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New cytotoxic cembranolides: isolation, biogenetic studies, and synthesis of analogues

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Abstract—Three new diterpene cembranes (**1**–**3**) along with eunilode (**4**) were isolated from the organic extracts of *Eunicea mammosa*. Biogenetic studies using a cell-free extract demonstrated that hydroxy cembrane **3** is the precursor to the ether-bridged **1**. In order to deduce further structure–activity relationships a series of analogues were synthesized and their cytotoxicity against several cancer cell lines was evaluated.
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1. Introduction

Specimens belonging to the genus *Eunicea* have been subjected to numerous chemical investigations yielding a wide variety of diterpenoids, most of them belonging to the oxygenated 14-membered ring cembranoid family.[1]

In our continuing investigations on the study of cytotoxic metabolites from gorgonians[2] and the biosynthetic origin of marine terpenes,[3] particularly on cembranes, we have focused our attention on the Caribbean gorgonian octocoral *Eunicea mammosa*, collected in Bahamas, as it is one of the richest sources of marine cembranoid diterpenoids. Both naturally occurring *Eunicea* cembranoids and some of their synthetic analogues have showed remarkable biological activity as cytotoxic agents.[4] In this paper, we wish to report the structural elucidation of three new cembranoid-diterpenes from *E. mammosa*, some biogenetic studies using its cell-free extract, and chemical transformations to obtain some analogues. Furthermore, the cytotoxic evaluation of both natural and synthetic cembranoids allowed us to enlarge the structure–activity relationships of this type of compounds.

2. Results and discussion

2.1. Isolation

Specimens of *E. mammosa* were collected by hand using SCUBA on Sweetings Cay in the Bahamas during the 1999 FAU expedition. Freshly collected animals were frozen on site and transported back to Florida. Cold specimens were thawed and exhaustively extracted with MeOH to obtain an extract that was fractionated using our standard partitioning procedure. The richest cembrane portion was chromatographed on a silica gel flash column using hexanes/acetone mixtures to yield an enriched diterpene fraction, which was then submitted repeatedly to reverse-phase HPLC (H_2O/MeOH mixtures) to give pure compounds **1**–**3** (Fig. 1).

The molecular formula of $C_{20}H_{30}O_4$ for **1** was obtained from the combination of the ^{13}C NMR data and the HRFABMS of the pseudomolecular ion $[M+Na]^+$ at *m/z* 357.2044, thus indicating six degrees of unsaturation in the molecule. Characteristic features of this new diterpene were defined by ^1H NMR, ^{13}C NMR, DEPT, gradient edited HSQC, HMBC, and DQCOSY experiments. Structural elucidation of **1** begun

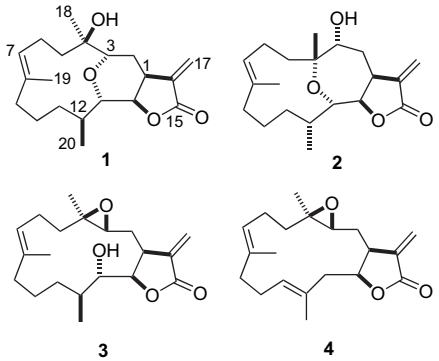

Figure 1. Structures of the cembranoid-diterpenes isolated from *E. mammosa*.

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with the identification of an α-methylene-γ-lactone group in **1**, which was suggested by the proton resonances at δ_H 6.38 (1H, d, J=3.4 Hz) and 5.60 (1H, d, J=3.4 Hz) due, respectively, to H-17α and H-17β, an allylic methine proton at δ_H 3.37 (1H, m), a lactone methine proton at δ_H 4.38 (1H, dd, J=8.0, 8.9 Hz), and a carbon resonance at δ_C 169.9 for a carbonyl lactone. Two ^{13}C NMR sp^2 signals at δ_C 130.9 (s, C-8) and 128.3 (d, C-7) and the methyl group at δ_C 15.9 (q, C-19) suggested the presence of a trisubstituted double bond, which was confirmed by the presence of an olefinic proton at δ_H 5.10 (1H, dd, J=5.9, 9.3 Hz) and an additional vinyl methyl group at δ_H 1.58 (3H, s, H-19). These functionalities accounted for four degrees of unsaturation.

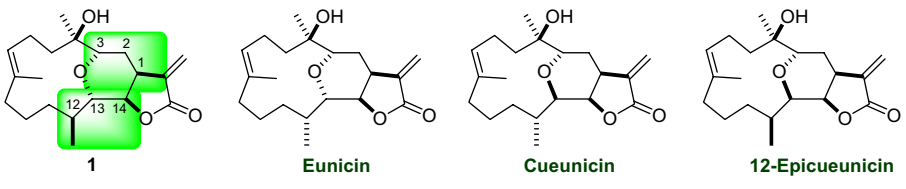
Three additional oxygenated carbons were deduced from the quaternary carbon signal at δ_C 73.4 (s) and the methine carbon signals at δ_C 76.7 (d) and 79.6 (d), which correlated the gradient edited HSQC responses to the oxygenated methines at δ_H 3.20 (1H, dd, J=3.7, 10.0 Hz) and 2.79 (1H, t, J=8.9 Hz). More direct proton–carbon correlations also showed the existence of two other methyl groups from the two quartet signals at δ_c 24.6 and 14.8 attached to the corresponding proton signals at δ_H 1.15 (s) and at δ_H 0.99 (d, J=6.8 Hz). The remaining unsaturations were assigned to a 14-membered ring, which bears an ether bridge between C-3 and C-13 positions. This connectivity was deduced by key HMBC correlations observed between the pairs H3–C13 and/or H13–C3. In this way the isolated H1–H2–H3/H1–H14–H13 spin-systems deduced by the DQCOSY experiment were assigned in a six-membered ring.

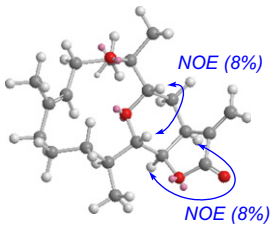
The comparison of these data to those of other cembranoids reported in the literature, indicated that **1** has the same planar structure as eunicin,[5] also isolated from *E. mammosa*, cueunicin isolated from *Eunicea succinea*,[6] and 12-*epi*cueunicin, a synthetic compound obtained from eupalmerin.[4]

To find out the relative stereochemistry of **1** proton–proton coupling constants, NOE differences, NOESY, GMMX calculations, and comparison with the above mentioned cembranes (see Fig. 2) were used. The presence of an observed NOE (8%) between H-1 and H-14 protons, and the coupling constant of 8.0 Hz, which was similar to that found in eunicin ($J_{1,14}$=7.8 Hz) and 12-*epi*cueunicin ($J_{1,14}$=8.7 Hz), clearly suggested a cis configuration for these protons. Also the presence of a NOE difference of 8% between H-13 and H-3 indicated that these protons are on the same face of the molecule assigned arbitrarily as β. This disposition ruled out the relative stereochemistry present in cueunicin and 12-*epi*cueunicin, where those protons are on the opposite faces of the molecule. Further comparison of the NMR data of compound **1** to those of eunicin suggested the same stereostructure, but epimeric at C-12. The proposed stereochemical assignment at this carbon was based on the observation of the different multiplicity of H-13. Thus, while the chemical shift of H-13 in **1** appears as a triplet (J=8.9 Hz), the same proton was observed in the ^{1}H NMR spectrum of eunicin as doublet with coupling constants of 9.5 Hz (J_{13-14}) and 0 Hz (J_{12-13}). This fact suggests an *anti* relative configuration between H-13 with H-12 in **1** instead of *syn* in eunicin. Besides, GMMX energy search minimization showed a low-energy conformer with similar values for the involved proton–proton coupling constants corresponding to the six-membered ring and H-12, and therefore confirmed the relative stereochemistry for all chiral centers (see Fig. 2). As a result of all these data, we have deduced the three dimensional arrangement of this compound, which has been named as 12-*epi*eunicin.

For the new cembranolide **2**, the molecular formula $C_{20}H_{30}O_4$ was obtained from its HRFABMS, which showed the pseudomolecular ion $[M+Na]^+$ at m/z 357.2040. The ^{1}H and ^{13}C NMR spectra of **2** had many features in common with those of **1**. Indeed, ^{1}H NMR, DQCOSY, edited HSQC, and HMBC experiments confirmed the presence of the α-methylene-γ-butyrolactone ring and the *E*-trisubstituted double bond Δ^7.

However, 2D NMR experiments suggested that the oxa-bridge was located between C-4 and C-13 instead of C-3 and C-13 as in **1**, forming now a seven-membered ring. Consequently, the hydroxyl group in **2** was located at C-3 rather than at C-4 as in **1**. This was also shown from an HSQC–TOCSY experiments where correlations from H-13 to H-3 can be found clearly from the TOCSY responses of every proton–carbon pair of this spin system. Also, comparison of the NMR data of compound **2** with those of other cembranes, showed the same planar structure but different relative stereochemistry to jeunicin,[7] its isomers 13αH,14βH-jeunicin[8] and 12,13-bis*epi*jeunicin.[4] The *E* geometry of the Δ^7 double





	1	Calculated by GMMX
^3J(H1-H2α)	3.7	2.1
^3J(H1-H2β)	10.0	11.6
^3J(H12-H13)	8.8	10.4
^3J(H13-H14)	8.8	8.4
^3J(H14-H1)	8.0	7.5

Figure 2. Conformation and key NOE's in the GMMX low-energy conformer of **1** and reported cembranoids with the same planar structure as **1**.

bond was confirmed by the high field position found for the ^{13}C NMR C-19 signal at δ_C 16.3.

In order to deduce the relative stereochemistry at C-1/C-3 and C-12/C-14 fragments, proton–proton coupling constant analysis revealed the same stereochemistry as in jeunicin. This was confirmed from the NOESY correlation between Me-20 and H-14. However, overlapping of the signals corresponding to the H-1 and H-3 protons in the ^1H NMR spectrum of **2** preclude us from determining the relative stereochemistry at C-3 and C-4 by NOE correlations on this compound. For that reason, compound **2** was acetylated to give **5**, whose ^1H NMR spectrum showed clearly H-1 and H-3 protons at different chemical shifts, allowing us to elucidate the relative stereochemistry by an NOESY experiment (Fig. 3).

Indeed, the NOE correlations between H-13 at 3.42 ppm and H-3 at 4.83 ppm and this in turn to Me-18 at 1.14 ppm, proved that those protons are on the same face of the molecule. On the other hand, NOE correlations between H-1 at 3.39 ppm and H-14 at 4.63 ppm and this in turn to Me-20 at 1.01 ppm in the NOESY of **5**, confirmed the relative configurations of these positions. With those last assignments and with the final structure in hand, we have named compound **2** as 4-*epi*jeunicin.

The molecular formula $C_{20}H_{30}O_4$ was established for compound **3** from its HRFABMS corresponding to the pseudomolecular ion $[M+Na]^+$ at *m/z* 357.2030. The ^1H and

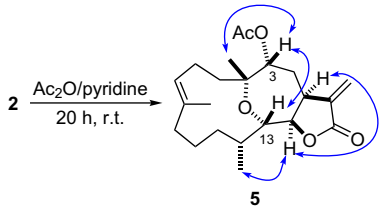


Figure 3. Acetylation of **2** and selected NOESY correlations in compound **5**.

^{13}C NMR spectra of **3** confirmed the presence of an α-methylene-γ-lactone group and a trisubstitued double bond as in **1**. The ^{13}C NMR signals at δ_c 58.4 (s) and 59.7 (d) suggested the presence of an epoxide group, which also was confirmed by the proton chemical shift at δ_H 3.07. Comparison of the NMR data of compound **3** with those of other epoxy-containing cembranes, showed a similar planar structure to eupalmerin,[9] its isomers 12-*epi*eupalmerin acetate[10] and 12,13-bis*epi*eupalmerin.[11] The high field resonance of the Me-19 signal in the ^{13}C NMR spectra of **3** (δ 15.1 ppm) indicated the *E* geometry of the double bond Δ^7. The relative stereochemistries of the epoxide group at C-3 and C-4 and the cis ring fusion at C-1 and C-14 in compound **3** were deduced by comparison of its spectroscopic data to those of cembranoid analogues. Nevertheless, the major differences between these compounds in the ^1H and ^{13}C NMR spectra were observed at the C-12 and C-13 positions and Me-20. The coupling constant of 6.8 Hz between H-13 and H-14 was indicative of an *anti* configuration between these protons as that occurring in 12,13-bis*epi*eupalmerin, which shows a *J* value of 10.0 Hz. However, eupalmerin and 12-*epi*eupalmerin acetate having a *syn* configuration between these protons showed very small coupling constants ($J<1$ Hz). Finally, the relative configuration of the remaining chiral center at C-12 was deduced by comparison of the NMR data of compound **3** to those of 12,13-bis*epi*eupalmerin showing that they have the same relative stereochemistry at all centers except at C-12 (Table 1).

This was confirmed when compound **3** was transformed to compound **1** under acidic conditions (TsOH, benzene) via transannular back-side attack of the C-13 hydroxyl group at C-3 of the epoxide. Thus, we named this as 13-*epi*eupalmerin with the structure as shown in **3**.

2.2. Biogenetic studies

To elucidate the metabolic origin of the terpenes isolated in this study, several incubations with ^3H-labeled metabolites

Table 1. ^{13}C NMR and DEPT data for **1–3** and **5–9** ($CDCl_3$)

Position	1	2	3	5	6	7	8	9
1	38.3 d	38.3 d	40.3 d	38.2 d	37.9 d	38.9 d	38.9 d	39.4 d
2	25.3 t	36.5 t	29.2 t	—	25.6 t	27.4 t	27.1 t	31.0 t
3	76.7 d	74.2 d	59.7 d	78.8 d	79.0 d	80.3 d	77.5 d	60.4 d
4	73.4 s	80.0 s	58.4 s	75.2 s	73.8 s	84.1 s	86.6 s	57.4 s
5	39.0 t	32.1 t	38.7 t	28.9 t	37.5 t	29.7 t	28.9 t	35.7 t
6	20.1 t	23.7 t	23.3 d	23.5 d	35.9 t	26.0 t[a]	36.3 t	24.7 t
7	128.3 d	126.4 d	124.2 d	126.6 d	65.2 d	85.3 d	91.5 s	62.3 d
8	130.9 s	134.7 s	135.7 s	138.6 s	61.1 s	35.2 d	213.2 s	62.6 s
9	36.7 t	40.3 t	36.7 t	40.4 t	36.7 t	25.9 t[a]	32.8 t	36.0 t
10	29.4 t	33.6 t	29.6 t	29.7 t	20.2 t	32.0 t	19.4 t	23.6 t
11	21.6 d	21.5 d	23.3 t	21.4 t	29.8 t	38.9 t	35.4 t	31.2 t
12	36.1 d	35.5 d	36.7 d	36.8 d	36.1 d	33.9 d	34.7 d	36.5 d
13	79.6 d	75.0 d	79.4 d	74.7 d	81.7 d	84.6 d	82.1 d	67.1 d
14	76.7 d	79.2 d	79.4 d	79.2 d	76.7 d	75.6 d	76.2 d	77.9 d
15	169.9 s	169.9 s	170.4 s	169.8 s	170.3 s	170.0 s	169.9 s	170.3 s
16	137.0 s	139.6 s	137.6 s	134.8 s	136.9 s	137.0 s	136.6 s	137.3 s
17	121.2 t	121.9 t	122.3 t	122.6 t	121.8 t	120.9 t	121.1 t	124.7 t
18	24.6 q	21.5 q	16.4 q	21.1 q	16.1 q	25.9 q[a]	24.2 q	15.7 q[a]
19	15.9 q	16.3 q	15.1 q	16.3 q	23.0 q	20.1 q	26.1 q	15.6 q[a]
20	14.8 q	14.2 q	14.6 q	14.2 q	14.7 q	17.1 q	16.6 q	14.0 q
$OCOCH_3$				170.4 s				
$OCOCH_3$				23.3 q				

[a] Interchangeable signals.

were performed using a cell-free extract of the gorgonian *E. mammosa*. We postulated that compound **3** would be the precursor of the ether-bridged terpene **1**. To test this hypothesis, the cell-free extract was incubated with ^{3}H-GGPP and the specific activity of the recovered terpenes (**1–3**) was measured. Biosynthetic intermediates early in a pathway have higher specific activity than those produced in subsequent steps. Thus, following an incubation of the cell-free extract for 24 h the quenched mixture was lyophilized and extracted with methanol. Partitioning of the methanol extract by our standard procedure gave a methylene chloride fraction. Purification of this fraction by RP-HPLC using 20% H_2O/MeOH yielded three radioactive compounds, which were identified as cembranes **1–3** by comparison of HPLC retention times with those of authentic samples. In order to assure that the observed radioactivity was due to these cembranes rather than unknown contaminants, compounds **1–3** were re-injected on HPLC, and a 25% aliquot of each of these cembranes was analyzed by a scintillation counter. Fractions collected prior to and following each of the cembrane peaks were at approximately background levels, indicating that the observed radioactivity was due to the cembranes. The recovered radioactivities and specific activities are summarized in Table 2.

Cembrane **3** has a significantly higher specific activity than **1** providing support for the proposed role of **3** as a precursor to the ether-bridged **1** as described in Figure 4. In addition to compounds **1–3**, euniolide (**4**) was also isolated from our extract of *E. mammosa* suggesting that cembrane **3** is derived from euniolide. The ether bridge of **1** is then derived from **3** by the attack of the hydroxyl substituent at the tertiary epoxide carbon. This biosynthetic proposal is further supported by the biomimetic transformation of **3** to **1**. Specifically, as it was mentioned before, compound **1** was generated from **3** by treatment with *p*-toluenesulfonic acid.

Table 2. Radioactivity recovered from incubation with 1 μCi [C_1–^{3}H]-GGPP

Compound	Recovered radioactivity (dpm)	Specific activity (dpm/mg)
1	11,280	9400
2	14,880	14,880
3	12,400	68,900

2.3. Chemical transformations

With the aim of identifying structure–cytotoxicity activity relationships of this class of terpene, we synthesized new analogues of cembranolides **1–3** using simple functional group interconversions in a concise fashion. The complete structural assignment of all the synthetic cembranoid analogues was accomplished on the basis of comprehensive 1D and 2D NMR and MS experiments.

2.3.1. Transformations of 12-*epi*eunicin (1). Several attempts of structural modification on the major compound isolated from this gorgonian, 12-*epi*eunicin (**1**), were performed (Fig. 5). Epoxidation of the Δ^7 double bond of **1** was achieved with *m*-CPBA in benzene at room temperature to afford, after reversed-phase HPLC separation, the new epoxide cembrane **6**. The (+)-HRESIMS data of the $[M+Na]^+$ pseudomolecular ion at *m*/*z* 373.1991 of **6** allowed us to determine its molecular formula as $C_{20}H_{30}O_5$. The difference of its atom composition in relation to that of **1** by one oxygen along with the carbon signals at 65.2 ppm (C-7) and 61.1 ppm (C-8) in the ^{13}C NMR spectrum, established the presence of an additional epoxide group in compound **6** instead of the Δ^7 double bond of **1**. Furthermore, the occurrence of a proton signal at 2.94 ppm in the ^{1}H NMR spectrum confirmed the trisubstituted epoxide function. The C-7*S**, C-8*S** (α) relative stereochemistry of this epoxide group was recognized by comparison of the ^{13}C NMR chemical shift values for C-7 and C-8 in **6** to those of α-(7*S*,8*S*)-epoxyeunicin (66.8 and 61.2 ppm, respectively) versus β-(7*R*,8*R*)-epoxyeunicin (60.5 and 59.8 ppm, respectively), obtained from epoxidation of euinicin.[12]

Treatment of compound **1** with iodine in CH_2Cl_2 at room temperature for 3 h, led to a complex product mixture from which could be isolated compounds **7** and **8** using a HPLC reversed-phase column. The (+)-HRESIMS data

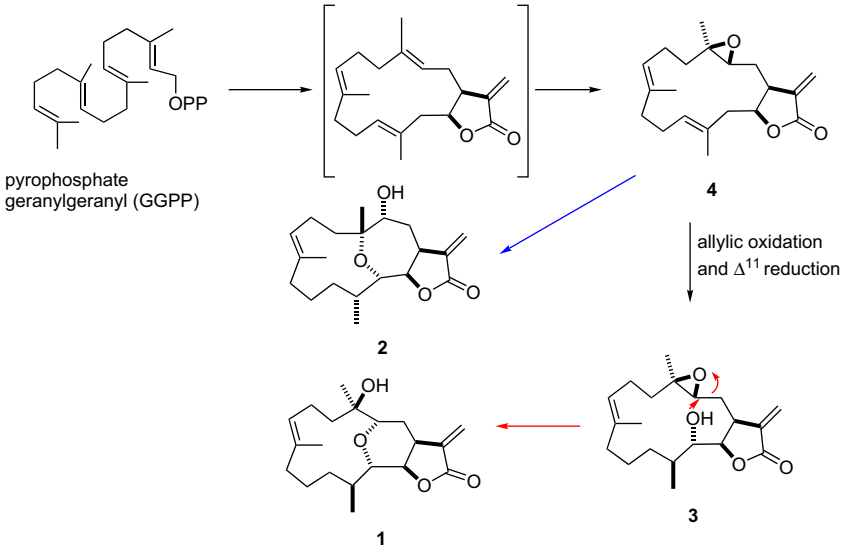


Figure 4. Biogenesis proposal.

of the $[M+Na]^+$ pseudomolecular ion at 377.2045 of compound **7** suggested the molecular formula of $C_{20}H_{30}O_4$ and six degrees of unsaturation. The structure of **7** presented an oxa-bridge function at the C-4/C-7 position, which was deduced from a standard gHMBC experiment. This new furanether **7** showed the same planar structure as inolides-A and B, obtained by others from the reaction of 12,13-bis-*epi*eupalmerin with iodine.[12] A combination of NOESY spectra and comparison of the NMR data of **7** to those of the synthetic analogues inolides-A and B, allowed us to establish the relative configuration of compound **7**. Thus, the NOE correlation between the Me-18 and H-7 indicated that they are on the same face of the molecule, assigned arbitrarily as β. Comparison of the ^{13}C and ^{1}H NMR spectral data of the C-3 to C-10 fragment of compound **7** to those of inolides-A and B allowed us to established the α disposition of the Me-20, which was very close to those of inolide-B.[12] Consequently, compound **7** was named as 12-*epi*inolide-B.

The structure of compound **8** was deduced from its 1D/2D NMR spectra and MS. Comparison of the ^{13}C NMR data of compound **8** to those of **1** indicated the major differences in the C-4/C-11 fragment. The carbon signal in the ^{13}C NMR of **8** at 213.2 ppm indicated the presence of a ketone functionality, which showed HMBC correlations to the H-6 proton at 1.96 ppm, to the H-9 protons at 1.83 ppm, and to the Me-19 at 2.20 ppm. On the other hand, HMBC correlations were observed from the H-5 proton at 1.52 ppm, the H_2-6 protons at 1.96/1.85 ppm, and H-9 protons at 1.83 ppm to the quaternary carbon at 91.5 ppm (C-7). Furthermore, the H_2-5 protons at 2.37 and 1.52 ppm also showed correlations to C-4 at 86.6 ppm and to Me-18 carbon at 24.2 ppm whose protons at 1.26 ppm were in turn HMBC correlated to C-3 carbon at 77.5 ppm. Finally, the H-6 proton at 1.85 ppm displayed a HMBC correlation to the C-9 carbon at 32.8 ppm (see Fig. 5). All of these correlations were in agreement with the presence of a five-membered cyclic ether bearing a methyl ketone group linked to the quaternary carbon at 91.5 ppm. The (+)-HRESIMS data for **8**, which showed the $[M+H]^+$ pseudomolecular ion at 349.2011 corresponding to the molecular formula $C_{20}H_{28}O_5$, confirmed the seven degrees of unsaturation present in the molecule. The lack of NOE correlations for the Me-19 precluded the determination of the relative stereochemistry of the chiral center at C-7.

2.3.2. Transformations of 13-*epi*eupalmerin (3). Conversion of **3** to diepoxide **9** was performed with *m*-CPBA in benzene at room temperature in 36% yield after separation by HPLC. The molecular formula $C_{20}H_{30}O_5$ of **9** was obtained by the (+)-HRESIMS data of the $[M+Na]^+$ pseudomolecular ion at 373.1994. In a similar way as that occurs with the epoxidation of 12,13-bis*epi*eupalmerin,[4] only one diastereoisomer was detected. The relative configuration of the additional epoxide group at C-7/C-8 position was determined by comparison of the NMR data of compound **9** with those reported for 12,13-bis*epi*eupalmerin epoxide, isolated from *E. succinea*,[13] which is epimeric at C-12. Finally, **3** was also transformed to compound **1** by treatment with *p*-toluenesulfonic acid in benzene (Fig. 6).

2.4. Biological studies

All these novel cembranes were studied in vitro with A549 (human lung carcinoma), H116 (human colon carcinoma), PSN1 (human pancreatic adrenocarcinoma), and T98G (human caucasian glioblastoma) tumor cells. The results, expressed as IC_{50} values in μg/mL, are displayed in Table 3. Most of the compounds showed moderate to strong cytotoxicity with the diepoxide **9** the most active and selective

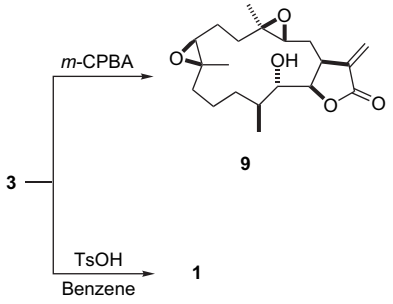


Figure 6. Chemical transformations of **3**.

Table 3. In vitro antitumor activities (IC_{50} in μg/mL) of the natural cembranes and their synthetic analogues

Compound	A-549	H116	PSN1	T98G
1	10	10	10	10
2	10	10	10	>10
3	1	5	5	0.5
5	5	5	5	>10
6	>10	10	>10	>10
7	5	1	1	5
8	10	5	5	>10
9	0.5	0.5	0.5	5

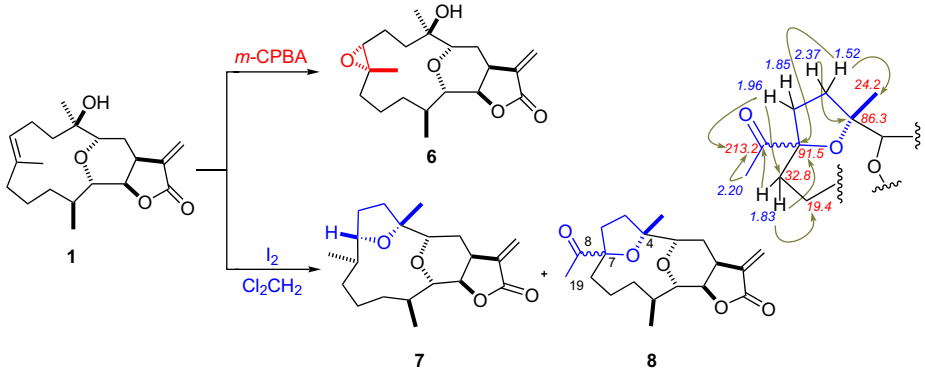


Figure 5. Chemical transformations of **1** and key HMBC correlations found in **8**.

against A-549, H116, and PSN1. It is noteworthy that the synthetic analogues (e.g., **7** and **9**) displayed greater potency than the parent natural products. The introduction of cyclic ether linkages across the cembrane skeleton results in an enhancement of cytotoxic activity. Thus, compound **7** is 10 times more active against H116 and PSN1 tumor cells than **1** (IC_{50}=10 μg/mL in **1** to IC_{50}=1 μg/mL in **7**). Furthermore, the cytotoxic activity present in compound **3** was significantly enhanced by the introduction of an extra epoxide functionality in compound **9** against H116 and PSN1 (IC_{50}=5 μg/mL in **3** to IC_{50}=0.5 μg/mL in **9**). These results corroborated the assessment that analogues of this series appear to be attractive targets for the development of antitumor agents.

3. Experimental

3.1. General methods

NMR spectra were recorded at 500/125 MHz ($^1H/^{13}C$), Bruker AVANCE 500; 200/50 MHz ($^1H/^{13}C$), Bruker AC-200 NMR spectrometer in $CDCl_3$. Carbon multiplicities were determined using DEPT-135 and DEPT-90 sequences. Atom connectivities were determined using gradient edited HSQC, HMBC, and DQCOSY experiments. Gradient NOESY experiments were carried out using a mixing time of 0.8 s and HSQC–TOCSY was run using a mixing time of 60 ms. (+)-LRAPCIMS and (+)-LRESIMS were measured on ThermoQuest Navigator spectrometer while (+)-HRESIMS were measured on VG Autospec and Bruker spectrometers. Optical rotations were determined on a JASCO DIP-1000 with an Hg lamp at 590 nm. Semipreparative HPLC was performed using Sharlau Nucleosil C18 column (300×8 mm) with RI detection.

3.2. Biological material

Specimens of *E. mammosa* were collected using SCUBA on Sweetings Cay in the Bahamas during the 1999 and 2000 FAU expeditions. Voucher samples are deposited at the Departamento de Química Fundamental, Universidade de A Coruña, under reference UDC 9951 and UDC 00EM.

3.3. Extraction and isolation

Specimens of the gorgonian collected in 1999 (337 g) were homogenized in MeOH (3×2.5 L), and the solvent was evaporated under reduced pressure. The crude extract was partitioned between CH_2Cl_2 and H_2O (1/1). The fraction soluble in CH_2Cl_2 was evaporated under pressure and partitioned between 10% aqueous MeOH (400 mL) and hexane (2×400 mL). Water was added to the polar fraction until the mixture became 50% in aqueous MeOH and then was extracted with CH_2Cl_2 (3×400 mL). The viscous oil (8.3 g) obtained from the CH_2Cl_2 fraction was submitted repeatedly to flash column chromatography (eluting with hexane/acetone mixtures of increasing polarity) to give several fractions. Fraction eluted with 15% acetone/hexane gave 505 mg of compound **1**. A second fraction eluted with 15–20% acetone/hexane (3 g) was separated by reversed-phase HPLC eluting with MeOH/H_2O (8/2) to obtain 22.4 mg of compound **3**, 11.3 mg of **2**, and 26.2 mg of **1**.

Specimens of the gorgonian collected in 2000 (350 g) were homogenized in MeOH (3×2.5 L), and the solvent was evaporated under reduced pressure. The crude extract was partitioned between 10% aqueous MeOH (400 mL) and hexane (2×400 mL) to give, after evaporation under pressure, 12.2 g of the hexane fraction. A portion of this fraction (500 mg) was submitted to flash column chromatography (eluting with hexane/acetone mixtures of increasing polarity) to give 2.8 mg of eunolide (**4**).

3.3.1. 12-*epi*Eunicin (1). Amorphous white solid. $[\alpha]_D$ −11.5 (*c* 1.20, $CHCl_3$). ^1H NMR (200 MHz, $CDCl_3$, δ_H ppm): 6.38 (1H, d, *J*=3.4 Hz, H-17α); 5.60 (1H, d, *J*=3.4 Hz, H-17β); 5.10 (1H, dd, *J*=5.9 and 9.3 Hz, H-7); 4.38 (1H, dd, *J*=8.0 and 8.9 Hz, H-14); 3.37 (1H, m, H-1); 3.20 (1H, dd, *J*=3.7 and 10.0 Hz, H-3); 2.79 (1H, t, *J*=8.9 Hz, H-13); 2.11 (2H, m, H-2); 1.58 (3H, s, H-19); 1.15 (3H, s, H-18); 0.99 (3H, d, *J*=6.8 Hz, H-20). ^{13}C NMR see Table 1. (+)-HR-ESIMS: *m/z* 357.2044 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_4Na$, 357.2036). (+)-LR-APCIMS *m/z*: 335 $[M+H]^+$, 357 $[M+Na]^+$.

3.3.2. 4-*epi*Jeunicin (2). Amorphous colorless oil. $[\alpha]_D$ −50.3 (*c* 0.53, $CHCl_3$). ^1H NMR (200 MHz, $CDCl_3$, δ_H ppm): 6.30 (1H, d, *J*=2.9 Hz, H-17); 5.61 (1H, d, *J*=2.9 Hz, H-17′); 5.23 (1H, dd, *J*=5.6 and 7.5 Hz, H-7); 4.59 (1H, dd, *J*=8.8 and 10.0 Hz, H-14); 3.57 (2H, m, H-1 and H-3); 3.38 (1H, dd, *J*=1.2 and 10.0 Hz, H-13); 1.68 (1H, m, H-12); 1.64 (3H, br s, H-19); 1.21 (3H, s, H-18); 0.97 (3H, d, *J*=7.3 Hz, H-20). ^{13}C NMR see Table 1. (+)-HR-ESIMS: *m/z* 357.2040 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_4Na$, 357.2036). (+)-LR-APCIMS *m/z*: 335 $[M+H]^+$, 357 $[M+Na]^+$, 317 $[M+H-H_2O]^+$.

3.3.3. 13-*epi*Eupalmerin (3). Amorphous colorless oil. $[\alpha]_D$ +15.8 (*c* 0.12, $CHCl_3$). ^1H NMR (200 MHz, $CDCl_3$, δ_H ppm): 6.33 (1H, d, *J*=2.4 Hz, H-17α); 5.82 (1H, d, *J*=2.4 Hz, H-17β); 5.18 (1H, dd, *J*=5.9 and 8.3 Hz, H-7); 4.56 (1H, t, *J*=6.8 Hz, H-14); 3.80 (1H, br t, *J*=5.2 Hz, H-13); 3.46 (1H, m, H-1); 3.07 (1H, br d, *J*=7.8 Hz, H-3); 1.58 (3H, s, H-19); 1.31 (3H, s, H-18); 1.02 (3H, d, *J*=6.8 Hz, H-20). ^{13}C NMR see Table 1. (+)-HR-ESIMS: *m/z* 357.2030 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_4Na$, 357.2036). (+)-LR-APCIMS *m/z*: 335 $[M+H]^+$, 357 $[M+Na]^+$.

3.4. Biogenetic studies

A cell-free extract was prepared from flash frozen *E. mammosa* (stored at −80 °C) by homogenizing in a phosphate buffer (pH 7.7 with EDTA and β-mercaptoethanol) with liquid nitrogen in a Waring blender. To remove cellular debris, the homogenate was centrifuged for 15 min at 9000×*g*. The supernatant was centrifuged at 18,000×*g* for 3 h and then passed through 0.45 μm nylon membrane filters. The cell-free extract (37 mL) was incubated with 1 μCi of ^3H-GGPP and $MgCl_2$ (7.3 mg, 1 mM) at room temperature for 24 h at 200 rpm. The incubation was lyophilized and extracted with methanol. The methanol extract was partitioned as before and the methylene chloride fraction was purified by reversed-phase HPLC eluting with 20% H_2O/MeOH. Compounds **1**–**3** were identified in the HPLC by comparison of retention times with those of an authentics samples. The radioactivity of these compounds was

determined using a scintillation counter. Compounds **1**–**3** were reinjected, and a small portion of each of these cembranes was analyzed by a scintillation counter. Fractions collected prior to and following each of the cembrane peaks were at approximately background levels, indicating that the observed radioactivity was due to the cembranes.

3.5. Acetylation of 4-*epi*jeunicin (2)

A solution of 4-*epi*jeunicin (**2**) (5 mg, 0.015 mmol) in Ac_2O (1 mL) and Py (1 mL) was stirred vigorously at room temperature overnight and concentrated to leave a residue identified as compound **5** (6 mg, quantitative yield).

3.5.1. Compound 5. Amorphous colorless oil. $[\alpha]_D$ +34.0 (*c* 0.09, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$, δ_H ppm): 6.36 (1H, d, *J*=2.5, H-17α); 5.68 (1H, d, *J*=2.5 Hz, H-17β); 5.24 (1H, dd, *J*=5.7 and 7.1 Hz, H-7); 4.83 (1H, d, *J*=7.4 Hz, H-3); 4.63 (1H, dd, *J*=8.6 and 10.1 Hz, H-14); 3.42 (1H, d, *J*=10.1 Hz, H-13); 3.39 (1H, m, H-1); 2.17 (3H, s, OAc); 1.65 (3H, br s, H-19); 1.14 (3H, s, H-18); 1.01 (3H, d, *J*=7.1 Hz, H-20). ^{13}C NMR see Table 1. (+)-LR-ESIMS *m/z* (rel int.): 415 $[M+K]^+$ (35), 399 $[M+Na]^+$ (100).

3.6. Reaction of 12-*epi*eunicin (1) with *m*-chloroperbenzoic acid

A solution of 12-*epi*eunicin (**1**) (100 mg, 0.299 mmol) in dry benzene (15 mL) was stirred vigorously with *m*-CPBA (67 mg, 0.39 mmol) at room temperature for 4 h and concentrated to afford a residue (112 mg) that was purified by reverse-phase HPLC (Sharlau C18, elution with MeOH/H_2O 65/35) to give compound **6** (11 mg, 11%).

3.6.1. Compound 6. Amorphous colorless oil. $[\alpha]_D$ +141 (*c* 0.08, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$, δ_H ppm): 6.40 (1H, d, *J*=2.9 Hz, H-17); 5.64 (1H, d, *J*=2.9 Hz, H-17′); 4.41 (1H, t, *J*=8.3 Hz, H-14); 3.38 (1H, m, H-1); 3.30 (1H, dd, *J*=4.5 and 9.1 Hz, H-3); 2.94 (1H, dd, *J*=1.2 and 9.3 Hz, H-7); 2.83 (1H, t, *J*=9.2 Hz, H-13); 2.05 (2H, m, H-2); 1.26 (3H, s, H-18); 1.26 (3H, s, H-19); 1.02 (3H, d, *J*=7.0 Hz, H-20). ^{13}C NMR see Table 1. (+)-HR-ESIMS *m/z* 373.1991 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1990).

3.7. Reaction of 12-*epi*eunicin (1) with iodine

A solution of iodine (49.2 mg, 0.196 mmol, 1.07 equiv) in Cl_2CH_2 (4 mL) was added drop wise over 3 min to a magnetically stirred solution of **1** (60 mg, 0.18 mmol) in Cl_2CH_2 (10 mL) and was stirred at room temperature for 3 h, concentrated and the resulting oil (108 mg) was purified by means of reverse-phase HPLC (Sharlau C18, elution with MeOH/H_2O 75/25) to give **7** (8 mg, 13%) and **8** (4 mg, 6%).

3.7.1. Compound 7. Amorphous colorless oil. $[\alpha]_D$ +91 (*c* 0.11, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$, δ_H ppm): 6.39 (1H, d, *J*=3.5 Hz, H-17α); 5.54 (1H, d, *J*=3.5 Hz, H-17β); 4.52 (1H, dd, *J*=7.9 and 9.6 Hz, H-14); 4.17 (1H, m, H-7); 3.41 (1H, m, H-1); 3.22 (1H, dd, *J*=2.4 and 11.8 Hz, H-3); 2.68 (1H, dd, *J*=8.3 and 9.5 Hz, H-13); 1.18 (3H, s, H-18); 1.00 (3H, d, *J*=7.2 Hz, H-20); 0.78 (3H, d, *J*=7.3 Hz, H-19). ^{13}C NMR see Table 1. (+)-HR-ESIMS *m/z* 357.2045 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_4Na$, 357.2036).

3.7.2. Compound 8. Amorphous colorless oil. $[\alpha]_D$ +34 (*c* 0.13, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$, δ_H ppm): 6.43 (1H, d, *J*=3.6 Hz, H-17α); 5.58 (1H, d, *J*=3.6 Hz, H-17β); 4.46 (1H, t, *J*=8.5 Hz, H-14); 3.43 (1H, m, H-1); 3.29 (1H, dd, *J*=2.0 and 12.3 Hz, H-3); 2.88 (1H, t, *J*=9.1 Hz, H-13); 2.37 (1H, ddd, *J*=8.4, 11.9 and 11.9 Hz, H-5); 2.20 (3H, s, H-19); 2.00 (1H, m, H-2); 1.96 (1H, m, H-6); 1.89 (1H, m, H-12); 1.85 (1H, m, H-6′); 1.83 (2H, m, H-9); 1.80 (1H, m, H-2′); 1.60 (2H, m, H-10 and H-11); 1.52 (1H, dd, *J*=8.4 and 11.9 Hz, H-5′); 1.26 (3H, s, H-18); 1.22 (1H, m, H-10′); 0.99 (3H, d, *J*=7.0 Hz, H-20). ^{13}C NMR see Table 1. (+)-HR-ESIMS: 349.2011 $[M+H]^+$ (calcd for $C_{20}H_{29}O_5$, 349.2010).

3.8. Reaction of 13-*epi*eupalmerin (3) with *m*-chloroperbenzoic acid

A solution of **3** (20 mg, 0.05 mmol) in dry benzene (3 mL) was stirred vigorously with *m*-CPBA (17 mg, 0.099 mmol, 1.3 mequiv) at room temperature for 4.5 h. Following it was concentrated to leave a residue that was purified by means of reverse-phase HPLC (Sharlau C18, elution MeOH/H_2O 65/35) to give **9** (6.3 mg, 36%).

3.8.1. Compound 9. Amorphous colorless oil. $[\alpha]_D$ +21 (*c* 0.03, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$, δ_H ppm): 6.47 (1H, d, *J*=1.4 Hz, H-17α); 5.84 (1H, d, *J*=1.4 Hz, H-17β); 4.41 (1H, m, H-14); 3.98 (1H, d, *J*=9.3 Hz, H-13); 3.45 (1H, ddd, *J*=1.9, 12.2 and 12.2 Hz, H-1); 2.76 (1H, dd, *J*=3.3 and 10.9 Hz, H-7); 2.73 (1H, m, H-2); 2.71 (1H, br s, H-3); 1.99 (1H, m, H-12); 1.67 (1H, m, H-2′); 1.33 (1H, s, H-18); 1.27 (1H, s, H-19); 1.09 (1H, d, *J*=6.8 Hz, H-20). ^{13}C see Tables 1 and 2. (+)-HR-ESIMS *m/z* 373.1994 $[M+H]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1985).

3.9. Reaction of 13-*epi*eupalmerin (3) with *p*-toluenesulfonic acid hydrate

A solution of compound **3** (1.3 mg, 0.003 mmol) in dry benzene (0.3 mL) was stirred vigorously with *p*-toluenesulfonic acid hydrate (0.022 mg, 0.0001 mmol, 0.03 mequiv) at room temperature for 24 h. Then, the reaction product was concentrated. The identification of compound **1** in the reaction products was carried out with analytical Hypersil® Elite C18, 150×4.6 mm 5μ, reverse-phase HPLC using MeOH/H_2O 75/25 as the mobile phase with a flow of 0.7 mL/min by comparison of the retention time (10.5 min) and co-injection with compound **1**.

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