

CHAPTER IV

RESULTS AND DISCUSSION


The preliminary bioactivity screening of the dichloromethane extract from the soft coral *Cladiella tuberosa* Tixier-Durivault, revealed its cytotoxicity (IC_{50} for P-388, A-549, and HT-29 : 2.0, 5.0, and 5.0 $\mu\text{g/ml}$, respectively) and brine shrimp lethality activity (LD_{50} 3.2 $\mu\text{g/ml}$). Therefore, this soft coral was selected for isolation and structure elucidation of the chemical constituents. Large-scale investigation of the soft coral guided by brine shrimp lethality test led to the isolation of four eunicellin diterpenoids.

Isolation of the chemical constituents

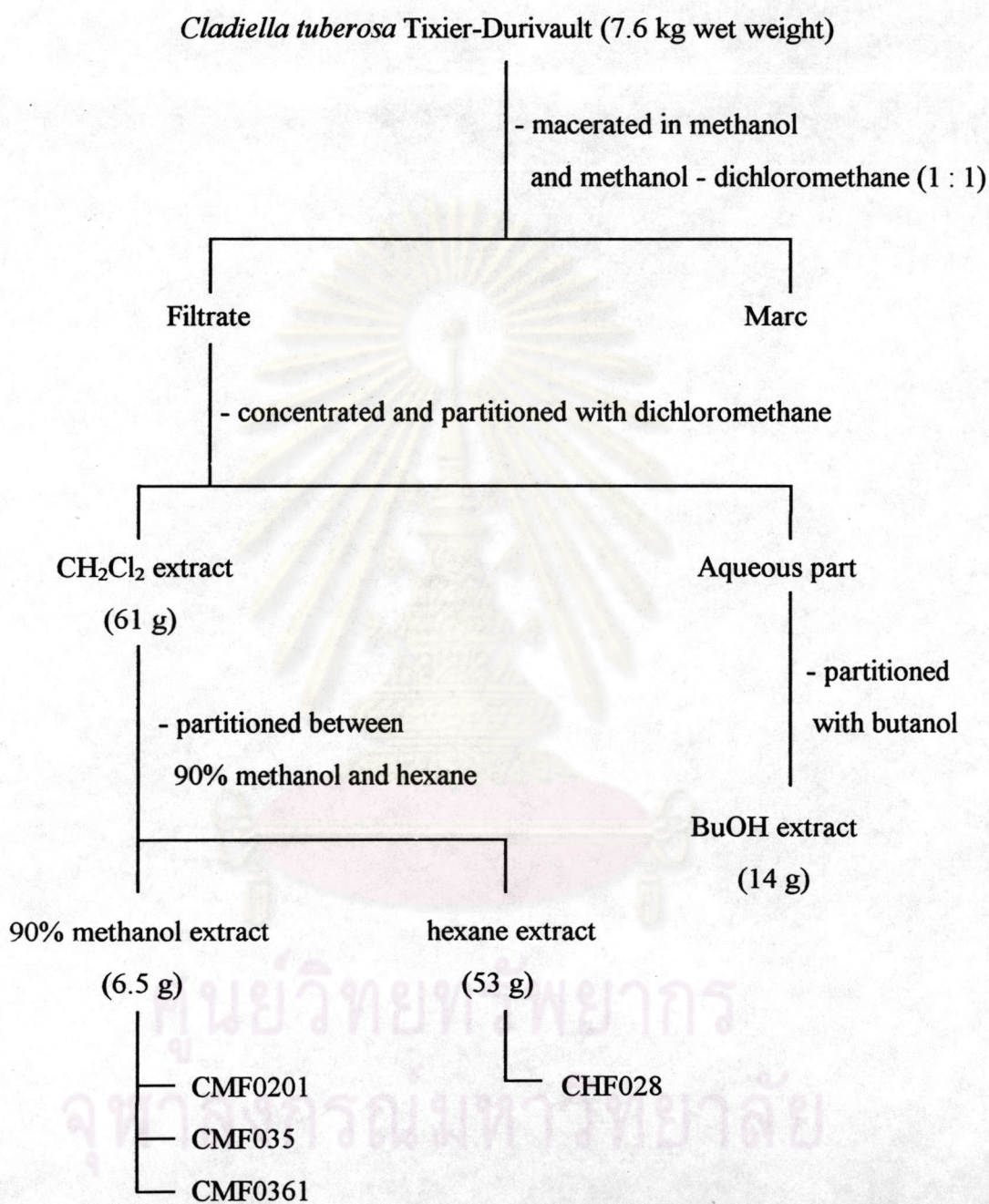
The repeat maceration of this soft coral (7.6 kg wet weight) in methanol and methanol : CH_2Cl_2 (1 : 1) and fractionation by solvent partition (Scheme 10) yielded the active hexane (53 g) and methanolic (6.5 g) extracts which were further fractionated by successive chromatographic techniques.

The hexane extract (17.5 g) was fractionated by silica gel quick column chromatography, silica gel flash column chromatography and then recrystallized to obtain a brine shrimp toxic compound (LD_{50} 0.6 $\mu\text{g/ml}$) which codenamed as CHF028 (140 mg, 5.5×10^{-3} % of wet weight). Compound CHF028 showed the cytotoxic activity against P-388 (mouse lymphoma) at IC_{50} 2.0 $\mu\text{g/ml}$; A-549 (human lung carcinoma), HT-29 (human colon carcinoma) and MEL-28 (human melanoma) at IC_{50} 5.0 $\mu\text{g/ml}$.

The methanolic extract (4.5 g) was further fractionated by chromatographic techniques to yield eight fractions, CMF006 - CMF013. Fraction CMF009 (335 mg), which exhibited the highest activity, was investigated by using repeated column chromatography to obtain compound CMF035 (4.2 mg, 8.0×10^{-5} % of wet weight). Fraction CMF008 (367 mg), which showed less cytotoxic activity was also investigated. After using several column chromatography, two compounds, CMF0201 (14.6 mg, 2.8×10^{-4} % of wet weight) and CMF0361 (5.0 mg, 9.4×10^{-5} % of wet weight), were obtained. The extraction and isolation of the compounds from the soft coral were present in Scheme 10.



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Structure Elucidation of the Isolated Compounds

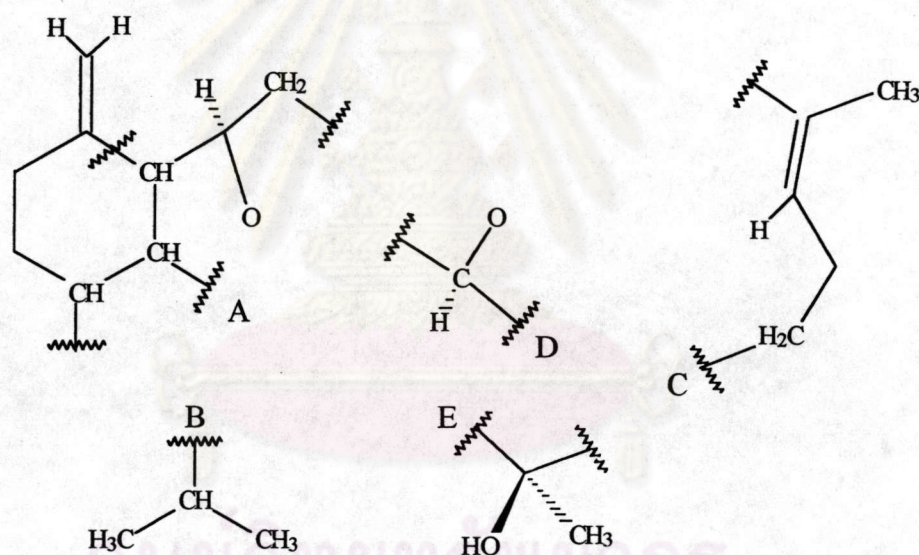
1. Compound CHF028

The electron impact mass spectrum (Figure 15) exhibited the molecular ion peak at m/z 304 and a fragment ion at m/z 286 due to the loss of one molecule of water. The ir spectrum (Figure 16) confirmed the presence of hydroxy group (ν_{\max} 3400 cm^{-1}) and also suggested the presence of exocyclic methylene (ν_{\max} 3080, 1660 and 900 cm^{-1}) and ether (ν_{\max} 1040 cm^{-1}) group. The ^{13}C nmr spectrum of CHF028 in CDCl_3 (Figure 20) showed 20 carbon peaks determined as four methyl, six methylene, seven methine and three quaternary carbons by High Sensitive Quantum Coherence (HSQC) (Figure 21 - 23) experiment. These data established its tentative molecular formula as $\text{C}_{20}\text{H}_{32}\text{O}_2$.

The ^1H nmr spectrum of CHF028 in CDCl_3 (Figure 17 - 19) showed the presence of two exocyclic methylene protons as two 1H broad triplets at δ 4.74 and 4.67 ppm, an isopropyl group as two 3H doublets at δ 0.93 and 0.73 ppm and a 1H multiplet at δ 1.80 ppm, two oxymethine protons at δ 3.98 and 3.70 ppm. A tertiary methyl attached to oxygen-bearing carbon appeared as three-proton singlet δ 1.14 ppm. A broad doublet at δ 5.55 ppm (1H, $J = 8.5$ Hz) and a methyl singlet at δ 1.84 ppm were attributable to a $\text{CH}_2\text{-CH=C-CH}_3$ group.

The $^1\text{H-}^1\text{H}$ COSY spectrum of CHF028 (Figure 24-25) which showed the correlations among the protons having geminal, vicinal and allylic coupling was summarized in Table 5. The vicinal couplings are observed as following : the proton at δ 2.42 ppm (H-8 α) to the proton at δ 3.98 ppm (H-9); H-9 proton to the proton at δ 2.67 ppm (H-10) which, in turn, coupled to the proton at δ 2.08 ppm (H-1); H-1 to the proton at δ 1.29 ppm (H-14); H-14 proton to two protons at δ 1.69 ppm (H-13 α) and 1.00 ppm (H-13 β), and these H-13 protons to the protons at δ 2.22 ppm (H-12 β)

and 2.08 ppm (H-12 α). H-12 α proton also showed the allylic coupling to two olefinic protons at δ 4.74 ppm and 4.67 ppm (H-20a and b). The above data suggested the fragment A as shown below. Two methyl protons at δ 0.93 ppm (H-15) and 0.73 ppm (H-16) were coupled to the proton at δ 1.80 ppm (H-17) suggesting the fragment B. The fragment C was proposed due to the proton at δ 1.64 ppm (H-4 α) was coupled to the proton at δ 2.38 ppm (H-5 β) which, in turn, coupled to the olefinic proton at δ 5.55 ppm (H-6) together with the exist of a methyl singlet at δ 1.84 ppm (19-CH₃). The methyl singlet at δ 1.14 ppm (18-CH₃) was suggested its connectivity to oxygenated-quaternary carbon as fragment D. Finally, the oxymethine proton at δ 3.70 (H-2) which appeared as singlet was assigned as fragment E.



The position of an isopropyl group was confirmed by the mass fragment peak at m/z 261 ($M^+ - 43$) and the assignment of the structure was finally accomplished by using Heteronuclear Multiple Bond Coherence (HMBC) experiment, optimized for 8 Hz coupling constant (Figure 26 - 28). Two exocyclic methylene protons at δ 4.74 and 4.67 ppm (H-20a and H-20b) were coupled to both C-10 (δ 47.9 ppm) and C-12 (δ 31.5 ppm) while a proton at δ 2.22 ppm (H-12 β) showed correlated peaks with both the methylene carbon signal at δ 110.1 ppm (C-20) and the quaternary olefinic carbon

signal at $\delta 147.0$ (C-11), establishing the connectivity of the exocyclic methylene part. Both proton signal at $\delta 2.42$ (H-8 α) and 1.99 ppm (H-8 β) were coupled to the olefinic carbon signal at $\delta 124.5$ ppm (C-7). The H-8 β signal also coupled to the methyl carbon signal at $\delta 20.5$ ppm (C-19), which proton signal, in turn, coupled to C-6 ($\delta 132.5$ ppm), C-7 ($\delta 124.5$ ppm) and C-8 ($\delta 38.5$ ppm) signals. Furthermore, the proton signal at $\delta 1.99$ ppm (H-8 β) and the methyl proton signals at $\delta 1.84$ ppm (H-19) showed the long-range coupling to the olefinic carbon signal at $\delta 132.5$ ppm (C-6) indicating the connectivity of C-7 and C-8 and placing another double bond between C-6 and C-7. The proton signals at $\delta 1.64$ (H-4 α) and 2.38 ppm (H-5 β) were coupled to the quaternary carbon signal at $\delta 75.1$ ppm (C-3), suggesting C-3 and C-4 connectivity.

The position of the isopropyl group was established by the correlation between the two methyl protons of the isopropyl group and the carbon signal at $\delta 42.6$ ppm (C-14). Finally, the proton signal at $\delta 3.70$ ppm (H-2) showed correlations with six carbon signals at $\delta 46.1$ (C-1), 39.0 (C-4), 80.5 (C-9), 47.9 (C-10), 42.6 (C-14) and 29.5 ppm (C-18), supporting the proposed structure. The long-range correlations between proton and carbon signals were shown in Figure 5.

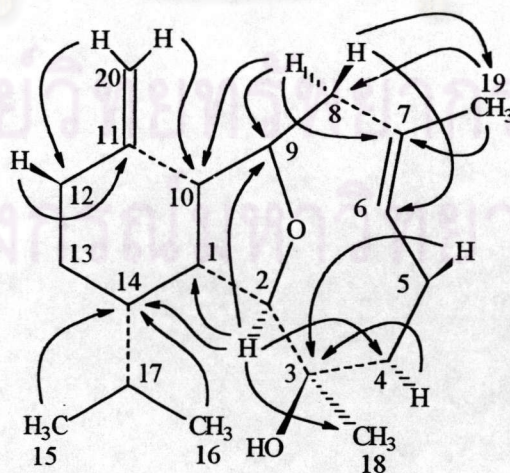


Figure 5. The long-range correlations in the HMBC spectrum of CHF028

Table 5. Carbon and proton chemical shift assignments and proton-proton correlations of CHF028

	δ C (ppm)	δ H (ppm)	Splitting pattern	multiplicity (<i>J</i> value, Hz)
1	46.1	2.08	dd	H-10 (7.6), H-14 (12.0)
2	92.2	3.70	br s	
3	75.1	-	-	-
4	39.0	β 1.78 α 1.64	m ddd	H-4 β (14.0), H-5 β (4.5), H-5 α (6.4)
5	24.3	β 2.38 α 2.10	tdd m	H-5 α (12.7), H-4 α (4.5), H-6 (8.5)
6	132.5	5.55	br d	H-5 β (8.5)
7	124.5	-	-	-
8	38.5	β 1.99 α 2.42	dd d	H-8 α (14.0) H-8 β (14.0), H-9 (5.3)
9	80.5	3.98	dd	H-8 α (5.3), H-10 (8.2)
10	47.9	2.67	dd	H-9 (8.2), H-1 (7.6)
11	147.0	-	-	-
12	31.5	β 2.22 α 2.08	dt m	H-12 α , H-13 α (3.3), H-13 β (3.3)
13	25.2	β 1.00 α 1.69	qd dq	H-13 α (12.8), H-12 β (3.3), H-12 α H-14 (12.8) H-13 β (12.8), H-12 β (3.3), H-12 α (3.3), H-14 (3.3)
14	42.6	1.29	tt	H-13 α (3.3), H-13 β (12.2), H-1 (12.2)
15	22.0	0.93	d	H-17 (6.8)
16	15.5	0.73	d	H-17 (6.8)
17	28.0	1.80	m	
18	29.5	1.14	br s	
19	20.5	1.84	s	
20	110.1	a 4.74 b 4.67	t br s	H-12 α (1.2) H-12 α (1.2)

The relative configuration of compound CHF028 was determined by analysis of its proton coupling constants, Nuclear Overhauser Effect Correlations (NOESY) spectrum (Figure 29 - 30), and Nuclear Overhauser Effect difference spectrum (NOEDS). The NOEDS showed correlations between protons through space by which the irradiated protons would affect the intensity of correlated proton signals, while NOESY spectrum showed correlations through space between protons in the whole molecule at the same time. The 90° dihedral angle between H-1 ($\delta 2.08$ ppm) and H-2 ($\delta 3.70$ ppm) produced virtually no coupling between the two protons. However, H-1 showed 2.2 % enhancement when H-2 was irradiated. The proton at position 1 was coupled to H-14 at $\delta 1.29$ ppm and H-10 at $\delta 2.67$ ppm with the coupling constants of 12.0 and 7.6 Hz, respectively, however, only the correlation to H-10 was visible in the NOESY spectrum and the NOEDS showed 3.0 % enhancement of H-1 upon irradiation of H-10 (Figure 31) suggesting a *trans* relationship between H-1 and H-14 and a *cis* relationship between H-1 and H-10. The proton at position 10, while coupling coupled to H-9 at $\delta 3.98$ ppm, also did not show any NOE to this proton, hence suggesting a *trans* relationship between H-10 and H-9. In the six-membered portion, the NOESY spectrum showed the correlations between H-12 α ($\delta 2.08$), H-13 α ($\delta 1.69$), and H-14 ($\delta 1.29$), indicating that these protons are in the same plane. NOESY spectrum also showed the correlation between H-12 β ($\delta 2.22$) and H-13 β ($\delta 1.00$); H-12 β and H-13 α ; H-12 β and H-20a ($\delta 4.74$) and between H-20b ($\delta 4.67$) and H-10, thus establishing the chair conformation of the six-membered ring. The NOESY correlations between each proton are shown in Figure 6.

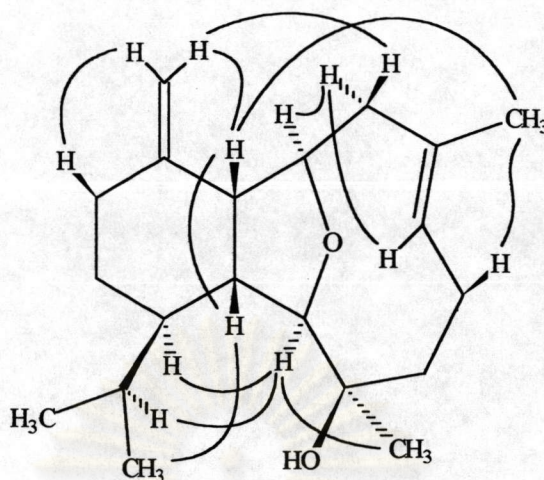


Figure 6. Correlations in the NOESY spectrum of CHF028

The H-15 methyl at δ 0.93 ppm and H-17 (δ 1.80 ppm) showed nOe to H-14 while the H-16 methyl at δ 0.73 ppm showed correlation to H-1, suggesting the same side between the H-16 methyl and H-1. The proton signal at position 17 was enhanced by 14 % and the methyl proton signals at δ 1.14 ppm by 2 % when H-2 was irradiated (Figure 32). Conversely, irradiation of the H-18 methyl protons at δ 1.14 ppm showed a magnetization transfer to H-2 (6 %) and H-6 (4 %), suggesting the 18-Me, H-2 and H-6 to be in the same plane. The trisubstituted double bond can be assigned the *trans* configuration since only negligible enhancement (0.7 %) of the NOE difference spectrum of H-6 was observed when the H-19 methyl was irradiated. The correlations observed in NOESY spectrum were summarized in Table 6.

Compound CHF028 was therefore identified as the known eunicellin diterpenoid, deacetylcladiellin [085], which has been obtained as a reduction product of cladiellin [038] (Kazlauskas, Murphy, and Wells, 1977). This is the first report of naturally occurring deacetylcladiellin and its bioactivities.

Table 6. The correlations between protons observed from the NOESY spectrum and long-range correlations between carbons and protons in the HMBC spectrum

	δC (ppm)	δH (ppm)	long-range correlations in HMBC spectrum	nOe observed between protons in NOESY spectrum
1	46.1	2.08	C-3, C-9, C-10, C-14, C-17	H-2, H-10, H-16
2	92.2	3.70	C-1, C-4, C-9, C-10, C-14, C-18	H-1, H-17, H-18
3	75.1	-	-	-
4	39.0	β 1.78 α 1.64	C-3, C-5	
5	24.3	β 2.38 α 2.10	C-3, C-4	H-19
6	132.5	5.54		H-8 α
7	124.5	-	-	-
8	38.5	β 1.99 α 2.42	C-6, C-7, C-19 C-7, C-9, C-10, C-19	Hb-20 H-6, H-9
9	80.5	3.98		H-8 α , H-12 α
10	47.9	2.67		H-1, Hb-20
11	147.0	-	-	-
12	31.5	β 2.22 α 2.08	C-11, C-13, C-14, C-20	H-13 α , H-13 β , Ha-20 H-9, H-13 α , H-14
13	25.2	α 1.69 β 1.00	C-11	H-12 α , H-12 β , H-14 H-12 β
14	42.6	1.29		H-2, H-12 α , H-13 α , H-15
15	22.0	0.93	C-14, C-15, C-17	H-14
16	15.5	0.73	C-14, C-16, C-17	H-1
17	28.0	1.80		H-2, H-14
18	29.5	1.14		H-2
19	20.5	1.84	C-6, C-7, C-8	H-5 β , Hb-20
20	110.1	a 4.74 b 4.67	C-10, C-12 C-10, C-12	H-12 β H-8 β , H-10, H-19

2 Compound CMF035

The electron impact mass spectrum (Figure 33) showed the molecular ion peak at m/z 320, loss of a molecule of water at m/z 302, an isopropyl group at m/z 277 and a further methyl at m/z 262. The ir spectrum (Figure 34) also confirmed the presence of hydroxy group (ν_{\max} 3425 cm^{-1}) and suggested the existence of exocyclic methylene (ν_{\max} 3080, 1650 and 900 cm^{-1}) and ether (ν_{\max} 1070 cm^{-1}) moieties in the structure. The ^{13}C nmr (Figure 37) and HMQC spectrum of CMF035 (Figure 38 - 40) exhibited 19 carbon peak : two sp^2 carbons at δ 147.9 and 109.2, four oxygen-bearing carbons at δ 90.5, 80.1, 78.1 and 74.9 ppm and the rest were thirteen sp^3 carbons. The HMBC spectrum of compound CMF035 (Figure 43) revealed another quaternary carbon signal hidden inside the solvent peaks at δ 77.0 ppm. These data suggested the tentative molecular formula as $\text{C}_{20}\text{H}_{32}\text{O}_3$.

The ^1H nmr spectrum of compound CMF035 (Figure 35 - 36) exhibited the signals of two exocyclic methylene protons (δ 4.67 and 4.64 ppm), three oxymethine protons (δ 4.57, 4.13, and 3.63 ppm) and four methyl groups (δ 1.16, 1.20, 0.97, and 0.80 ppm). Judging from the characteristic of eunicellin diterpenoid, the exocyclic methylene protons of compound CMF035 could be assigned to the position 20. The carbon and proton assignments and the multiplicity of protons were summarized in Table 7.

Table 7. The carbon and proton chemical shift assignments and the multiplicity of protons of CMF035.

	δC (ppm)	δH (ppm)	multiplicity and J value (Hz)
1	45.1	2.17	ddd; $J = 11.0, 7.3$
2	90.5	3.63	br s
3	74.9	-	-
4	39.9	α 1.86	dd; $J = 14.6, 10.4$
		β 1.80	dd; $J = 14.6, 8.8$

Table 7. (continued)

	δC (ppm)	δH (ppm)	multiplicity and J value (Hz)
5	29.3	a 1.99	m
		b 1.55	m
6	80.1	4.57	br d; $J = 5.8$
7	77.0	-	-
8	45.3	α 1.73	dd; $J = 15.0, 3.6$
		β 2.26	dd; $J = 15.0, 11.6$
9	78.0	4.13	ddd; $J = 11.6, 7.0, 3.6$
10	53.0	2.97	t; $J = 7.0$
11	147.9	-	-
12	31.6	α 2.04	br t; $J = 13.7$
		β 2.25	dt; $J = 13.7, 3.6$
13	24.8	α 1.70	dq; $J = 12.8, 3.6$
		β 1.06	qd; $J = 12.8, 3.2$
14	43.7	1.29	br t; $J = 11.0$
15	22.0	0.97	d; $J = 6.7$
16	15.9	0.80	d; $J = 6.7$
17	29.1	1.74	m
18	30.3	1.16	s
19	23.0	1.20	s
20	109.2	a 4.67	br s
		b 4.64	br s

The HMBC spectrum of compound CMF035 (Figure 41 - 43) revealed a quaternary carbon signal (C-7) at $\delta 77.0$ ppm resonated at the same chemical shift as the solvent (chloroform-d) and long-range correlated to the protons at $\delta 1.73$ (H-8 α) and 1.20 ppm (H-19). The ethereal bridge between C-2 and C-9 was confirmed by the long-range correlation between the proton at $\delta 3.63$ ppm (H-2) and C-9 ($\delta 78.1$ ppm)

and also between H-9 (δ 4.13) and C-2 (δ 90.5). In addition, two methyl signals at δ 1.16 and 1.20 ppm can be assigned as H-18 and H-19, respectively. The correlations from the HMBC spectral data were shown in Figure 7 and summarized in Table 8.

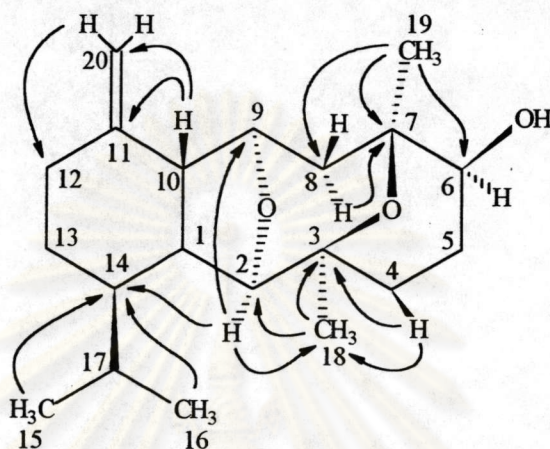


Figure 7. The long-range correlations in the HMBC spectrum of compound CMF035

The relative configuration of compound CMF035 was also determined by the NOESY experiment. The NOESY correlations from NOESY spectrum (Figure 44) were summarized in Figure 8 and Table 8.

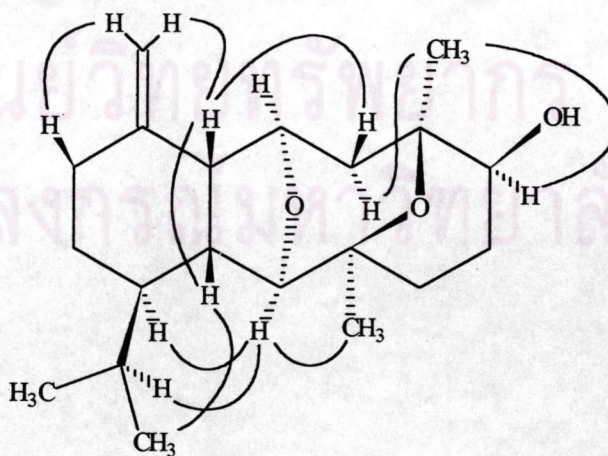


Figure 8. The correlations in the NOESY spectrum of compound CMF035

Table 8. The long-range correlations between protons and carbons and nOe observed between protons of CMF035

	δC (ppm)	δH (ppm)	long-range correlations in HMBC spectrum	nOe observed between protons in NOESY spectrum
1	45.1	2.17	C-9, C-14, C-17	H-2, H-10, H-16, H-18
2	90.5	3.63	C-3, C-9, C-10, C-14, C-18	H-1, H-4 α , H-14, H-17, H-18
3	74.9	-	-	-
4	39.9	α 1.86 β 1.80	C-3, C-6 C-2, C-3, C-6, C-18	H-2, H-5b, H-6
5	29.3	a 1.99 b 1.55	C-3, C-7	H-4 α
6	80.1	4.57		H-4 α
7	77.0	-	-	-
8	45.3	α 1.73 β 2.26	C-6, C-7 C-9, C-10	H-19 H-10
9	78.0	4.13	C-2, C-11	H-12 α , H-14, H-20b
10	53.0	2.97	C-1, C-9, C-11, C-12, C-14, C-20	H-1, H-8 β , H-12 β , H-20b
11	147.9			
12	31.6	α 2.04 β 2.25	C-10	H-9 H-10, H-20a
13	24.8	α 1.70 β 1.06	C-14	H-15 H-17
14	43.7	1.29		H-2, H-15
15	22.0	0.97	C-14, C-17	H-13 α , H-14
16	15.9	0.80	C-14, C-17	H-1
17	29.1	1.74		H-2
18	30.3	1.16	C-2, C-3, C-4	H-1, H-2
19	23.0	1.20	C-6, C-7, C-8	H-6, H-8 α
20	109.2	a 4.67 b 4.64	C-10 C-12	H-12 β H-9, H-10

Finally, comparison of the nmr spectral data of compound CMF035 with reported values of related compounds revealed to be the known eunicellin diterpenoid, sclerophytin A [052], which exhibited cytotoxicity to L1210 cell line at the concentration of 0.001 $\mu\text{g/ml}$ (Sharma and Alam, 1988). The compound was initially reported as a constituent of the soft coral *Sclerophyllum capitalis*.

3 Compound CMF0201

The ir spectrum (Figure 45) exhibited absorption peak at ν_{max} 3500 cm^{-1} (OH stretching), 1650 cm^{-1} (C=C stretching), 1070 cm^{-1} (C-O stretching), 910 cm^{-1} (C-O-C stretching). The electron impact mass spectrum (Figure 46) showed fragments indication of successive losses of two molecules of water at m/z 302 and 284 and an isopropyl group at m/z 259. The ^{13}C nmr spectrum (Figure 50) displayed 20 carbon signals which were determined as three methyl, seven methylene, seven methine, and three quaternary carbons from the HSQC (Figure 53 - 54) and DEPT (Distortionless Enhancement by Polarization Transfer) spectra (Figure 51 - 52). The above data established the tentative molecular formula of CMF0201 as $\text{C}_{20}\text{H}_{32}\text{O}_3$.

The complete assignment of CMF0201 was accomplished by analysis of its ^1H and ^{13}C nmr spectra and the absolute configuration was established by using the Mosher's method. The ^1H nmr spectrum (Figure 47 - 49) gave the signals of four olefinic protons at δ 5.52, 5.14, 4.80 and 4.67 ppm, three oxymethine protons at δ 4.40, 4.12 and 3.66 ppm and three methyl groups at δ 1.23, 0.96 and 0.74 ppm.

According to the ^1H - ^1H COSY spectrum of CMF0201 (Figure 55-58), the vicinal couplings are observed as followed : the proton at δ 2.79 ppm (H-8 α) to the proton at δ 4.12 ppm (H-9); H-9 proton to the proton at δ 2.96 ppm (H-10) which further coupled to the proton at δ 2.22 ppm (H-1); H-1 proton to the proton at δ 1.29 ppm (H-14) which, in turn, coupled to the protons at δ 1.73 (H-13 α) and 1.04 ppm (H-13 α), and H-13 α to two protons at δ 2.05 (H-12 α) and 2.27 ppm (H-12 β). H-12 α

proton showed allylic coupling to two olefinic protons at δ 4.80 and 4.67 ppm (H-20a and b). The above data suggested the fragment A as shown below. The fragment B was suggested because of the vicinal coupling between two methyl protons at δ 0.96 (H-15) and 0.74 ppm (H-16) and the proton at δ 1.81 ppm (H-17). The proton at δ 4.40 ppm (H-6) was coupled to the protons at δ 2.08 (H-5 α) and 1.86 ppm (H-5 β) which, in turn, coupled to the protons at δ 1.62 (H-4 α) and 1.79 ppm (H-4 β) indicating the fragment C as shown below. The remained fragments D, E, and F were assigned by analysing the splitting patterns of protons and the chemical shifts of ^1H and ^{13}C . The carbon and proton chemical shift assignments and proton-proton correlations of CMF0201 were summarized in Table 9.

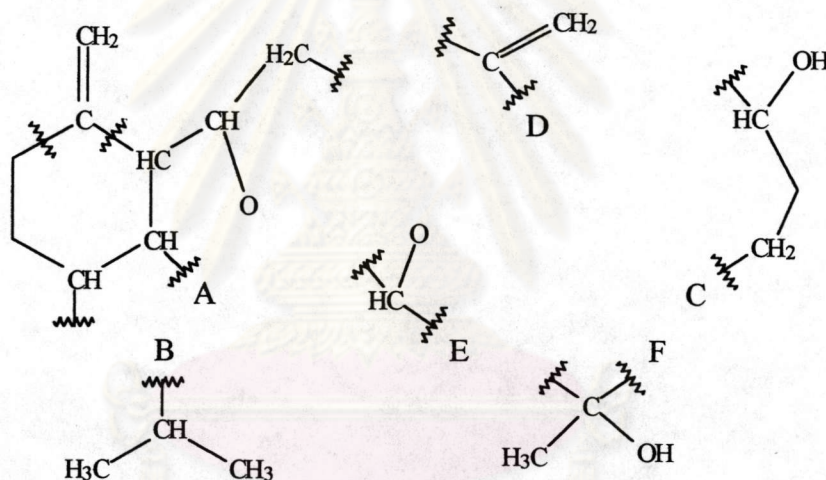


Table 9. Carbon and proton chemical shift assignments and proton-proton correlations of CMF0201

	δ C (ppm)	δ H (ppm)	Splitting pattern	multiplicity (J value, Hz)
1	44.1	2.22	dd	H-10 (7.6), H-14 (12.2)
2	91.9	3.66	br s	
3	74.1	-	-	-
4	35.1	β 1.79	ddd	H-4 α (15.0), H-5 β (3.6), H-5 α (11.0)
		α 1.62	ddd	H-4 β (15.0), H-5 β (7.0), H-5 α (4.0)

Table 9. (continued)

	δ C (ppm)	δ H (ppm)	Splitting pattern	multiplicity (<i>J</i> value, Hz)
5	35.6	β 1.86	tdd	H-5 α (11.0), H-4 β (3.6), H-4 α (7.0), H-6 (11.0)
		α 2.08	tt	H-5 β (11.0), H-4 β (11.0), H-4 α (4.4), H-6 (4.0)
6	72.9	4.40	dd	H-5 β (11.0), H-5 α (4.0)
7	152.2	-	-	-
8	39.3	β 2.28	br d	H-8 α (13.5)
		α 2.79	ddd	H-8 β (13.5), H-9 (5.5)
9	79.7	4.12	dd	H-10 (10.3), H-8 α (5.5)
10	47.7	2.96	dd	H-9 (10.3), H-1(7.6)
11	146.3	-	-	-
12	31.8	β 2.27	dt	H-12 α (13.0), H-13 β (3.3), H-13 α (3.0)
		α 2.05	br t	H-12 β (13.0), H-13 β (12.8)
13	25.3	β 1.04	qd	H-13 α (12.8), H-12 β (3.3), H-12 α (12.8), H-14 (12.8)
		α 1.73	dq	H-13 β (12.8), H-12 β (3.0), H-12 α (3.0), H-14 (3.3)
14	44.4	1.29	tt	H-1 (12.2), H-13 β (12.8), H-13 α (3.3), H-17 (3.0)
15	22.0	0.96	d	H-17 (6.7)
16	15.2	0.74	d	H-17 (6.7)
17	27.9	1.81	m	-
18	27.0	1.23	br s	-
19	116.6	a 5.52	br s	-
		b 5.14	br s	-
20	111.2	a 4.80	br t	-
		b 4.67	br t	-

The unambiguous assignment of this compound was accomplished by HMBC experiment optimized for coupling constant of 8 Hz (Figure 57). Two olefinic protons at δ 5.52 and 5.14 ppm showed long-range coupling to the carbons at δ 72.9 (C-6) and 39.3 ppm (C-8), suggesting the position of an exocyclic methylene moiety (C-19). Two more olefinic protons at δ 4.80 and 4.67 ppm (H-20a and b), which coupled to carbon signal at δ 31.8 (C-12) and 47.7 ppm (C-10), indicated the position of the other exomethylene to be at C-11. The proton at δ 3.66 ppm (H-2) showed long-range correlation through a heteroatom to a carbon signal at δ 79.7 ppm (C-9) supporting the position of an ethereal bridge between C-2 and C-9. This proton also correlated to carbon signal at δ 27.0 and 44.1 ppm suggesting the position of 18-methyl group and the connectivity between C-1 and C-2, respectively. The position of C-4 at δ 35.1 ppm was proven from the long-range correlation of the H-2 proton at δ 3.66 ppm and the 18-Me protons at δ 1.23 ppm to C-4 signal. The position of 18-methyl group was confirmed by its correlation to carbon signals at δ 74.1 (C-3) and δ 91.9 ppm (C-2). An isopropyl group, appearing as a one-proton multiplet at δ 1.81 (H-17) and two methyl doublets at δ 0.96 and 0.74 ppm, exhibited long-range correlation to carbon signal at δ 44.4 ppm, indicating its position as at C-14. The long-range correlations between protons and carbons were shown in Figure 9.

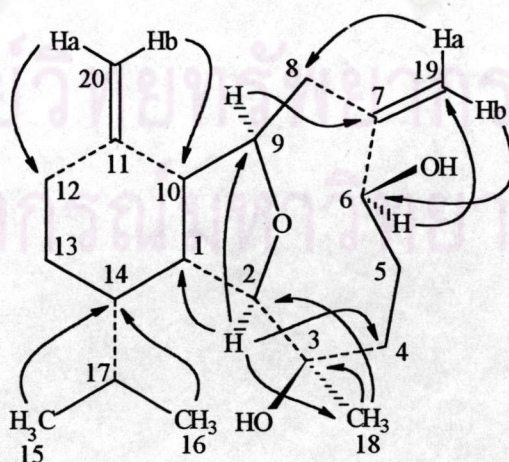
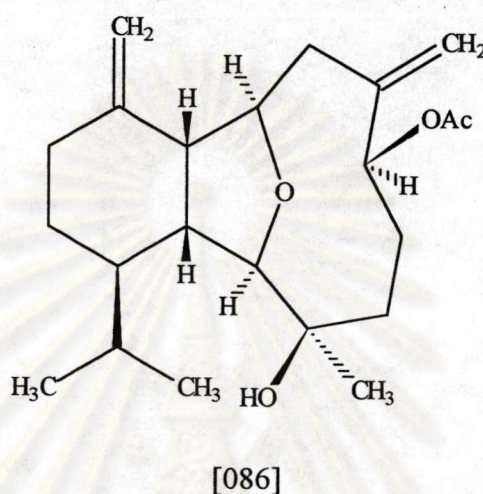


Figure 9. The long-range correlations in the HMBC spectrum of CMF0201

Upon treatment with acetic anhydride in pyridine, the compound CMF0201 formed a monoacetyl derivative [086] at 6-OH as indicated by the downfield shift of H-6 from δ 4.40 ppm to δ 5.32 ppm (Figure 58) thus confirming the presence of a secondary alcohol moiety at C-6.



The relative configuration of compound CMF0201 was proposed by analysis of NOESY spectrum (Figure 59 - 60) and proton coupling constants. For the six-membered ring part of the molecule, the H-1 proton signal at δ 2.22 ppm coupled to the H-10 proton signal at δ 2.96 and also showed the nOe indicating the *cis* relationship at the ring junction which were assigned as β orientation. The H-1 proton also coupled to the H-14 proton at δ 1.29 ppm ($J_{1,14} = 11.5$ Hz) without nOe observed, suggesting the α -axial of H-14, thus, the configuration of an isopropyl assigned as β -equatorial. In the ten-membered ring part including the 2,9 ethereal bridge, the 90° dihedral angle between H-1 and H-2 exhibited virtually no coupling between these two protons, therefore, H-2 appeared as singlet and its configuration assigned as α -equatorial. This H-2 proton signal showed nOe to proton signals at δ 1.23 (18-CH₃), 1.81 (H-17), and 4.40 ppm (H-6) suggesting similar configuration for these protons. H-10 proton (δ 2.96 ppm) was coupled to the proton at δ 4.12 (H-9) with the coupling constant of 10.3 Hz but did not show any nOe suggesting a *trans* relationship between H-9 and H-10. However, H-10 showed nOe to proton at δ 2.28

ppm (H-8) indicating β -configuration of this proton. The dihedral angle between H-8 β and H-9 protons was about 90° , so no coupling was observed between these protons and H-8 β appeared as doublet. On the other hand, H-9 was coupled to the proton at $\delta 2.79$ ppm with the coupling constant of 5.5 Hz corresponding to the dihedral angle of about 30° . Thus the configuration of H-9 was assigned as α , while the proton signal at $\delta 2.28$ ppm was assigned as H-8 β -axial and at $\delta 2.79$ ppm as H-8 α -equatorial. The correlations between protons from the NOESY spectrum were shown in Figure 10 and were summarized in Table 10.

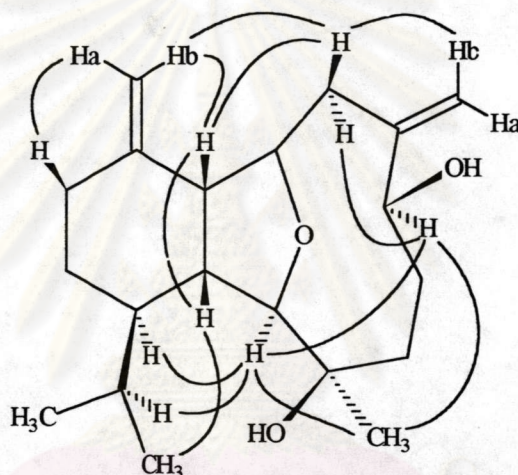


Figure 10. The correlations in the NOESY spectrum of CMF0201

Table 10. Long-range correlations in the HMBC spectrum and nOe observed in the NOESY spectrum of CMF0201

	δC (ppm)	δH (ppm)	long-range correlations in HMBC spectrum	nOe observed between protons in NOESY spectrum
1	44.1	2.22	C-9	H-2, H-16
2	91.9	3.66	C-1, C-4, C-9, C-14, C-18	H-1, H-6, H-14, H-17, H-18
3	74.1	-	-	-
4	35.1	α 1.62 β 1.79		H-10
5	35.6	α 2.08 β 1.86		Ha-19

Table 10. (continued)

	δC (ppm)	δH (ppm)	long-range correlations in HMBC spectrum	nOe observed between protons in NOESY spectrum
6	72.9	4.40	C-19	H-2, H-5 α , H-8 α , H-18
7	152.2	-	-	-
8	39.3	α 2.79 β 2.28	C-6, C-7, C-9, C-10, C-19 C-6, C-7, C-19	H-6 H-10, Hb-19, Hb-20
9	79.9	4.12	C-7, C-11	H-12 α
10	47.7	2.96	C-8, C-9, C-11, C-14, C-20	H-4 β , H-12 β , Hb-19, Hb-20
11	146.3	-	-	-
12	31.8	α 2.05 β 2.27		H-9, H-14 Ha-20
13	25.3	α 1.73 β 1.04		H-15 H-12 β , H-16
14	44.4	1.29		H-2, H-12 α , H-15
15	22.0	0.96	C-14, C-15, C-17	H-13 α , H-14, H-16
16	15.2	0.74	C-14, C-16, C-17	H-1, H-13 β , H-15
17	27.9	1.81	C-13, C-14, C-15, C-16	H-2, H-18
18	27.0	1.23	C-2, C-3, C-4	H-2, H-6, H-17
19	116.6	a 5.52 b 5.14	C-6, C-8 C-6, C-8	H-5 β H-8 β , Hb-20
20	111.2	a 4.80 b 4.67	C-10, C-12 C-10, C-12	H-12 β H-10, H-8 β

The absolute configuration of compound CMF0201 was established utilizing modified Mosher's method since this compound has a secondary hydroxy group at C-6. The (*R*) - and (*S*) - MTPA esters were prepared by treatment the alcohol with (*S*) - and (*R*) - α -methoxy α -trifluoromethyl-phenylacetic chloride, respectively. Each product was further purified by using silica gel column chromatography and CHCl₃ : ethylacetate (1 : 1) as an eluent. The result was achieved by comparison the proton chemical shifts between these two derivatives (Figure 61-63 and 64-66) The $\Delta\delta$ ($\delta_S - \delta_R$) values were shown in Table 11.

Table 11. The $\Delta\delta(\delta_S-\delta_R)$ values of the MTPA derivatives of compound CMF0201

proton	δH (ppm)		$\Delta\delta$ ($\delta_S-\delta_R$)
	(<i>S</i>)-MTPA	(<i>R</i>)-MTPA	
2	3.651	3.652	-0.001
4 α	1.560	1.590	-0.030
4 β	1.815	1.826	-0.011
5 α	1.968	2.082	-0.114
5 β	1.945	1.982	-0.037
9	4.120	4.110	+0.010
10	2.892	2.878	+0.014
19a	5.195	5.100	+0.095
19b	5.441	5.253	+0.188
20a	4.633	4.622	+0.011
20b	4.785	4.780	+0.005

Following the MTPA rules, these data indicated an *S* configuration for C-6 and therefore the absolute stereochemistry for compound CMF0201 was assigned as (1*R*, 2*R*, 3*R*, 6*S*, 9*S*, 10*R*, 14*R*)-cladiell-7(19),11(20)-dien-3,6-diol. This compound was identical with a known eunicellin diterpenoid first isolated from *Cladiella australis* (Rao *et al.*, 1994b).

4 Compound CMF0361

The molecular formula $C_{23}H_{40}O_6$ of compound CMF0361 was deduced from the high resolution fabms measurement ($[M+H]^+$ observed : m/z 413.2916, calculated : m/z 413.2904) (Figure 68). The eims spectrum (Figure 67) showed the $[M-H_2O]^+$ and $[M-H_2O-HOAc]^+$ fragments at m/z 394 and 334, respectively. The ir spectrum (Figure 69) exhibited the presence of hydroxy groups (ν_{max} 3500 cm^{-1}), carbonyl (1720 cm^{-1}) and ether moieties (910 cm^{-1}).

The ^1H nmr spectrum of CMF0361 (Figure 70 - 71) showed the presence of three oxymethine protons at δ 4.08 (dd, $J = 11.0, 3.6$ Hz), 4.06 (ddd, $J = 11.0, 7.5, 3.6$ Hz) and 3.55 (s). Seven methyl peaks were observed including a methoxy group δ 3.36 (s), an acetoxy group δ 2.00 (s), a methyl geminal to the acetoxy group δ 1.46 (s), two methyls of an isopropyl group δ 0.95 and 0.82 (d, $J = 6.7$ Hz), and two tertiary methyl signals attached to oxygen-bearing carbons and resonated at the same chemical shift of δ 1.16 ppm.

The ^{13}C nmr (Figure 72) and HMQC spectrum of compound CMF0361 (Figure 73 - 74) indicated 23 carbons assignable as three quaternary, seven methine, five methylene, seven methyl and one carbonyl carbon.

The ^1H - ^1H COSY spectrum of compound CMF0361 (Figure 75) displayed the correlations between protons as followed : the H-8 β proton at δ 2.30 to H-9 at δ 4.06 ppm with the coupling constant of 11.0 Hz, H-9 to the proton signal at δ 2.82 (H-10) with the coupling constant of 7.5 Hz, H-10 to the proton signal at δ 2.18 (H-1) with the coupling constant of 7.5 Hz. In turn, H-1 coupled to the proton signal at δ 1.22 (H-14) which finally coupled to the H-13 proton at δ 1.40 ppm. The above data indicated that compound CMF0361 had a eunicellin skeleton with no olefinic moieties. The carbon and proton chemical shift assignments and the multiplicity of protons were summarized in Table 12.

Table 12. The carbon and proton chemical shift assignments and the multiplicity of protons of CMF0361.

	δC (ppm)	δH (ppm)	multiplicity and J value (Hz)
1	42.0	2.18	dd; $J = 10.4, 7.5$
2	90.8	3.55	br s
3	74.6	-	-
4	41.0	1.85	ddd; $J = 14.6, 8.0, 2.0$
		1.83	ddd; $J = 14.6, 10.3, 2.0$

Table 12. (continued)

	δC (ppm)	δH (ppm)	multiplicity and J value (Hz)
5	25.7	1.97	m
		2.40	m
6	90.8	4.08	dd; $J = 11.0, 3.6$
7	76.8	-	-
8	46.4	α 1.74	dd; $J = 15.0, 3.6$
		β 2.30	ddd; $J = 15.0, 11.0, 1.22$
9	75.6	4.06	ddd; $J = 11.0, 7.5, 3.6$
10	54.4	2.82	t; $J = 7.5$
11	82.9	-	-
12	30.6	2.40	br d; $J = 11.6$
		1.40	br t; $J = 11.6$
13	18.0	α 1.41	m
		β 1.20	m
14	42.6	1.22	m
15	21.8	0.95	d; $J = 6.7$
16	15.4	0.82	d; $J = 6.7$
17	29.1	1.71	m
18	30.4	1.16	s
19	24.0	1.16	s
20	24.5	1.46	s
-OMe	57.1	3.36	s
-OOCMe	170.2	-	-
-OOCMe	22.6	2.00	s

The connectivity and the assignment of the quaternary carbons of each fragment were determined by the HMBC spectrum (Figure 76 - 78). The methyl protons of the isopropyl showed long-range coupling to the carbon at $\delta 42.6$ ppm

revealed that the isopropyl group was connected to C-14. The methine proton H-2 at δ 3.55 ppm showing long-range coupling to carbons at δ 42.0 (C-1), 54.4 (C-10), and 42.6 ppm (C-14) confirmed the connectivity of C-2 to C-1. Furthermore, this proton showed long-range coupling to the quaternary carbon at δ 74.6 ppm which was assigned as C-3. The proton at δ 1.85 ppm which showed long-range coupling to the carbon at δ 74.6 (C-3) and 90.8 (C-2) was assigned as H-4. This proton showed long-range coupling to the carbon at δ 25.7 ppm which was assigned as C-5. The proton at δ 1.74 (H-8 α) showed long-range correlation to the quaternary carbon at δ 76.8 ppm which was assigned as C-7. The methoxy group was placed at C-6 based on the long-range correlation of the methoxy protons at δ 3.36 ppm to C-6 (δ 90.8 ppm). Furthermore, H-9 (δ 4.06 ppm) showed long-range correlation to the carbon at δ 90.8 (C-2) through heteroatom and in turn, H-2 also showed long-range correlation to C-9 suggesting the etheral bridge between C-2 and C-9 which was the characteristic of the eunicellin diterpenoids. The proton at δ 2.82 (H-10) showed long-range correlations to two carbons at δ 82.9 and 30.6 ppm which were assigned as C-11 and C-12, respectively.

The HMBC spectrum also showed the location of methyl group. The methyl carbon at δ 30.4 ppm was assigned as C-18 since the proton at δ 3.55 (H-2) and 1.85 ppm (H-4) showed long-range correlation to this carbon, in turn, the 18-CH₃ protons showed long-range correlation to C-2 and C-3. The methyl carbon at δ 24.0 ppm was assigned as C-19 because the methyl protons showed long-range correlation to C-8, C-7, and C-6. Finally, the methyl carbon at δ 24.5 ppm was assigned as C-20 since H-10 showed long-range correlation to this methyl carbon and this methyl proton (δ 1.46) showed long-range correlation to C-10, C-11, and C-12. The downfield signal of C-11 (δ 82.9 ppm) comparing to those of C-3 (δ 74.6 ppm) and C-7 (δ 76.5 ppm) suggested the placement of the acetoxy group at C-11 and the hydroxy groups at C-3 and C-7. The long-range correlations between protons and carbons from the HMBC spectrum were shown in Figure 11 and summarized in Table 13.

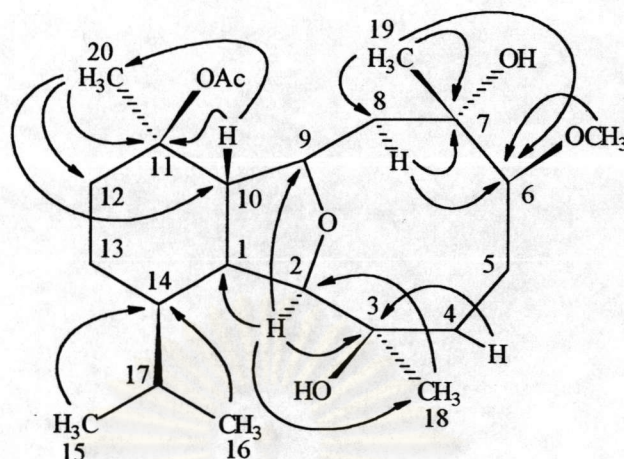


Figure 11. The correlations in the MHBC spectrum of CMF0361

The relative stereochemistry of CMF0361 was assigned by analysing the coupling constants between protons and the NOESY spectrum (Figure 79-81). The coupling constant 7.2 Hz of H-1 and H-10 and its nOe correlation suggested the (β)-*cis*-relation at the ring junction which was similar to other eunicellin derivatives. The 90° dihedral angle between H-1 and H-2 without nOe suggested α -configuration of H-2. The coupling constant 7.6 Hz of H-9 and H-10 without nOe suggested α -configuration of H-9. Observation of nOes among 19-CH₃, H-8 α , and H-8 β defined the β -configuration of the methyl group. H-6 α and pseudo- β -axial of 6-OCH₃ were defined because of no nOe observation between 19-CH₃ and H-6. Observation of nOes among H-2, H-4, H-14, and H-17 suggested the α -configuration of these protons. Finally, the observation of nOes among H-9, H-8 α , and 20-CH₃ defined the α -configuration of these protons. The results from NOESY spectrum and the relative stereochemistry of CMF0361 were shown in Figure 12 and summarized in Table 13.

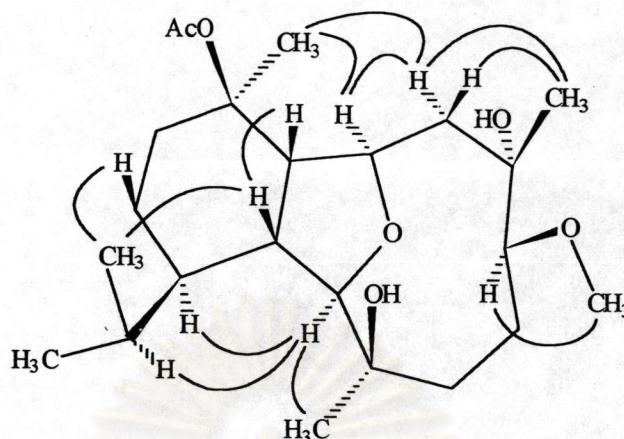


Figure 12. The correlations in the NOESY spectrum of CMF0361

Table 13. The long-range correlations between protons and carbons and nOe observed between protons of CMF0361.

	δC (ppm)	δH (ppm)	long-range correlations in HMBC spectrum	nOe observed between protons in NOESY spectrum
1	42.0	2.18	C-9, C-10, C-14, C-17	H-2, H-10, H-16,
2	90.8	3.55	C-1, C-3, C-9, C-10, C-14, C-18	H-1, H-14, H-17, H-18, C-4 α , H-21
3	74.6	-	-	-
4	41.0	1.85 1.83	C-2, C-3, C-5, C-18	H-18 H-2, H-18
5	25.7	1.97 2.40		H-21
6	90.8	4.08		H-21
7	76.8	-	-	-
8	46.4	α 1.74 β 2.30	C-6, C-7 C-9, C-10	H-9, H-19, H-20 H-19
9	75.6	4.06	C-2, C-11	H-8 α , H-20
10	54.4	2.82	C-1, C-8, C-9, C-11, C- 12, C-14	H-1, H-20



Table 13. (continued)

	δ C (ppm)	δ H (ppm)	long-range correlations in HMBC spectrum	nOe observed between protons in NOESY spectrum
11	82.9	-	-	-
12	30.6	2.40 1.40	C-10, C-11 C-13	
13	18.0	α 1.40 β 1.20		H-15 H-16
14	42.6	1.22		H-2
15	21.8	0.95	C-14, C-16, C-17	H-13 α
16	15.4	0.82	C-14, C-15, C-17	H-13 β , H-1
17	29.1	1.71	C-1, C-13, C-14, C-15, C-16	H-2
18	30.4	1.16	C-2, C-3	H-2, H-4 α , H-4 β
19	24.0	1.16	C-6, C-7	H-8 α , H-8 β
20	24.5	1.46	C-10, C-11, C-12	H-8 α , H-9, H-10
-OMe	57.1	3.36	C-6	H-5, H-6
-OOCMe	170.2	-	-	-
-OOCMe	22.6	2.00	C-23	

The compound CMF0361 is proposed to be a new eunicellin diterpenoid which the structure is similar to palmonin A, a compound isolated from the gorgonian, *Eunicella verrucosa* (Ortega *et al.*, 1993). Compound CMF0361 was named as 3-deacetylpalmonin A.