.5 ($CH_2$, C-12), 37.3 (CH, C-8), 36.4 ($CH_2$, C-4), 36.0 ($CH_2$, C-22), 35.4 (CH, C-20), 30.4 ($CH_2$, C-2), 29.8 ($CH_2$, C-1), 28.0 ($CH_2$, C-16), 24.5 ($CH_2$, C-23), 23.9 ($CH_2$, C-15), 21.5 ($CH_3$, C-26), 21.4 ($CH_2$, C-11), 21.0 ($CH_3$, OAc), 18.5 ($CH_3$, C-21), 14.1 ($CH_3$, C-19), 12.0 ($CH_3$, C-18); HREIMS $(M)^+$ *m/z* obsd 474.3339; calcd for $C_{29}H_{46}O_5$, 474.3345.

**Compound 2** (**2**): white amorphous solid; $[\alpha]^{25}_{D}$ -6.1° (*c* 0.4, MeOH); IR (KBr) $\nu_{max}$ 3400, 2940, 2870, 1740, 1710, 1470, 1375, 1240, 980 cm$^{-1}$; $^1$H NMR ($CDCl_3$) $\delta$ 3.98 (1H, m, H-3), 3.96 (dd, $J$ = 10.7, 7.3 Hz, min. of H-27), 3.95 (dd, $J$ = 10.7, 7.3 Hz, maj. of H-27), 3.84 (dd, $J$ = 10.7, 2.4 Hz, maj. of H-27), 3.83 (dd, $J$ = 10.7, 1.9 Hz, min. of H-27), 2.72 (1H, dd, $J$ = 13.2, 12.2 Hz, H-7), 2.12 (1H, dd, $J$ = 13.2, 4.4 Hz, H-7), 2.06 (3H, s, OAc), 2.02 (1H, ddd, $J$ = 12.2, 3.4, 2.4 Hz, H-12), 1.87 (1H, m, H-2), 1.86-1.81 (3H, m, H-4, H-9, H-16), 1.79 (1H, m, H-4), 1.77 (1H, m, H-25), 1.70 (2H, m, H-1, H-8), 1.54 (1H, br d, $J$ = 13.7 Hz, H-1), 1.51 (1H, m, H-15), 1.46 (2H, m, H-2, H-11), 1.35 (3H, m, H-20, H-23, H-24), 1.31 (1H, m, H-14), 1.30 (1H, m, H-22), 1.29 (1H, m, H-11), 1.25 (1H, m, H-16), 1.23 (1H, ddd, $J$ = 12.8, 12.2, 3.4 Hz, H-12), 1.21 (1H, m, H-22), 1.19 (1H, m, H-23), 1.12 (1H, m, H-17), 1.04 (1H, m, H-15), 1.02 (1H, m, H-24), 0.93 (d, $J$ = 6.8 Hz, min. of H-26), 0.92 (d, $J$ = 6.8 Hz, maj. of H-26), 0.91 (3H, d, $J$ = 6.8 Hz, H-21), 0.81 (3H, s, H-19), 0.65 (3H, s, H-18); $^{13}$C NMR ($CDCl_3$) $\delta$ 212.3 (C, C-6), 171.3 (C, OAc), 80.9 (C, C-5), 69.59/69.45 ($CH_2$, C-27), 67.3 (CH, C-3), 56.3 (CH, C-14), 56.05/56.02 (CH, C-17), 44.5 (CH, C-9), 43.1 (C, C-13), 42.4 (C, C-10), 41.8 ($CH_2$, C-7), 39.6 ($CH_2$, C-12), 37.3 (CH, C-8), 36.4 ($CH_2$, C-4), 36.05/35.95 ($CH_2$, C-24), 35.68/35.62 (CH, C-20), 33.85/33.70 ($CH_2$, C-22), 32.54/32.48 (CH, C-25), 30.3 ($CH_2$, C-2), 29.8 ($CH_2$, C-1), 28.1 ($CH_2$, C-16), 23.9 ($CH_2$, C-15), 23.30/23.26 ($CH_2$, C-23), 21.4 ($CH_2$, C-11), 21.0 ($CH_3$, OAc), 18.6 ($CH_3$, C-21), 17.01/16.78 ($CH_3$, C-26), 14.1 ($CH_3$, C-19), 12.0 ($CH_3$, C-18); HREIMS $(M)^+$ *m/z* obsd 476.3496; calcd for $C_{29}H_{48}O_5$, 476.3502.

**Compound 3** (**3**): white amorphous solid; mp 202-205 °C; $[\alpha]^{25}_{D}$ -28.3° (*c* 0.1, MeOH); IR (KBr) $\nu_{max}$ 3400, 2940, 2865, 1955, 1740, 1710, 1465, 1375, 1160 cm$^{-1}$; $^1$H NMR ($CDCl_3$) $\delta$ 3.97 (1H, dddd, $J$ = 11.2, 11.2, 5.4, 5.4 Hz, H-3), 3.67 (3H, s, OMe), 2.70 (1H, dd, $J$ = 13.2, 12.2 Hz, H-7), 2.43 (1H, tq, $J$ = 6.8, 7.3 Hz, H-25), 2.11 (1H, dd, $J$ = 13.2, 4.4 Hz, H-7), 2.02 (1H, ddd, $J$ = 12.2, 3.4, 2.5 Hz, H-12), 1.88 (1H, m, H-2), 1.86-1.82 (3H, m, H-4, H-9, H-16), 1.80 (1H, dd, $J$ = 14.2, 11.2 Hz, H-4), 1.70 (2H, m, H-1, H-8), 1.57 (1H, m, H-24), 1.53 (2H, m, H-1, H-15), 1.45 (2H, m, H-2, H-11), 1.39 (1H, m, H-24), 1.37 (1H, m, H-22), 1.34 (2H, m, H-20, H-23), 1.30 (1H, m, H-14), 1.28 (1H, m, H-11), 1.23 (1H, m, H-12), 1.17 (1H, m, H-23), 1.14 (3H, d, $J$ = 7.3 Hz, H-26), 1.11 (1H, m, H-17), 1.05 (1H, m, H-15), 1.01 (1H, m, H-22), 0.89 (3H, d, $J$ = 6.4 Hz, H-21), 0.80 (3H, s, H-19), 0.64 (3H, s, H-18); $^{13}$C NMR ($CDCl_3$) $\delta$ 212.0 (C, C-6), 177.4 (C, C-27), 80.9 (C, C-5), 67.3 (CH, C-3), 56.3 (CH, C-14), 56.0 (CH, C-17), 51.5 ($CH_3$, OMe), 44.5 (CH, C-9), 43.1 (C, C-13), 42.4 (C, C-10), 41.8 ($CH_2$, C-7), 39.6 ($CH_2$, C-12), 39.4 (CH, C-25), 37.3 (CH, C-8), 36.4 ($CH_2$, C-4), 35.7 ($CH_2$, C-22), 35.6 (CH, C-20), 34.1 ($CH_2$, C-24), 30.4 ($CH_2$, C-2), 29.8 ($CH_2$, C-1), 28.0 ($CH_2$, C-16), 23.9 ($CH_2$, C-15), 23.7 ($CH_2$, C-23), 21.4 ($CH_2$, C-11), 18.5 ($CH_3$, C-21), 17.0 ($CH_3$, C-26), 14.1 ($CH_3$, C-19), 12.0 ($CH_3$, C-18); HREIMS $(M)^+$ *m/z* obsd 462.3347; calcd for $C_{28}H_{46}O_5$, 462.3345.

**Acknowledgment**. The authors thank Professor Jun-Im Song, Department of Biology, Ewha Womans University, Seoul, Korea, for taxonomic assignment. Thanks are also extended to Dr. Young Hwan Kim, Korea Basic Science Institute, Taejeon, Korea for providing mass data. This research was financially supported by grants (BSPE -98702 and -99749) from Ministry of Maritime Affairs and Fisheries.

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