Natural Product Sciences
26(4) : 303-310 (2020)
https://doi.org/10.20307/nps.2020.26.4.303

Calyxaprenols A-D, New Merohexaprenoid Metabolites from the Marine Sponge *Calyx* sp.

Chang-Kwon Kim and Kirk R. Gustafson*

Molecular Targets Program, Center for Cancer Research, National Cancer Institute, Frederick, Maryland, USA, 21702-1201

Abstract – Four new merohexapenoids named calyxaprenols A-D (**1** - **4**), together with the known compound haliclotriol A (**5**), have been isolated from the marine sponge *Calyx* sp. which was collected from the southwest islands of Palau. Based on comprehensive spectroscopic analyses, calyxaprenols A (**1**) and B (**2**) were determined to be pentacyclic hexaprenoids that are appended to a glycolic acid-substituted phenol moiety, whereas calyxaprenols C (**3**) and D (**4**) possess a tricyclic hexaprenoid skeleton joined to a hydroquinone ring. Identification of new merohexaprenoids from a *Calyx* sponge expands the known taxonomic distribution of this sparsely distributed class of marine metabolites and increases the chemical diversity described for this genus of marine sponge.
Keywords – *Calyx* sp., marine sponge, merohexaprenoid, calyxaprenol

Introduction

Marine merohexapenoids are a relatively rare family of metabolites derived from mixed terpenoid and polyketide biogenesis which have demonstrated a variety of important biological activities. Since the isolation of the mokupalides,[1] the first sponge-derived head-to-tail type triterpenes, approximately 30 head-to-tail merotriterpenoids which incorporate benzoic subunits appended to a variety of triterpene scaffolds have been identifed from diverse sponge sources.[2] These compounds include the shaagrockols, toxicols, and toxiusol from the Red Sea sponge *Toxiclona toxius*,[3,4] along with akaterpin, isoakaterpin, ilhabelanol, and ilhabrene from a *Callyspongia* sp. sponge.[5,6] Adociasulfates 1-12 and adociaquinol,[7-11] haliclotriols A and B,[12] and halicloic acids A and B,[13] have been reported from *Haliclona* (aka *Adocia*) sp. sponges, while adociasulfates 13 and 14 were isolated from the sponge *Cladocroce aculeata*.[14] These merohexapenoid metabolites have been reported to exhibit potent and diverse biological properties including inhibiting the enzymatic activities of phosphatidylinositol-specific phospholipase C (PI-PLC),[5] adenosine phosphoribosyl transferase (APRT),[6] indoleamine 2,3-dioxygenase,[13] and HIV reverse transcriptase,[15] as well as modulation of the H^+-ATPase protein pump,[8] and reduction of the microtubule-stimulated ATPase activity of kinesin.[14]

In conjunction with long-term and ongoing natural products discovery efforts at the NCI,[16,17] an extract of the marine sponge *Calyx* sp. which had been collected around Palau, was selected for detailed chemical study. Prior investigations of *Calyx* sponges have provided a variety of new metabolites including unusual steroids,[18-21] fatty acids,[22,23] alkaloids,[24-26] and diphenylbutanoids.[27] Chromatographic separation of the *Calyx* extract provided four new merohexaprenoid metabolites that were named calyxaprenols A-D (**1** - **4**), along with the known compound haliclotriol A (**5**).[12] Based on extensive NMR and MS analyses, calyxaprenols A (**1**) and B (**2**) were determined to consist of a core hexacyclic terpenoid ring system attached to a glycolic acid moiety at the benzylic position. The triterpene component of calyxaprenols C (**3**) and D (**4**) was established to have a bicyclic rearanged drimane skeleton and a prenyl-substituted cyclohexene ring that are linked via an ethylene bridge, and this C_{30} scaffold was joined with a hydroquinone moiety (Fig. 1).

*Author for correspondence
K. R. Gustafson, Molecular Targets Program, Center for Cancer Research, National Cancer Institute, Frederick, Maryland, USA, 21702-1201
Tel: +1-301-846-5197; E-mail: gustafki@mail.nih.gov.

ORCID
Kirk R. Gustafson: 0000-0001-6821-4943
Chang-Kwon Kim: 0000-0002-4637-3764

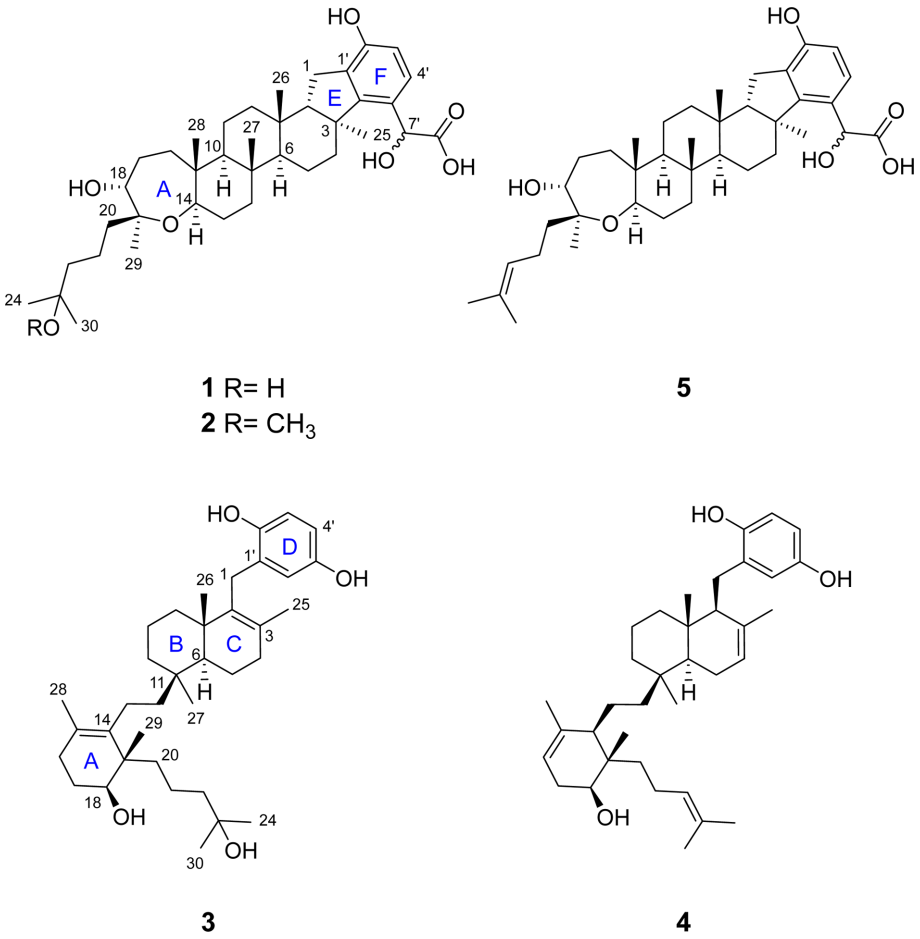

Fig. 1. Structures of calyxaprenols A-D (**1** – **4**) and haliclotriol A (**5**) isolated from the marine sponge *Calyx* sp.

Experimental

General Experimental Procedures – Optical rotations were measured on a Rudolph research analytical AUTOPOL IV automatic polarimeter, IR spectra were recorded with a Bruker ALPHA II FT-IR spectrometer, and UV spectra were measured with a Thermo Scientific Nanodrop 2000C spectrophotometer. NMR spectra were obtained with a Bruker Avance III NMR spectrometer equipped with a 3 mm cryogenic probe and operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Spectra were calibrated to residual solvent signals at δ_H 3.31 and δ_C 49.0 in MeOH-d_4. All 2D NMR experiments were acquired with nonuniform sampling (NUS) set to 50% or 25%. HRESIMS data were acquired on an Agilent Technology 6530 Accurate-mass Q-TOF LC/MS. High-performance liquid chromatography (HPLC) was performed using a Varian ProStar 215 solvent delivery module equipped with a Varian Prostar 320 UV-Vis detector, operating under Star 6.41 chromatography workstation software.

Animal material – Samples of the marine sponge *Calyx* sp. were collected by scuba at a depth of 12 feet, in the southwest islands of Palau in 2008, and kept frozen until extraction. The collection was carried out by the Coral Reef Research Foundation, under contract with the National Products Branch, U.S. National Cancer Institute. A voucher specimen (voucher ID # 0CDN 9731) was deposited at the Smithsonian Institution, Washington, D. C.

Extraction and isolation – The frozen sponge sample (127 g wet wt.) was ground and extracted using the standard NCI method for marine samples to provide 4.4 g of organic solvent extract (NSC C029729).[28] A 3.0 g aliquot of the *Calyx* sp. organic extract was subjected to C_{18} reversed-phase flash column chromatography using a step gradient elution with 100% hexane (fraction A, 348 mg), 100% CH_2Cl_2 (fraction B, 217 mg), 100% EtOAc (fraction C, 75 mg), 100% acetone (fraction D, 397 mg), and 100% MeOH (fraction E, 1.1 g). Fraction D was further separated by preparative reversed-phase HPLC

(Agilent Dynamax C18 column, 21.4 mm × 250 mm; 9.0 mL/min, CH_3CN-H_2O gradient from 30:70 to 100:0), yielding 7 peaks rich in secondary metabolites. Purification of sub-fraction 1 was accomplished by semi-preparative HPLC (Phenomenex Luna C18 column, 10 mm × 250 mm; 2.0 mL/min, MeCN-H_2O gradient from 35:65 to 80:20) to yield calyxaprenol C (**3**, 1.1 mg), as an amorphous solid. Fraction E was separated by preparative reversed-phase HPLC (Agilent Dynamax C18 column, 21.4 mm × 250 mm; 9 mL/min, MeCN-H_2O gradient from 20:80 to 100:0), yielding 10 peaks rich in secondary metabolites. Further purification of the sub-fraction 1 by semi-preparative HPLC (Phenomenex Luna C18 column, 10 mm × 250 mm; 3.0 mL/min, MeCN-H_2O gradient from 50:50 to 100:0) afforded calyxaprenol D (**4**, 0.5 mg), while fractionation of sub-fraction 6 by the same semi-preparative HPLC method but with a MeCN-H_2O gradient from 55:45 to 100:0 yielded, in order of elution, calyxaprenol A (**1**, 2.0 mg), calyxaprenol B (**2**, 1.8 mg), and haliclotriol A (**5**, 4.2 mg) as amorphous solids.

Calyxaprenol A (1) – white, amorphous powder; $[\alpha]_D^{25}$ +7.5 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 215 (3.86), 271 (2.84) nm; IR (film) ν_{max} 3412, 2932, 1632, 1485, 1233, 1022 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 625.4108 $[M-H]^-$ (calcd for $C_{38}H_{57}O_7$, 625.4104).

Calyxaprenol B (2) – white, amorphous powder; $[\alpha]_D^{25}$ +6.9 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.19), 272 (3.96) nm; IR (film) ν_{max} 3339, 2938, 1649, 1483, 1240, 1020 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 639.4270 $[M-H]^-$ (calcd for $C_{39}H_{59}O_7$, 639.4261).

Calyxaprenol C (3) – colorless, amorphous solid; $[\alpha]_D^{25}$ -12.1 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 211 (3.48), 277 (2.81) nm; IR (film) ν_{max} 3450, 2930, 1642, 1597, 1243, 1040 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 575.4075 $[M+Na]^+$ (calcd for $C_{36}H_{56}O_4Na$, 575.4076).

Calyxaprenol D (4) – colorless, amorphous solid; $[\alpha]_D^{25}$ -10.9 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.12), 274 (2.74) nm; IR (film) ν_{max} 3402, 2935, 1677, 1592, 1242, 1045 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 557.3973 $[M+Na]^+$ (calcd for $C_{36}H_{54}O_3Na$, 557.3971).

Result and Discussion

The molecular formula of calyxaprenol A (**1**) was determined by HRESIMS to be $C_{38}H_{58}O_7$, with 10 degrees of unsaturation. Detailed examination of the ^1H and ^{13}C NMR data (Table 1) aided by 2D NMR experiments, revealed this compound possessed a 7/6/6/6/5/6-hexacyclic fused ring system (rings A-F) and an additional prenylated side chain. The partial structure of the indane ring system (rings E and F) was elucidated from *ortho* coupled aromatic protons H-3′ (δ_H 6.53)/H-4′ (δ_H 6.90) and HMBC correlations between H-1 (δ_H 2.61)/C-2, C-3, C-1′, C-2′, and C-6′, H-4′ (δ_H 6.90)/C-2′, C-6′, and C-7′, and H-7′ (δ_H 5.52)/C-4′, C-5′, C-6′, and C-8′ (Fig. 2). Collectively these correlations supported substitution of a hydroxy group at C-2′ (δ_C 154.7) and a glycolic acid residue at C-5′ (δ_C 126.0) in the tetrasubstituted aromatic ring. A partial structure of the decalin system (rings C and D) was established by COSY correlations between H_2-8/H_2-9, H_2-9/H-10, H_2-4/H_2-5, and H_2-5/H-6, and HMBC correlations from H_3-26 (δ_H 1.09)/C-2, C-6, C-7, and C-8. Fusion of the 5-membered ring (E ring) with the cyclohexane ring (D ring) was supported by HMBC correlations from the singlet methyl H_3-25 (δ_H 1.16) to C-2, C-6, C-7, and C-8. In the same manner, assignment of the fused oxepane-cyclohexane rings (A and B rings) was confirmed by COSY correlations between H_2-12/H_2-13, H_2-13/H-14, H_2-16/H_2-17, and H_2-17/H-18, and HMBC correlations from H_3-27 (δ_H 0.92)/C-6, C-10, C-11, and C-12, and H_3-28 (δ_H 0.84)/C-10, C-14, C-15, and C-16 (Fig. 2). Additional support for assignment of the oxepane ring (A ring) was provided by HMBC correlations from the singlet methyl H_3-29 (δ_H 1.17) to the C-18 (δ_C 75.8) oxymethine, the C-19 (δ_C 81.6) oxygenated quaternary carbon, and the C-20 (δ_C 43.1) methylene. The ether linkage in ring A was secured by an HMBC cross-peak from H-14 (δ_H 3.53) to C-19 (δ_C 81.6). Thus, the hexacyclic ring system of **1** was established. The terminal isoprene unit of the molecule was defined by COSY correlations between H_2-20/H_2-21 and H_2-21/H_2-22, as

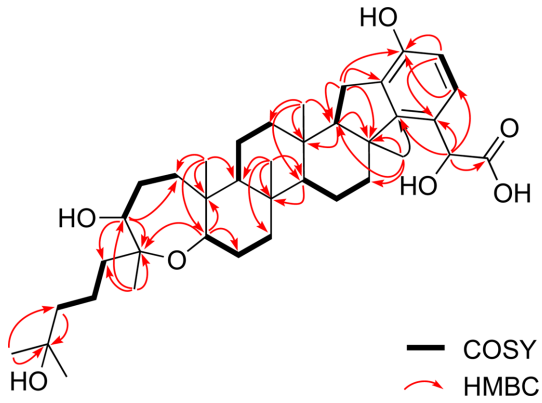

Fig. 2. Selected COSY and HMBC correlations for calyxaprenol A (**1**).

Table 1. ^{13}C NMR (150 MHz) and ^{1}H NMR (600 MHz) Data for Calyxaprenols A (**1**) and B (**2**) in MeOH-d_4

	1		**2**	
Position	δ_C (type)	δ_H mult (*J* in Hz)	δ_C (type)	δ_H mult (*J* in Hz)
1	25.4, CH_2	2.61, dd (14.6, 6.2)	25.3, CH_2	2.61, dd (14.4, 6.2)
		2.42, t (14.6)		2.42, t (14.4)
2	65.5, CH	1.70, m	65.5, CH	1.70, m
3	49.8, C		49.8, C	
4	40.4, CH_2	2.39, m	40.4, CH_2	2.39, m
		1.90, m		1.90, m
5	18.7, CH_2	1.63, m	18.7, CH_2	1.63, m
		1.54, m		1.58, m
6	62.5, CH	0.94, m	62.5, CH	0.95, m
7	38.4, C		38.4, C	
8	43.3, CH_2	1.71, m	43.3, CH_2	1.72, m
		1.15, m		1.14, m
9	19.8, CH_2	1.72, m	19.8, CH_2	1.71, m
		1.45, m		1.42, m
10	58.8, CH	0.89, m	58.8, CH	0.90, m
11	38.0, C		38.0, C	
12	39.9, CH_2	1.69, m	39.9, CH_2	1.70, m
		0.98, m		0.99, m
13	28.1, CH_2	1.57, m	28.1, CH_2	1.56, m
		1.37, m		1.38, m
14	78.2, CH	3.53, dd (11.4, 4.6)	78.2, CH	3.53, dd (11.5, 4.6)
15	42.1, C		42.1, C	
16	36.7, CH_2	1.50, m	36.7, CH_2	1.52, m
		1.43, m		1.44, m
17	27.0, CH_2	1.95, m	27.0, CH_2	1.95, m
		1.66, m		1.66, m
18	75.8, CH	3.78, d (6.7)	75.9, CH	3.76, d (6.8)
19	81.6, C		81.5, C	
20	43.1, CH_2	1.36, m	42.9, CH_2	1.36, m
		1.29, m		1.29, m
21	20.4, CH_2	1.45, m	18.7, CH_2	1.53, m
		1.34, m		1.42, m
22	45.4, CH_2	1.43, m	41.3, CH_2	1.48, m
		1.39, m		1.43, m
23	71.4, C		76.4, C	
24	29.2, CH_3	1.18, s	25.4, CH_3	1.15, s
25	22.4, CH_3	1.16, s	22.4, CH_3	1.16, s
26	17.7, CH_3	1.09, s	17.7, CH_3	1.09, s
27	16.8, CH_3	0.92, s	16.8, CH_3	0.92, s
28	14.2, CH_3	0.84, s	14.2, CH_3	0.84, s
29	19.4, CH_3	1.17, s	19.4, CH_3	1.17, s
30	29.2, CH_3	1.18, s	25.4, CH_3	1.15, s
1′	129.6, C		129.6, C	
2′	154.7, C		154.8, C	
3′	114.2, CH	6.53, d (8.3)	114.2, CH	6.53, d (8.3)
4′	127.5, CH	6.90, d (8.3)	127.5, CH	6.90, d (8.3)
5′	126.0, C		126.0, C	
6′	155.2, C		155.2, C	
7′	69.5, CH	5.52, s	69.5, CH	5.52, s
8′	175.9, C		175.9, C	
OCH_3			49.1, CH_3	3.18, s

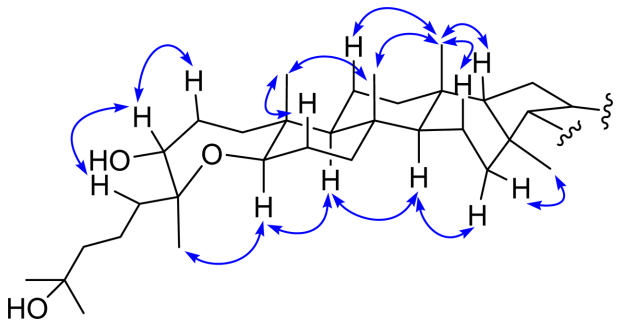

Fig. 3. Selected ROESY correlations for calyxaprenol A (**1**).

well as HMBC correlations from the *gem*-dimethyl group H_3-24 and H_3-30 (δ_H 1.18) to the C-23 (δ_C 71.4) oxygen-bearing quaternary carbon. The 6H singlet for these two methyl groups showed HMBC correlations to the C-24/C-30 (δ_C 29.2) carbon signal, which confirmed that they were germinal. Thus, the planar structure of calyxaprenol A (**1**) was established.

The relative configuration of **1** was elucidated from 2D ROESY NMR data (Fig. 3) and comparison with the NMR data for haliclotriol A (**5**).[12] The all *trans* fused A-B-C-D-E ring system was established by ROESY cross-peaks between H_3-28/H-13β and H_3-27, H_3-27/H_3-26, and H_3-26/H-9β, H-5β and H-2 which indicated all ring junction methyl groups were located on the β face of the molecule, with the exception of H_3-25. Additional ROESY cross-peaks at H-6/H-10 and H-4α, H-10/H-14, and H-14/H_3-29 were supportive of α-orientations for the A/B/C/D ring junction protons, as well as the H_3-29 methyl group. Around the D/E ring juncture, the carbon chemical shifts of C-1 (δ_C 25.4), C-2 (δ_C 65.5), C-3 (δ_C 49.8), C-4 (δ_C 40.4), C-7 (δ_C 38.4), C-25 (δ_C 22.4) and C-26 (δ_C 17.7) were virtually identical to those of haliclotriol A (**5**).[12] These similarities imply that **1** and **5** have the same boat and envelop conformations in the D and E rings, respectively. This relative relationship was further supported by ROESY correlations between H_3-26/H-2 and H_3-25/H-4β. Finally, the β-orientation of hydroxy methine proton H-18 was confirmed by H-18/H-20 and H-17β ROESY correlations. Thus, the relative configurations were assigned to be 2*S**, 3*S**, 6*R**, 7*S**, 10*S**, 11*S**, 14*R**, 15*R**, 18*R**, and 19*S**. Attempts to obtain additional stereochemical information were unsuccessful as efforts to generate MTPA esters of **1** resulted in decomposition. This completed the structural assignment of calyxaprenol A (**1**).

The molecular formula of calyxaprenol B (**2**) was established as $C_{39}H_{60}O_7$ through HRESIMS analysis, which differed by addition of CH_2 from the molecular formula of **1**. Based on the close similarity of its ^{1}H and ^{13}C NMR data (Table 1), the planar structure of **2** was concluded to be the same as that of **1**, and all of the subsequent 2D NMR data supported this assignment. The presence of a methoxy group in place of a hydroxy group in **2** was apparent from an additional three proton singlet at δ_H 3.18 that showed an HSQC correlation to a new carbon signal at δ_C 49.1. The deshielded chemical shift of the C-23 quaternary carbon (δ_C 76.4 in **2** and δ_C 71.4 in **1**) suggested the methoxy group was substituted at C-23, which was confirmed by HMBC cross-peaks from OCH_3, H_3-24, and H_3-30 to C-23. The ROESY data of **2** exhibited very similar proton-proton cross-peaks to those of **1**, which allowed assignment of all *trans* fused A-B-C-D-E ring junctions and an *anti* orientation between H_3-29 and H-18. Thus, the relative configuration of calyxaprenol B (**2**) was assigned as 2*S**, 3*S**, 6*R**, 7*S**, 10*S**, 11*S**, 14*R**, 15*R**, 18*R**, and 19*S**.

The molecular formula of calyxaprenol C (**3**) was determined by HRESIMS measurements to be $C_{36}H_{56}O_4$, with 9 degrees of unsaturation. Initial inspection of the ^{13}C NMR data (Table 2) revealed 10 carbons in the aromatic/olefinic region of the spectrum and 26 aliphatic carbon signals. These data suggested that calyxaprenol C (**3**) consisted of an aromatic ring joined to a tricyclic hexaprenoid which had two olefinic bonds. Analysis of the ^{1}H and ^{13}C NMR data revealed the aromatic portion of the molecule (ring D) had signals appropriate for a monosubstituted hydroquinone with C-4′, δ_C 113.2, δ_H 6.41 (1H, dd, J = 8.4, 3.1 Hz); C-3′, δ_C 116.1, δ_H 6.57 (1H, d, J = 8.4 Hz); C-6′, δ_C 116.7, δ_H 6.49 (1H, d, J = 3.1 Hz), C-1′ δ_C 129.8, C-2′ δ_C 148.3, and C-5′ δ_C 150.4, respectively. The position of the aromatic hydroxy groups was established by the following HMBC correlations: H-3′/C-1′ and C-5′, H-4′/C-2′ and C-6′, and H-6′/C-2′ and C-4′.

A decalin ring system (B and C rings) in **3** was assigned from characteristic ^{1}H and ^{13}C NMR signals and the COSY and HMBC data (Fig. 4). A connection between the hydroquinone ring and the hexaprenoid scaffold was established by HMBC correlations from the allylic and benzylic methylene proton signals of H_2-1 δ_H 3.33 (1H, d, J = 17.5 Hz) and δ_H 3.18 (1H, d, J = 17.5 Hz) to three aromatic carbons (C-1′, C-2′, and C-6′), two olefinic quaternary carbons C-2 (δ_C 139.0) and C-3 (δ_C 129.5), and a fully substituted aliphatic carbon C-7 (δ_C 40.2). Two deshielded signals at δ_C 139.0 and 129.5 were assigned to the C-2/C-3 tetrasubstituted olefin by HMBC correlations from H_3-25 (δ_H 1.50) to C-2, C-3, and the C-4 methylene signal (δ_C 34.8) and from H_3-26 (δ_H 1.05) to C-2, C-6, C-7, and C-8. Furthermore, the partial structure of the bicyclic ring system was confirmed by COSY couplings

Table 2. ^{13}C NMR (150 MHz) and ^{1}H NMR (600 MHz) Data for Calyxaprenols C (**3**) and D (**4**) in MeOH-d_4

	3		**4**	
Position	δ_C (type)	δ_H mult (*J* in Hz)	δ_C (type)	δ_H mult (*J* in Hz)
1	27.8, CH_2	3.33, d (17.5)	27.2, CH_2	2.65, dd (15.3, 9.6)
		3.18, d (17.5)		2.48, br d (15.3)
2	139.0, C		56.3, CH	2.36, br d (9.6)
3	129.5, C		137.2, C	
4	34.8, CH_2	2.19, m	122.6, CH	5.33, br s
		2.08, m		
5	20.3, CH_2	1.76, m	24.3, CH_2	1.96, m
		1.57, m		1.93, m
6	55.4, CH	1.35, m	54.1, CH	1.32, m
7	40.2, C		38.2, C	
8	37.4, CH_2	1.58, m	40.8, CH_2	1.95, m
		1.03, m		1.19, m
9	20.1, CH_2	1.54, m	19.9, CH_2	1.49, m
		1.32, m		1.43, m
10	38.1, CH_2	1.77, m	38.1, CH_2	1.75, m
		0.90, m		0.96, m
11	37.7, C		36.8, C	
12	34.0, CH_2	1.71, m	32.9, CH_2	1.78, m
		1.24, m		1.15, m
13	24.2, CH_2	2.03, m	25.7, CH_2	1.48, m
		1.80, m		1.25, m
14	137.2, C		49.9, CH	1.79, m
15	128.6, C		137.8, C	
16	31.9, CH_2	2.04, m	120.1, CH	5.26, br s
		1.99, m		
17	28.0, CH_2	1.69, m	33.4, CH_2	2.26, m
		1.67, m		1.92, m
18	72.1, CH	3.66, dd (9.6, 4.4)	71.8, CH	3.70, dd (8.8, 6.7)
19	44.9, C		41.4, C	
20	39.1, CH_2	1.61, m	36.4, CH_2	1.69, m
		1.31, m		1.27, m
21	19.9, CH_2	1.33, m	23.1, CH_2	1.99, m
22	45.6, CH_2	1.40, m	126.4, CH	5.14, t (6.7)
23	71.4, C		131.7, C	
24	29.1, CH_3	1.15, s	25.9, CH_3	1.69, s
25	20.3, CH_3	1.50, s	22.5, CH_3	1.44, s
26	21.6, CH_3	1.05, s	15.4, CH_3	0.95, s
27	29.4, CH_3	0.98, s	29.2, CH_3	0.86, s
28	20.1, CH_3	1.62, s	24.1, CH_3	1.71, s
29	21.9, CH_3	0.96, s	17.4, CH_3	0.85, s
30	29.2, CH_3	1.15, s	17.9, CH_3	1.65, s
1′	129.8, C		132.3, C	
2′	148.3, C		148.8, C	
3′	116.1, CH	6.57, d (8.4)	116.7, CH	6.55, d (8.5)
4′	113.2, CH	6.41, dd (8.4, 3.1)	113.5, CH	6.41, dd (8.5, 3.1)
5′	150.4, C		150.9, C	
6′	116.7, CH	6.49, d (3.1)	116.9, CH	6.64, d (3.1)

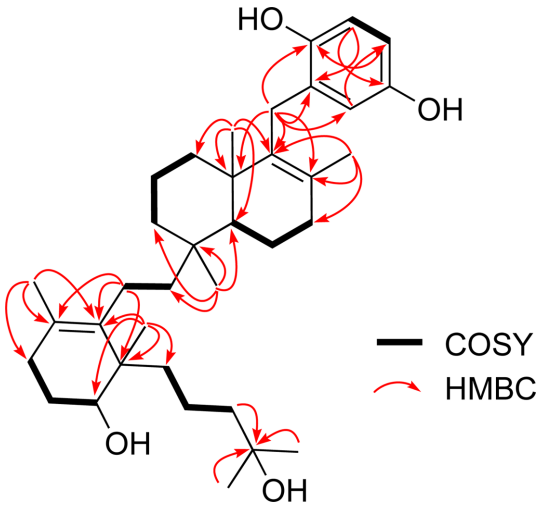

Fig. 4. Selected COSY and HMBC correlations for calyxaprenol C (**3**).

between H_2-4/H_2-5, H_2-5/H-6, H_2-8/H_2-9, and H_2-9/H_2-10, and additional HMBC correlations between H_3-27 (δ_H 0.98)/C-6, C-10, C-11, and a methylene signal at C-12 (δ_C 34.0). An ethylene bridge connecting ring B and a cyclohexene ring (A ring) was supported by H_2-12/H_2-13 COSY cross-peaks and HMBC correlations between H_2-13 and two quaternary olefinic carbons C-14 (δ_C 137.2)/C-15 (δ_C 128.6), as well as a fully substituted aliphatic carbon at C-19 (δ_C 44.9). The cyclohexene ring was further defined by HMBC correlations between H_3-28 (δ_H 1.62)/C-14, C-15, and a methylene signal at C-16 (δ_C 31.9), and H_3-29 (δ_H 0.96)/C-14, an oxymethine carbon at C-18 (δ_C 72.1), C-19, and a methylene carbon at C-20 (δ_C 39.1). A terminal alkyl chain was apparent from COSY correlations between H_2-20/H_2-21 and H_2-21/H_2-22, and HMBC correlations from H_2-22 as well as a *gem*-dimethyl group H_3-24 and H_3-30 (δ_H 1.15) to the C-23 (δ_C 71.4) oxygen-bonded quaternary carbon. Molecular formula considerations required hydroxy groups at C-18 and C-23, thus the planar structure of calyxaprenol C (**3**) was established.

The relative stereochemistry of **3** was assigned from 2D ROESY data (Fig. 5). ROESY correlations from H_3-26 to H-5β (δ_H 1.57), H-1 (δ_H 3.33), and H-12a (δ_H 1.24), together with correlations from H-6 (δ_H 1.35) to H-5α (δ_H 1.76), H-8α (δ_H 1.58), and H_3-27, establish the *trans*-fused decalin ring system and the axial orientation of the ethylene bridge. Additional cross-peaks from H-18 δ_H 3.66 (dd, J = 9.6, 4.4 Hz) to H-16α (δ_H 2.04) and H-20a (δ_H 1.31) were supportive of an α-orientation for the oxymethine proton. Moreover, a β-orientation of the H_3-29 (δ_H 0.96) methyl group was confirmed by H_3-29/H-17β

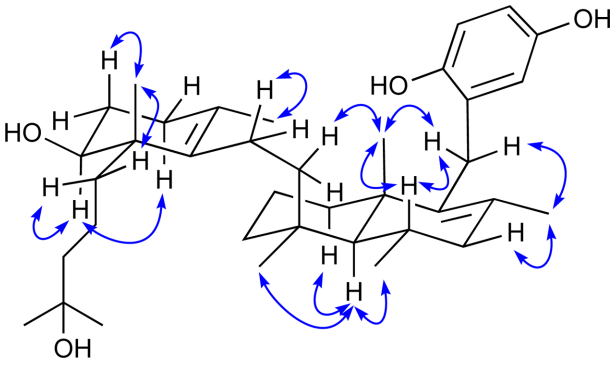

Fig. 5. Selected ROESY correlations for calyxaprenol C (**3**).

(δ_H 1.67) and H-20b (δ_H 1.61) ROESY correlations. (Fig. 5). From these data the relative configuration was assigned to be 6*S**, 7*S**, 11*R**, 18*S**, and 19*S**, which completed the structural elucidation of calyxaprenol C (**3**).

The molecular formula of calyxaprenol D (**4**), deduced to be $C_{36}H_{54}O_3$ from HRESIMS analysis, represented a loss of H_2O from the molecular formula of **3**. The ^1H and ^{13}C NMR data of **4** were similar to those of **3** except for the addition of three proton signals at δ_H 5.33 (1H, br s), δ_H 5.26 (1H, br s), and δ_H 5.14 (1H, t, J = 6.7 Hz) corresponding to three different trisubstituted olefins. The position of H-4 (δ_H 5.33) in the C ring was determined by HMBC correlations from the olefinic methyl signal at H_3-25 (δ_H 1.44) to carbon signals at C-2 (δ_C 56.3), C-3, and C-4 and the methyl signal at H_3-26 (δ_H 0.95) to C-2, C-6, C-7, and C-8. These correlations indicated that C-2 was flanked by a trisubstituted olefin on one side and a ring junction bearing a methyl group on the other side. In a similar manner, comparison with the NMR data of **3** revealed a double bond migration in ring A had occurred from C-14/C-15 to C-15/C-16 in **4**, and this was supported by HMBC correlations from the methyl signal at H_3-28 (δ_H 1.71) to an sp^3 methine carbon C-14 (δ_C 49.9), a fully substituted olefinic carbon C-15 (δ_C 137.8), and a protonated olefin carbon C-16 (δ_C 120.1). The 4-methyl-3-pentenyl substructure was elaborated based on COSY correlations between the allylic H_2-21 protons and the vinyl methine H-22 (δ_H 5.14, t, J = 6.7 Hz), as well as H_2-20. The partial structure was completed through HMBC correlations from the geminal vinylic methyls H_3-24 (δ_H 1.69) and H_3-30 (δ_H 1.69) to C-22, C-23, C-24, and C-30. The ROESY data of **4** showed very similar proton-proton cross-peaks to those of **3**. ROESY correlations from H_3-26 to H-5β (δ_H 1.93), H-1 (δ_H 2.48), and H-12a (δ_H 1.78), together with correlations from H-6 (δ_H 1.32) to H-2 (δ_H 2.36) and H_3-27 (δ_H 0.86), established the *trans*-fused B/C ring system and the axial position of the

ethylene bridge substituted at C-11. Additional cross-peaks between H-18 (δ_H 3.70)/H-14 (δ_H 1.79) and H-20a (δ_H 1.27), and H_3-29 (δ_H 0.85)/H-17β (δ_H 1.92) and H-20b (δ_H 1.69) were supportive of α-orientations for H-14 and H-18 and a β-orientation for H_3-29, respectively. The relative configuration was thus assigned to be 2*S**, 6*S**, 7*S**, 11*R**, 14*R**, 18*S**, and 19*S** and the structure of calyxaprenol D (**4**) was established.

The known compound haliclotriol A (**5**) was also purified from the *Calyx* sp. extract and it was identified by comparison of its NMR data with published values.[12] Calyxaprenols A (**1**) and B (**2**) are related to **5** by addition of H_2O and MeOH, respectively, to the trisubstituted sidechain olefin in **5**. They are more distantly related to halicolic acid A, which is also a glycolic acid-containing merohexaprenoid that incorporates an oxepane ring, but it has a different carbon skeleton than the calyxaprenols.[13] Identification of the calyxaprenol merohexanprenoids from a sponge in the genus *Calyx* expands the known taxonomic distribution of this important class of marine metabolites. It also increases the chemical diversity of *Calyx* sponges and suggests that further chemical investigation of these sponges may prove fruitful. Merohexaprenoids such as the calyxaprenols represent hybrid biosynthetic products that incorporate components of both terpenoid and polyketide biosynthesis. While there have been numerous studies of the biosynthesis of hybrid nonribosomal peptide and polyketide metabolites, the biogenesis of hybrid terpenoid and polyketide compounds remains relatively under explored. Considering the range of important biological activities associated with merohexaprenoids, a better understanding of the biosynthetic processes and pathways associated with their production could provide future opportunities for enhanced natural product discovery efforts.

Acknowledgments

Acknowledgement goes to the Natural Products Support Group (Frederick National Laboratory for Cancer Research) for extract preparation. This project has been funded in whole or in part by the Intramural Research Program of NIH, Frederick National Laboratory for Cancer Research, Center for Cancer Research. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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Received September 19, 2020
Revised November 24, 2020
Accepted November 26, 2020