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THE STRUCTURE OF EUNIOLIDE, A NEW CEMBRANOID DITERPENE FROM THE CARIBBEAN GORGONIANS *EUNICEA SUCCINEA* AND *EUNICEA MAMMOSA*.[1]

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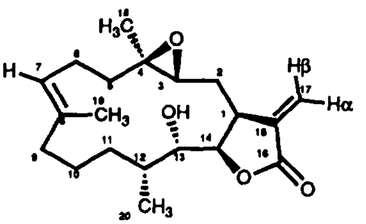
Abstract: Three simple γ-lactonic cembranolides were isolated from the gorgonian *Eunicea succinea* collected near Palomino Key, Puerto Rico on July, 1989. The structure of two were determined from spectral data and chemical correlation experiments to be those of known 12,13-bisepieupalmerin (**1**) and eunicin (**3**). The structure of the third metabolite was deduced from spectroscopic data and from chemical degradation experiments to be that of euniolide (**2**), a previously undescribed γ-lactonic cembranolide. Extraction of a fresh specimen of *Eunicea mammosa* collected near Desecheo Island, Puerto Rico on March, 1989 also afforded euniolide in addition to known cembranoid diterpene eupalmerin acetate (**4**).

The diterpene skeleton most frequently encountered in coelenterates is the cembranoid system, which contains a 14-membered carbocycle.[2] Cembrane-type diterpenes are generally the major components of the lipid fraction of marine coelenterates such as soft corals (alcyonarians) and, to lesser extent, gorgonians.[3] In the course of our studies of the secondary metabolites of Caribbean gorgonians, we undertook an extensive re-investigation of the genus *Eunicea* (family Plexauridae) with particular reference to the distribution of terpenes across various species collected at various locations around Puerto Rico and throughout the Caribbean Sea. We have earlier reported the isolation of diterpenes of the dolabellane class from several specimens of the genus *Eunicea* .[4] We now wish to report the isolation and structural elucidation of a further cembranolide diterpene, euniolide (**2**), from two distinct species of *Eunicea* isolated in the course of these investigations.

Extraction of a freshly collected specimen of the gorgonian *Eunicea succinea* collected at Palomino Key, Puerto Rico on July, 1989 afforded a rich chloroform extract (8.5 g, 3.4% of the dry weight of the animal), from which the major non-lipid component **1** was isolated after successive chromatography on a Sephadex LH-20 column (1:1 $CHCl_3$/MeOH) and silica gel column (70:30 hexane/EtOAc). This component (1.04 g), a white crystalline solid, mp 144-147°C, represents 4.8% of the total organic extract, and after further spectral analyses (^1H- and ^{13}C-NMR, IR, UV, and MS) was identified as previously reported 12,13-bisepieupalmerin (**1**).[5] Table I shows the complete assignment of all carbon and hydrogen atoms in **1** as deduced from heteronuclear correlation methods, COSY spectra and double resonance NMR spectroscopy.

Repeated chromatography of the less polar fraction on silica gel (95:5 hexane/EtOAc) afforded a colorless oil (296 mg, 1.4%) which after spectroscopic and chemical degradation studies was subsequently identified as the new γ-lactonic cembranolide euniolide (**2**).[6] Further elution yielded a homogeneous oily component (181 mg, 0.83%) which after detailed spectroscopic analyses (^1H- and ^{13}C-NMR, UV, MS, and IR) and chemical correlation experiments was characterized as known oxa-bridged cembranoid diterpene eunicin (**3**).[7]

A fresh specimen (March, 1989) of *Eunicea mammosa* from Desecheo Island, Puerto Rico, collected at a depth of 42-52 ft, was extracted with methanol. TLC assays indicated that this gorgonian contained only two significant metabolites. The initial extract (MeOH) was partitioned after concentration between hexane and water and the crude oily residue obtained after filtration and concentration of the hexane was successively purified by size exclusion chromatography on a Bio-Beads S-X2 column (toluene) and Sephadex LH-20 column (1:1 $CHCl_3$/MeOH). Silica gel chromatography (95:5 hexane/EtOAc) of a portion (4.08 g) of the terpene-containing fractions gave euniolide (**2**) (705 mg, 8.3% of the total organic extract; identified by comparison of its UV, IR, ^1H- and ^{13}C-NMR, and mass spectra with material isolated earlier from *E. succinea*) followed by the known cembranoid diterpene eupalmerin acetate (**4**)[8] (1.41 g, 16.7%; identified by two-dimensional NMR techniques, including 2D-homo- and heteronuclear chemical shift correlation spectroscopy, UV, IR, ^1H- and ^{13}C-NMR, and mass spectra).



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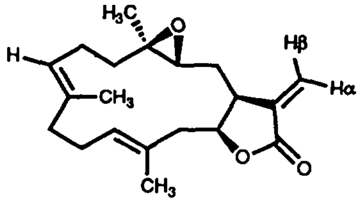

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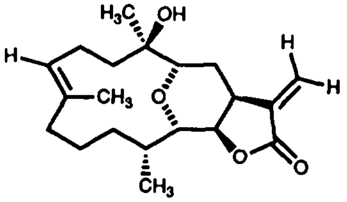

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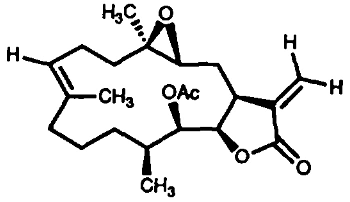

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Characterization of euniolide (2) was carried out on a chromatographically homogeneous sample: an optically active oil, $[\alpha]_D^{26}$ = +30°, of formula $C_{20}H_{28}O_3$ as determined by high-resolution mass measurement of its molecular ion. After prolonged storage at -10°C, however, euniolide crystallized from a $CHCl_3$/MeOH mixture, mp 122-125°C. The molecular formula required seven degrees of unsaturation: the ^{13}C-NMR spectrum contained a singlet at δ 169.29 ppm (lactone carbonyl) and six additional sp^2 carbons (three singlets, two doublets and one triplet). The molecule is thus tricyclic and contains a vinyl methylene group. The IR and ^{1}H-NMR spectra indicate the presence of an α-methylene-γ-lactone: IR 1760, 1666 cm^{-1}; ^{1}H-NMR δ 6.25, 1H, d, J = 1.6 Hz and 5.65, 1H, d, J = 1.6 Hz. In addition, the ^{1}H-NMR spectrum ($CDCl_3$) showed two methyl-bearing trisubstituted double bonds (δ 1.64, 3H, br s; 1.56, 3H, br s; 5.07, 1H, br t, J = 6.5 Hz; 4.91, 1H, br d, J = 10.1 Hz), a methyl group on a carbon bearing oxygen (δ 1.27, 3H, s), an allylic methine proton (δ 3.06, 1H, ddd, J = 1.6, 5.2, 11.0 Hz), a lactonic methine proton (δ 4.76, 1H, ddd, J = 2.8, 5.7, 10.6 Hz) and an epoxymethine proton (δ 2.80, 1H, dd, J = 3.8, 9.6 Hz). Irradiation of the proton at δ 3.06 (H1) collapsed the doublets at δ 5.65 (H17β) and 6.25 (H17α) ppm to sharp singlets and the lactonic methine proton at δ 4.76 ppm (H14) to a doublet of doublets (J = 2.8, 10.6 Hz). These findings revealed that the lactonic methine proton (H14) at δ 4.76 ppm is also coupled to the methylene protons at position 13 (δ 2.38 and 2.56) and since the connectivity of the C13 protons stopped there, thus C12 (δ 130.31) must be a quaternary carbon. These results confirm the homoallylic nature and ring size of the lactone. The C13-C14 connectivity was confirmed by double resonance experiments on the H13 resonances and by autocorrelated proton two-dimensional NMR since crosspeaks connecting the C13 protons and H14 are observed in the contour plot of the ^{1}H-^{1}H COSY spectrum of euniolide (2). Additional experimental evidence supporting the C13-C14 connectivity is based on strong responses visible in the contour plot of the NOESY spectrum of 2. During irradiation of the proton at δ 3.06 (H1) modified absorption patterns were also observed distinctively around δ 2.28 and 1.40 ppm, thereby confirming the location of the methylene protons in position C2. A nearby epoxide ring was indicated (δ 60.36 (s), 59.40 (d); δ 2.80, dd, J = 3.8, 9.6 Hz, epoximethine) also by double resonance NMR experiments. Irradiation of the protons at δ 2.28 and 1.40 ppm (H2) collapsed the doublet of doublets at δ 2.80 ppm (H3) to a doublet (J =9 Hz) and a broad triplet, respectively. These double resonance experiments confirmed the chain of coupling between H3 and the protons around the α-methylene-γ-lactone ring including H13 and H14 and the more remote exomethylene protons H17αβ. The presence of a 3,4-epoxide was thus established as shown in partial structure **A**. Microozonolysis of euniolide afforded one mole of levulinic acid which confirmed the remaining ring to be cembranoid and also established unequivocally the position of the double bonds within the polyisoprenoid chain as shown. With this chemical degradation experiment the structure of euniolide devoid of stereochemistry was unambiguously confirmed as **2**.

Table I. ^{1}H- and ^{13}C-NMR Spectral Data Obtained for 12,13-Bisepieupalmerin (1) and Euniolide (2) in Deuteriochloroform at Observation Frequencies of 300.07 and 75.46 MHz, Respectively, at 24°C.

	12,13-bisepieupalmerin (1)[a]		euniolide (2)[a]	
position	^{13}C(mult)[b,c]	^{1}H (mult, J(Hz), intgrtn)[c]	^{13}C(mult)[b,c]	^{1}H (mult, J(Hz), intgrtn)[c]
1	39.37(d)	3.38(m,1H)	41.42(d)	3.06(ddd,1.6,5.2,11.0,1H)
2	30.79(t)	2.15(m,1H)	30.85(t)	2.28(m,1H)
		2.01(m,1H)		1.40(m,1H)
3	60.01(d)	2.57(m,1H)	59.40(d)	2.80(dd,3.8,9.6,1H)
4	59.83(s)		60.36(s)	
5	37.98(t)	2.08(m,1H)	37.65(t)	2.05(m,1H)
		1.17(m,1H)		1.35(m,1H)
6	23.47(t)	2.18(m,2H)	22.73(t)	2.18(m,1H)
				2.01(m,1H)
7	124.56(d)	4.99(t,5.1,1H)	124.87(d)	5.07(brt,6.5,1H)
8	136.01(s)		134.97(s)	
9	37.19(t)	2.07(m,2H)	38.95(t)	2.25(m,2H)
10	31.43(t)	1.58(m,2H)	25.09(t)	2.35(m,1H)
				2.12(m,1H)
11	23.24(t)	1.78(m,1H)	126.08(d)	4.91(brd,10.1,1H)
		1.42(m,1H)		
12	31.61(d)	2.10(m,1H)	130.31(s)	
13	71.94(d)	3.75(m,1H)	37.56(t)	2.56(brd,16.8,1H)
				2.38(brd,16.0,1H)
14	78.52(d)	4.27(brt,1H)	79.28(d)	4.76(ddd,2.8,5.7,10.6,1H)
15	138.96(s)		141.19(s)	
16	169.96(s)		169.29(s)	
17	123.59(t)	6.41(brs,1H)	120.86(t)	6.25(d,1.6,1H)
		5.77(brs,1H)		5.65(d,1.6,1H)
18	16.44(q)	1.22(s,3H)	17.14(q)	1.27(s,3H)
19	15.61(q)	1.55(s,3H)	15.84(q)	1.56(s,3H)
20	12.19(q)	0.89(d,6.8,3H)	17.41(q)	1.64(s,3H)

[a] Chemical shifts are reported in ppm downfield from tetramethylsilane.

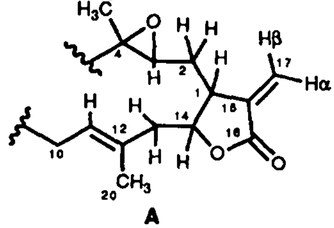
[b] Resonance multiplicities were determined using the APT experiment and are denoted as s, d, t and q for singlet, doublet, triplet and quartet, respectively.

[c] Assignments were aided by ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, Long-Range HETCOR and homonuclear spin-decoupling experiments.

Table II. Cytotoxicity of Compounds 1 - 4 from *Eunicea* against CHO-K1 Cells.

Agent	ED_{50} (µg/mL)
12,13-Bisepieupalmerin (1)	4.80
Euniolide (2)	3.80
Eunicin (3)	4.14
Eupalmerin acetate (4)	9.30

Previous investigations of the cembranolide diterpene series have indicated that it is not possible by NMR analysis to define the relative stereochemistries around the α-methylene-γ-lactone ring based on the magnitude of the $J_{1,14}$ coupling constant.[9] Because of the high flexibility of the cembrane ring, coupling constants around the five-membered lactone ring do not permit unequivocal stereochemical assignments. Moreover, the same studies have shown that the chemical shift differences for protons in the vicinity of the γ-lactone ring do not reflect configuration changes either. Therefore, in the absence of X-ray crystallographic evidence for structure **2**,[10] it seems unwise to speculate further on the relative configuration of these groups on the basis of coupling constant and chemical shift considerations. However, the relative stereochemical orientation of pairs of protons H1/H14, H1/H3 and H3/H18 was established through one-dimensional nuclear Overhauser difference spectra (nOeds). Thus, the H1/H14, H1/H3 and H3/H18 pairs are oriented as shown on the basis of 8.3%, 3.0%, and 2.0% nOe's, respectively. The absolute orientation shown is taken from the X-ray diffraction study reported previously for synthetic **2** by Marshall.[6] Finally, in the ^{13}C-NMR spectrum of euniolide the methyl resonances occur upfield of 20 ppm indicating the E-configuration of the trisubstituted double bonds.[11]



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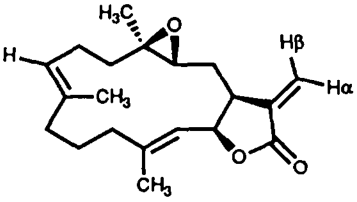

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In an effort to provide additional evidence on the stereostructure as well as an understanding of the conformational properties of **2** we undertook a series of experiments to correlate the structure of euniolide with those of **1** and **4**. Attempts to convert 12,13-bisepieupalmerin (**1**) into euniolide (**2**) via formation of intermediate **5** by successive dehydration and double bond migration resulted in concomitant transannular back-side attack of the C13 hydroxyl group at C3 of the epoxide leading exclusively to eunicin (**3**). Thus, cembranolide **1**, the principal metabolite from *E. succinea* has the proper stereochemistry to be the logical biosynthetic precursor to eunicin (**3**).[5] Further attempts to dehydrate **1** into **5** under mild acidic conditions (SiO_2, P_2O_5) led also to exclusive formation of the oxa-bridged diterpene. Additional attempts to convert eupalmerin acetate (**4**) into **5** by base-induced β-elimination led instead to intractable polar mixtures.

In the present work we have demonstrated the presence of new cembranoid diterpene **2** in two Caribbean gorgonians of the genus *Eunicea* and provided evidence to establish **1** as a logical biosynthetic precursor to eunicin (**3**). Cytotoxic activities (ED_{50}, μg/mL) of the present cembranolide diterpenes against CHO-K1 cells are shown in Table II. Further comparative analyses of the distribution of terpenes across various species of the genus *Eunicea* are currently underway.

Experimental

General Experimental Procedures. The ^{1}H- and ^{13}C-NMR spectra were recorded on a General Electric Multinuclear QE-300 in $CDCl_3$. ^{1}H chemical shifts are referenced to the residual $CHCl_3$ signal (7.24 ppm) and ^{13}C chemical shifts are referenced to the solvent peak (77.0 ppm). IR spectra were recorded in KBr or as neat films on a Nicolet 600 FT-IR spectrophotometer. UV spectra were recorded on a Hewlett-Packard Chem Station 8452A spectrometer. Optical rotations were determined on a Perkin-Elmer Polarimeter Model 243B. Low-resolution mass spectra were recorded at 70eV on a Hewlett-Packard 5995A mass spectrometer. Ozone was generated with a Polymetrics, Inc. Ozonator Model T-408. GLC-MS analyses were performed on a Hewlett-Packard 59970 MS Chem Station. Analtech Uniplate Si gel analytical TLC precoated plates (250μ) were used for routine TLC assays. Either Aldrich Si gel (230-400 mesh) or Analtech 35-75 micron Si gel were used for flash chromatography. All solvents used were either spectral grade or were distilled from glass prior to use.

Isolation.- ***Eunicea succinea*** was collected from Palomino Key, Puerto Rico at a depth of 20-40 ft. The freeze-dried gorgonian (249.5 g) was extracted with MeOH (3 X 1500 mL) to give 36.9 g of a dark green oil. After prior extraction with hexane the MeOH extract was partitioned between $CHCl_3$ and H_2O and the residue obtained from $CHCl_3$ after filtration and concentration (8.5 g) was fractionated by size exclusion chromatography on a 185 cm X 150 mm column of Sephadex LH-20 using $CHCl_3$-MeOH (1:1). The diterpene-containing fractions were combined on the basis of TLC assay considerations and after concentration, the colorless oily residue (5.0 g) was fractionated further by flash chromatography on a 120 cm X 50 mm column of Si gel using successively 10% EtOAc/hexane, 30% EtOAc/hexane, 50% EtOAc/hexane, and 100% EtOAc (250 mL of each fraction). The fraction eluting with 10% EtOAc/hexane contained euniolide (**2**) and eunicin (**3**), and the fraction eluting with 30% EtOAc/hexane contained 12,13-bisepieupalmerin (**1**). Concentration of the latter fraction gave an amorphous white solid which after crystallization from hexane/benzene mixtures afforded pure crystals of **1** [1.04 g, mp 144-147°C; $[\alpha]_D^{29}$ = -149.5° (c = 3.86, $CHCl_3$)] [lit.[5] mp 144-145°C; $[\alpha]_D$ = -158.8° (c = 1.57, $CHCl_3$)]. The crystals were identified further as **1** by comparison of their IR, ^1H-NMR, ^{13}C-NMR, and mass spectral data with reported values. Rechromatography of the less polar fraction on Si gel using 5% EtOAc/hexane yielded euniolide (296 mg) and eunicin (181 mg), both as colorless oils. However, after prolonged storage at -10°C both natural products crystallized slowly. Identification of eunicin [mp 149-150°C; $[\alpha]_D^{29}$ = -84.7° (c = 2.44, EtOH)] [lit.[7] mp 150-152°C; $[\alpha]_D^{29}$ = -95° (c = 2.0, EtOH)] was established also by comparison of the IR, ^1H-NMR, ^{13}C-NMR, and mass spectral data of the crystals with values reported elsewhere.[7]

Physical and Spectral Data for Euniolide (2).- Crystalline solid: mp 122-125°C; $[\alpha]_D^{26}$= +30° (c = 0.15, $CHCl_3$); UV ($CHCl_3$) λ_{max} 242 nm (ε 350); IR (neat) 2900, 1778, 1760, 1666, 1437, 1406, 1338, 1265, 1154, 1119, 989, 939, 873, 807 cm^{-1}; ^1H- and ^{13}C-NMR see Table I; EIMS m/z (rel. int.) 316(2), 283(1), 248(1), 164(7), 131(21), 119(26), 107(31), 79(19), 69(100); HREIMS m/z 316.2027 ($C_{20}H_{28}O_3$, $[M]^+$, -3.4 mmu error), 301.1785 ($C_{19}H_{25}O_3$, $[M-CH_3]^+$, -6.1 mmu error), 298.1940 ($C_{20}H_{26}O_2$, $[M-H_2O]^+$, 2.2 mmu error).

Extraction of *Eunicea mammosa*. - A single specimen was collected from Desecheo Island, Puerto Rico, at a depth of 42-52 ft. The wet animal (384.5 g) was extracted with MeOH (3 X 500 mL) and the resulting suspension was filtered and concentrated to give 17.46 g of a green oily residue. After partitioning between hexane and water, the crude hexane extract (13.42 g) was fractionated successively on a 185 cm X 150 mm column of Bio-Beads SX-2 (toluene) (8.64 g) and a 185 cm X 150 mm column of Sephadex LH-20 using $CHCl_3$/MeOH (1:1). A portion (4.08 g) of the cembranolide-containing fractions was fractionated further by column chromatography on a 230 cm X 50 mm column of Si gel using successively 5% EtOAc/hexane, 10% EtOAc/hexane, 30% EtOAc/hexane and 100% EtOAc. Combination of like fractions on the basis of TLC analyses eluting with 5% EtOAc/hexane afforded pure euniolide (0.71 g, colorless oil) and eupalmerin acetate (**4**) as an amorphous white solid. Recrystallization of **4** from hexane/benzene mixtures afforded the pure cembranolide [1.41 g, mp 151-153°C; $[\alpha]_D^{25}$ = +8.3° (c = 1.76, $CHCl_3$)] [lit.[2] mp 157-159°C; $[\alpha]_D^{25}$ = +8.0°]. Since the spectral data of eupalmerin acetate have not been recorded in the literature we established its identity by a careful and detailed spectral investigation of its molecular structure.[12]

Ozonolysis of Euniolide (2).- Ozone in oxygen (2%) was bubbled for 4-5 min through a solution of euniolide (**2**) (23 mg) in EtOAc (10 mL) at -78°C. The ozonide was decomposed after removal of the solvent under reduced pressure upon addition of H_2O (10mL) followed by heating to 80°C for 1h in the presence of a few drops of H_2O_2. After extraction with ether and concentration *in vacuo* the various oxo compounds were analyzed by GC-MS on a capillary HP-1 (cross-linked methyl silicone gum) column (12 m, 0.20 mm) and the temperature was programmed from 35 to 250°C at 10°C/min. The retention time of the major component was 9.7 min. Under identical conditions an authentic sample of levulinic acid showed identical retention time and mass spectral fragmentation patterns.

Conversion of 12,13-Bisepieupalmerin (1) into Eunicin (3).- To a solution of cembranolide **1** (30 mg, 0.09 mmole) in anhydrous benzene (15 mL) was added P_2O_5 (15 mg). The resulting suspension was stirred at 25°C for 10 min. After concentration *in vacuo* at 35°C, the residue was taken up with 10% EtOAc/hexane and passed through a short (25 cm X 35 mm) column of Si gel. The solvent was evaporated to yield a homogeneous residue by TLC assay (15 mg) which was subsequently identified as eunicin by comparison of its IR, ^1H-NMR, mass spectrum and R_f data with values recorded on authentic material isolated earlier from *E. succinea* . The conversion of **1** into **3** was also accomplished under the following reaction conditions: (a) $SiO_2/CHCl_3$ at 25°C for 3-4 days and (b) TsCl/Py in benzene at reflux for 12h.

Acknowledgements

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