Isolation of New Steroidal Hemiacetals from the Gorgonian *Euplexaura anastomosans*

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Anastomosacetals A−D (**1**−**4**), four novel steroids of the cholestane class, have been isolated from the gorgonian *Euplexaura anastomosans*. The structures of these compounds have been determined by spectroscopic methods as possessing an unusual hemiacetal linkage in the side chain.

Coelenterates (phylum Cnidaria) have produced a wide variety of both biologically active and structurally unique secondary metabolites; terpenoids and steroids are the major groups of metabolites from these animals.[1] Steroids of coelenterates are recognized to possess unusual carbon skeletons and functionalities and exhibit various bioactivities.[2] In our recent investigation of benthic organisms of Korean waters, these animals have also proved to be a rich source of novel steroids.[3−6] As a part of our continuing search for novel compounds from marine organisms, we collected the gorgonian *Euplexaura anastomosans* Brundin (Plexauridae, order Gorgonacea) off the shore of Keomun Island, Korea.[7] Silica vacuum flash chromatography of the combined CH_2Cl_2 and MeOH extracts followed by silica and C_{18} reversed-phase HPLC yielded several unusual steroids of the cholestane class. Herein we report the isolation and structure determination of anastomosacetals A−D (**1**−**4**), novel steroidal hemiacetals possessing a tetrahydropyran ring in the side chain.

Anastomosacetal A (**1**) was isolated as a white solid with the composition $C_{27}H_{40}O_4$ by HRMS and ^{13}C-NMR spectroscopic methods. The presence of an α,β,α′,β′-unsaturated carbonyl group was readily recognized by a carbon signal at δ 186.30 in the ^{13}C-NMR spectrum and a strong absorption band at 1660 cm^{-1} in the IR spectrum. Careful examination of the NMR spectral data revealed that compound **1** was a steroid possessing a 1,4-cyclohexadien-3-one moiety as ring A. A characteristic absorption maximum at 240 nm in the UV spectrum supported this interpretation. Furthermore, comparison of the ^{1}H- and ^{13}C-NMR assignments aided by a combination of ^{1}H−COSY and HMQC experiments with known metabolites revealed that **1** possessed the same A−C rings as the minabeolides, a series of withanolides isolated from the soft coral *Minabea* sp. (Table 1).[8]

The molecular formula and the NMR data showed that, in addition to an oxygen of the C-3 carbonyl group, **1** possessed three oxygen atoms at ring D and side chain. The functionalities of these oxygens were determined as a hemiacetal and a hydroxyl group by carbon resonances at δ 93.14 (d), 84.03 (s), and 73.51 (d) in the ^{13}C-NMR spectrum and corresponding proton signals at δ 5.50 (1H, br s) and 3.60 (1H, ddd, J = 11.7, 6.4, 2.0 Hz) in the ^{1}H-NMR spectrum. This interpretation was supported by the disappearance of the signals at δ 3.48 (1H, s) and 2.93 (1H, br s) in the ^{1}H-NMR spectrum in a D_2O exchange experiment, while the splitting pattern of the proton at δ 5.50 changed from a broad singlet to a doublet (J = 2.3 Hz) in the same experiment. The position of the hemiacetal group was assigned to C-21 of the cholestane skeleton because four methyl signals in the NMR spectra of **1** were confidently assigned to C-18, -19, -26, and -27 by a combination of the ^{1}H−COSY and HMQC experiments (Table 1).

Table 1. Carbon NMR Assignments for Compounds **1**−**4**[a]

C no.	**1**	**2**	**3**	**4**
1	155.81	158.44	35.89	38.53
2	127.49	127.41	34.33	38.16
3	186.30	200.25	196.92	212.08
4	123.83	40.97	124.18	44.68
5	169.27	44.22	168.54	46.60
6	32.93	27.63	32.75	28.94
7	33.61	31.22	32.16	31.64
8	35.69	35.80	35.83	35.55
9	51.80	50.21	53.34	53.28
10	43.55	38.91	38.34	35.60
11	22.72	21.15	21.08	21.33
12	32.24	32.46	32.48	32.51
13	47.58	47.64	47.40	47.53
14	49.38	49.43	49.93	50.17
15	23.48	23.19	23.66	23.31
16	36.80	36.93	37.14	36.93
17	84.03	84.14	83.79	84.28
18	14.76	14.90	14.70	14.80
19	18.74	12.98	17.13	11.47
20	44.52	44.60	45.04	44.61
21	93.14	93.22	93.17	93.21
22	19.47	19.48	19.98	19.49
23	28.17	28.18	28.76	28.19
24	73.51	73.51	73.12	73.48
25	32.66	32.65	33.16	32.65
26	18.65[b]	18.65[b]	18.80[b]	18.66[b]
27	18.36[b]	18.36[b]	18.72[b]	18.35[b]

[a] NMR spectra were obtained at 125 MHz in $CDCl_3$ (**1**, **2**, and **4**) and C_6D_6 (**3**) solutions. Assignments were aided by DEPT, HMQC, and HMBC experiments. [b] Interchangeable signals.

Initiated from the H-26 and H-27 methyl protons, tracing of the proton spin-couplings by the ^{1}H−COSY data readily determined the H-24 proton as a methine signal at δ 3.60. The carbon at δ 73.51 bearing this proton exhibited a long-range ^{1}H−^{13}C correlation with the H-21 hemiacetal proton at δ 5.50 in HMBC experiments (Experimental Section). In addition, the hydroxyl proton of C-21 at δ 2.93 showed a long-range correlation with the C-20 carbon at δ 44.52. Therefore, the C-21

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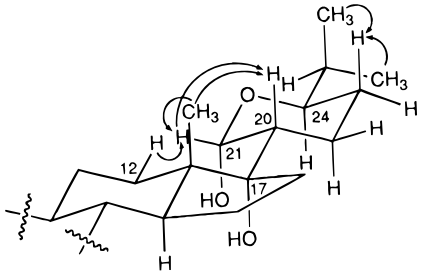


Figure 1. Stereochemistry of **1** depicted on the basis of proton couplings and NOESY (→) correlations.

and C-24 carbons were connected to each other by an ether linkage forming a tetrahydropyran ring.

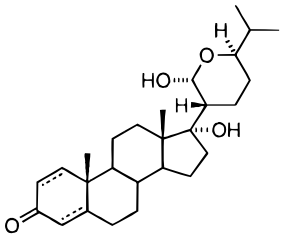
The position of the remaining tertiary hydroxyl group was assigned to C-17 on the basis of the ^{1}H−COSY and proton-decoupling experiments in which the H-20 proton was spin-coupled to only the H-21 and H-22 protons ($J_{20,21}$ = 2.9 Hz, $J_{20,22}$ = 12.7, 3.4 Hz). HMBC correlations of the C-17 carbon at δ 84.03 with the H-18 methyl protons at δ 0.87 and the hydroxyl proton at δ 3.48 supported this interpretation. Thus, the structure of anastomosacetal A (**1**) was unambiguously determined as a steroidal hemiacetal of the cholestane class.

Compound **1** possessed four asymmetric carbon centers (C-17, -20, -21, and -24) at the tetrahydropyran ring and vicinity. The stereochemistry of these centers was determined by analysis of proton coupling constants and NOESY experiments. Both the H-20 and H-21 protons showed strong NOESY correlations with the H-18 methyl protons. The orientations of these protons were assigned as axial and equatorial for the H-20 and H-21 protons, respectively, on the basis of the splitting pattern and coupling constants of the H-20 proton ($J_{20,21}$ = 2.9 Hz, $J_{20,22}$ = 12.7, 3.4 Hz) (Figure 1). NOESY correlations of the H-21 proton with the H-12β (δ 1.54) and H-20 protons supported this interpretation. To exhibit these correlations, the hydroxyl group of the C-17 carbon must be α-oriented to the ring D. Similarly, the orientation of the H-24 proton was determined as axial from the coupling constants between the H-23 and H-24 protons ($J_{23,24}$ = 11.7, 2.0 Hz) and strong NOESY correlations between the H-23β (δ 1.33) proton and the H-26 and H-27 protons. Thus, the configuration of **1** was assigned as 17α,20*R*,21*R*,24*R*.

A closely related metabolite, anastomosacetal B (**2**) was isolated as a white solid that was analyzed for $C_{27}H_{42}O_4$ by HRMS and ^{13}C-NMR spectrometry. Spectral data for this compound were very similar to those derived from **1**. The only significant differences in the NMR data were for carbon and proton signals corresponding to ring A (Table 1). Most noticeable were replacements of the signals of the C-4 and C-5 olefinic carbons in the ^{13}C-NMR spectrum and the H-4 proton in the ^{1}H-NMR spectrum of **1** by upfield signals. IR and UV data (1680 cm^{-1}; 230 nm) of **2** were also indicative of the hydrogenation of the C-4 double bond that was confirmed by ^{1}H−COSY and HMQC experiments. Thus, the structure of anastomosacetal B (**2**) was defined as the 4,5-dihydro derivative of **1**.

An apparently related steroid, anastomosacetal C (**3**) was isolated as a white solid that had the identical molecular formula as **2**. Due to the highly unstable nature of this compound in acidic condition (either in $CHCl_3$ or in silica), the NMR data of **3** were obtained in C_6D_6 solutions and were still highly compatible with those of **1** and **2** (Table 1). IR (1665 cm^{-1}), UV (241 nm), and especially the ^{1}H- and ^{13}C-NMR data revealed that the C-1 double bond of **1** was hydrogenated in **3**. The structure of the other part was determined to be identical to **1** and **2** by a combination of ^{1}H−COSY and HMQC experiments. Thus, the structure of **3** was defined as the 1,2-dihydro derivative of **1**.

Another related compound, anastomosacetal D (**4**) was isolated as a white solid, and a molecular formula of $C_{27}H_{44}O_4$ was deduced by a combination of HRMS and ^{13}C-NMR spectroscopic data. The loss of signals for the olefinic carbons and protons in the NMR spectra revealed that **4** possessed a 3-cyclohexanone moiety as ring A that was confirmed by IR (1710 cm^{-1}) and UV (no λ max) data. Combination of ^{1}H−COSY and HMQC experiments defined the structure of **4** as the 1,2,4,5-tetrahydro derivative of **1**.



1

2 (4,5-dihydro)

3 (1,2-dihydro)

4 (1,2,4,5-tetrahydro)

Marine animals have produced a wide variety of polyoxygenated steroids, some of which possess acetal, hemiacetal, ketal, or hemiketal groups in the side chain.[1,2,9−12] To the best of our knowledge, however, anastomosacetals are the first examples of marine steroids possessing a hemiacetal functionality formed by oxidation of the C-21 methyl group.

The crude extracts of *E. anastomosans* exhibited moderate cytotoxicity against the P-388 cell-line (LC_{50} 73.3 μg/mL) and toxicity to brine-shrimp larvae (LC_{50} 303 ppm). However, the same tests using pure compounds revealed that anastomosacetals were not toxic to either cancer cell-lines or brine-shrimp larvae. The substances responsible for the toxicity of the crude extracts are currently under investigation.

Experimental Section

General Experimental Procedures. NMR spectra were recorded in $CDCl_3$ (**1**, **2**, and **4**) and C_6D_6 (**3**) solutions on a Varian Unity-500 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured at 500 and 125 MHz, respectively. All of the chemical shifts were recorded with respect to internal TMS. IR spectra were recorded on a Mattson GALAXY spectrophotometer. UV spectra were obtained by using a Milton-Roy spectrophotometer. Optical rotations were measured on a JASCO digital polarimeter using a 2-mL microcell. Mass spectra were provided by the Mass Spectrometry Facility, Department of Chemistry, University of California, Riverside, by the FABMS method. Melting points were measured on a Fisher-Johns apparatus and are reported uncor-

rected. All solvents used were spectral grade or were distilled from glass prior to use.

Animal Material. *Euplexaura anastomosans* (sample number 91K-1) was collected by hand using scuba at 15−35 m depth in November 1991, along the shore of Keomun Island, South Sea, Korea.[7] The collected samples were briefly dried under shade and kept at −25 °C until chemically investigated.

Extraction and Isolation. The animals (2.5 kg) were thawed, macerated, and repeatedly extracted with CH_2Cl_2 (3 L × 3) and MeOH (3 L × 2). The combined crude extracts (10.2 g) were separated by silica vacuum flash chromatography by using sequential mixtures of *n*-hexane and EtOAc as eluents. Fractions eluted with moderately polar solvents (30−45% EtOAc in hexane) were combined and separated by silica semi-preparative HPLC (YMC Si column, 1 cm × 25 cm, 30% EtOAc in hexane) to yield partially pure compounds **1**−**4** in the order of **2**, **4**, **3**, and **1**. Final purification was made by semi-preparative C_{18} reversed-phase HPLC (YMC ODS column, 1 cm × 25 cm, 100% CH_3CN for **1** and **2**; 10% aqueous MeOH for **3** and **4**).

Anastomosacetal A (1): isolated as a white solid (21 mg, 0.2% of crude extract); mp 134−136 °C; $[\alpha]^{25}_D$ +22.3° (*c* 0.4, MeOH); IR (KBr) ν_{max} 3400, 2930, 2870, 1660, 1620, 1460, 1120, 1095, 990 cm^{-1}; UV (MeOH) λ max 244 nm (ϵ 17 500); ^1H NMR ($CDCl_3$) δ 7.05 (1H, d, J = 10.0 Hz, H-1), 6.22 (1H, dd, J = 10.0, 2.0 Hz, H-2), 6.07 (1H, br s, H-4), 5.50 (1H, br s, H-21), 3.60 (1H, ddd, J = 11.7, 6.4, 2.0 Hz, H-24), 3.48 (1H, s, OH-17), 2.93 (1H, br s, OH-21), 2.46 (1H, ddd, J = 13.4, 13.2, 4.2 Hz, H-6), 2.36 (1H, ddd, J = 13.4, 3.0, 3.0 Hz, H-6), 2.02 (1H, dddd, J = 13.2, 13.2, 12.7, 3.5 Hz, H-22), 1.95 (1H, m, H-7), 1.89 (1H, ddd, J = 12.7, 3.4, 2.9 Hz, H-20), 1.76 (1H, m, H-14), 1.74 (2H, m, H-12, −23), 1.71 (2H, m, H-11), 1.67 (2H, m, H-16), 1.64 (1H, m, H-8), 1.62 (2H, m, H-15, -22), 1.60 (1H, m, H-25), 1.54 (1H, m, H-12), 1.33 (1H, dddd, J = 13.2, 13.2, 11.7, 3.9 Hz, H-23), 1.24 (3H, s, Me-19), 1.17 (1H, m, H-15), 1.10 (1H, m, H-7), 1.08 (1H, ddd, J = 11.7, 11.7, 3.9 Hz, H-9), 0.91 (3H, d, J = 6.8 Hz, Me-26), 0.88 (3H, d, J = 6.8 Hz, Me-27), 0.87 (3H, s, Me-18); HMBC correlations (optimized for 6 and 8 Hz) *H*-1−*C*-3, -5, -10, -19, *H*-2−*C*-4, -10, *H*-4−*C*-2, -6, -10, *H*-6−*C*-4, -5, -7, *H*-18−*C*-13, -14, -17, *H*-19−*C*-1, -5, -9, -10, *H*-21−*C*-22, -24, *H*-24−*C*-27, *H*-26−*C*-24, -25, *H*-27−*C*-24, -25, -26, *OH*-17−*C*-16, -17, *OH*-21−*C*-20; HRFABMS $[M + H]^+$ m/z obsd 429.2997, $C_{27}H_{41}O_4$ requires 429.3005.

Anastomosacetal B (2): isolated as a white solid (18 mg, 0.2% of crude extract): mp 107−108 °C; $[\alpha]^{25}_D$ +15.6° (*c* 0.3, MeOH); IR (KBr) ν max 3450, 2930, 2870, 1680, 1630, 1440, 1370, 1170, 1095 cm^{-1}; UV (MeOH) λ_{max} 230 nm (ϵ 14 000); ^1H NMR ($CDCl_3$) δ 7.13 (1H, d, J = 10.3 Hz, H-1), 5.85 (1H, d, J = 10.3 Hz, H-2), 5.51 (1H, br s, H-21), 3.61 (1H, ddd, J = 11.7, 6.4, 2.0 Hz, H-24), 3.47 (1H, s, OH-17), 2.92 (1H, br s, OH-21), 2.36 (1H, dd, J = 18.1, 14.2 Hz, H-4), 2.22 (1H, br dd, J = 18.1, 3.9 Hz, H-4), 2.01 (1H, dddd, J = 13.2, 13.2, 12.7, 3.9 Hz, H-22), 1.92 (1H, m, H-5), 1.88 (1H, m, H-20), 1.82 (1H, m, H-11), 1.78 (1H, m, H-14), 1.75 (1H, m, H-12), 1.72 (1H, m, H-23), 1.69 (3H, m, H-7, -16, -16), 1.65 (1H, m, H-15), 1.61 (2H, m, H-22, -25), 1.57 (1H, m, H-12), 1.50 (1H, m, H-8), 1.46−1.41 (3H, m, H-6, -6, -11), 1.34 (1H, dddd, J = 13.2, 13.2, 11.7, 3.9 Hz, H-23), 1.10 (1H, m, H-15), 1.02 (2H, m, H-7, -9), 1.00 (3H, s, Me-19), 0.93 (3H, d, J = 6.8 Hz, Me-26), 0.88 (3H, d, J = 6.8 Hz, Me-27), 0.84 (3H, s, Me-18); HRFABMS $[M + H]^+$ m/z obsd 431.3169, $C_{27}H_{43}O_4$ requires 431.3161.

Anastomosacetal C (3): isolated as a white solid (11 mg, 0.1% of crude extract); mp 187−188 °C; $[\alpha]^{25}_D$ +23.3° (*c* 0.3, MeOH); IR (KBr) ν_{max} 3400, 2920, 2870, 1665, 1615, 1460, 1385, 1240, 1125, 1100, 1000 cm^{-1}; UV (MeOH) λ max 241 nm (ϵ 18 000); ^1H NMR (C_6D_6) δ 5.83 (1H, br s, H-4), 5.25 (1H, br s, H-21), 3.57 (1H, br dd, J = 11.7, 6.2 Hz, H-24), 3.44 (1H, s, OH-17), 3.00 (1H, br s, OH-21), 2.31 (1H, br ddd, J = 16.6, 3.9, 3.4 Hz, H-2), 2.20 (1H, ddd, J = 16.6, 14.7, 4.9 Hz, H-2), 2.14 (1H, br ddd, J = 13.1, 12.7, 12.7 Hz, H-22), 1.93 (1H, dddd, J = 14.2, 13.8, 4.4, 1.9 Hz, H-6), 1.84 (1H, m, H-6), 1.79 (1H, m, H-14), 1.75 (2H, m, H-16, -20), 1.65 (1H, m, H-25), 1.60 (1H, m, H-15), 1.57 (3H, m, H-1, -12, -23), 1.54 (2H, m, H-16, -22), 1.48 (1H, m, H-7), 1.34 (1H, m, H-1), 1.29 (1H, m, H-11), 1.22 (1H, m, H-12), 1.18 (1H, m, H-23), 1.16 (1H, m, H-8), 1.12 (1H, m, H-11), 1.04 (3H, d, J = 6.8 Hz, Me-26), 0.93 (3H, d, J = 6.8 Hz, Me-27), 0.89 (1H, m, H-15), 0.78 (1H, m, H-7), 0.77 (3H, s, Me-19), 0.66 (1H, ddd, J = 11.7, 11.2, 3.4 Hz, H-9), 0.57 (3H, s, Me-18); HRFABMS $[M + H]^+$ m/z obsd 431.3156, $C_{27}H_{43}O_4$ requires 431.3161.

Anastomosacetal D (4): isolated as a white solid (29 mg, 0.3% of crude extract); mp 75−76 °C; $[\alpha]^{25}_D$ −9.1° (*c* 0.5, MeOH); IR (KBr) ν_{max} 3400, 2920, 2860, 1710, 1620, 1435, 1390, 1225, 1170, 1100 cm^{-1}; UV (MeOH) no λ_{max}; ^1H NMR ($CDCl_3$) δ 5.51 (1H, br s, H-21), 3.60 (1H, ddd, J = 11.7, 6.4, 2.0 Hz, H-24), 3.45 (1H, s, OH-17), 3.03 (1H, br s, OH-21), 2.37 (1H, ddd, J = 15.6, 13.7, 6.3 Hz, H-2), 2.29 (1H, dddd, J = 15.6, 4.9, 2.4, 2.4 Hz, H-2), 2.26 (1H, dd, J = 14.7, 14.2 Hz, H-4), 2.07 (1H, ddd, J = 14.7, 3.9, 2.0 Hz, H-4), 2.01 (2H, m, H-1, -22), 1.88 (1H, ddd, J = 12.7, 2.4, 2.4 Hz, H-20), 1.76 (1H, m, H-14), 1.72 (1H, m, H-23), 1.71−1.65 (4 H, m, H-7, -15, -16, -16), 1.62 (1H, m, H-11), 1.60 (2H, m, H-22, -25), 1.58 (2H, m, H-12), 1.53 (1H, m, H-5), 1.43 (1H, dddd, J = 11.7, 10.7, 10.7, 3.9 Hz, H-8), 1.38 (1H, m, H-11), 1.37−1.26 (4H, m, H-1, -6, -6, -23), 1.09 (1H, m, H-15), 1.01 (3H, s, Me-19), 0.96 (1H, dddd, J = 12.7, 12.2, 12.2, 4.9 Hz, H-7), 0.91 (3H, d, J = 6.8 Hz, Me-26), 0.88 (3H, d, J = 6.8 Hz, Me-27), 0.81 (3H, s, Me-18), 0.78 (1H, ddd, J = 12.2, 10.7, 3.4 Hz, H-9); HRFABMS $[M + H]^+$ m/z obsd 433.3327, $C_{27}H_{45}O_4$ requires 433.3318.

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