

Diterpenes from the Soft Coral *Clavularia inflata* (Coelenterata, Octocorallia, Stoloniifera)

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Three diterpenes were isolated from the dichloromethane extract of the Stoloniifer *Clavularia inflata*. The structure of (3*S*,4*S*)-clavulara-1(15),17-diene-3,4-diol **1** was elucidated by 1D and 2D NMR spectroscopy while the structures of (4*R*)-clavulara-1(15),17-diene-4-ol **2** and (1*R*,4*R*)-clavulara-17-ene-1,4-diol **3** were determined by comparing their proton NMR spectra with those previously reported.

INTRODUCTION

Soft corals are a major source of terpenoids. Studies conducted on these marine organisms led to the isolation of novel terpenoid metabolites of varying biological activities. The genus *Clavularia*, for example, gave a number of cytotoxic compounds exhibiting anti-tumor and anti-inflammatory activities¹⁻³. Five diterpenes have been isolated from *Clavularia inflata*. They are (3*S*,4*S*)-clavulara-1(15),17-diene-3,4-diol **1**^{4,5}, (4*R*)-clavulara-1(15),17-diene-4-ol **2**^{4,5}, (1*R*,4*R*)-clavulara-17-ene-1,4-diol **3**^{4,5}, (3*E*,5*R*,7*E*,11*S*,12*S*,14*Z*)-11-methyl-15-nordolabella-3,7,14-trien-5-ol **4**⁶ and (3*Z*,5*E*,9*E*,1*R**,12*aR**)-1-isopropyl-6,10,12a-trimethyl-1,2,4,7,8,11,12,12a-octahydrocyclopentacycloundecen-7-ol, **5**⁷. The

structures of **3**, **4**, and **5** were determined by x-ray crystallography while the structures of **1** and **2** were determined by chemical intercorrelation with **3**. Compounds **1**, **2** and **3** were first reported as 3 α ,4 β -dihydroxy-clavulara-1(15),17-diene, 4 β -hydroxycavulara-1(15),17-diene and 1 α ,4 β -dihydroxycavular-17-ene respectively⁴. However, the compounds were renamed after the absolute configurations of compounds **2** and **3** were determined by the modified Horeau method.⁵

In this paper, we report the isolation and structure elucidation of the diterpenes **1**, **2**, and **3** (Figure 1) from *Clavularia inflata*. This is the first report on the structure elucidation of **1** using 1D and 2D NMR analysis.

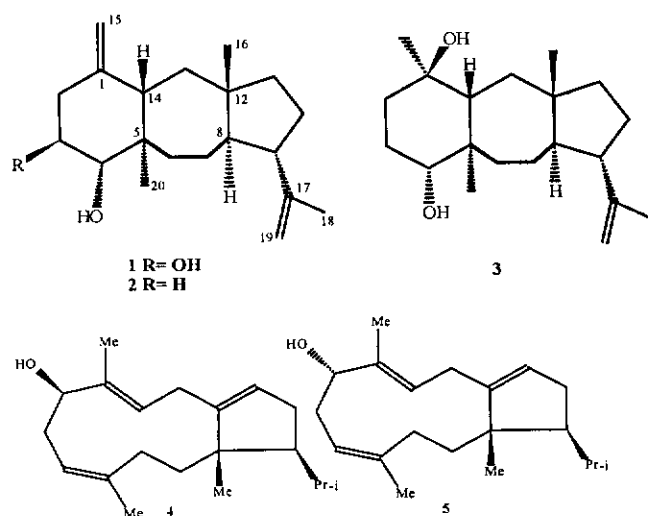


Figure 1. Structures of Compounds 1-5

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extract of freeze-dried *Clavularia inflata* afforded the diterpenes 1-3. The structure of 1 was elucidated by 1D and 2D NMR spectroscopy as follows.

The ^1H NMR spectrum of 1 (Table 1) indicated resonances for three methyl singlets, one of which belong to an allylic methyl at δ 1.65 (3H, s); two carbinyl protons at δ 3.02 (1H, d, $J=9$ Hz) and 3.46 (1H, m); and four vinylidene double bond protons at δ 4.70 (1H, s, br), 4.87 (1H, s, br), and 4.64 (2H, m). The relatively shielded region (δ 1.4-2.6) indicated the resonances for overlapping methylene and methine protons of 1.

Table 1. 300 MHz ^1H NMR and 75 MHz ^{13}C NMR Spectral Data of 1 in CDCl_3

Carbon No.	^{13}C , δ	^1H , δ
C-1	147.2	—
C-2	43.7	2.07 (H-2, 1H, m), 2.58 (H-2', 1H, dd, $J=3$ Hz, 14 Hz)
C-3	71.5	3.46 (1H, m)
C-4	82.4	3.02 (1H, d, $J=9$ Hz)
C-5	43.8*	—
C-6	34.8	1.58 (H-6, 1H, m), 2.10 (H-6', 1H, m)
C-7	25.2	1.29 (H-7, 1H, m), 1.62 (H-7', 1H, m)
C-8	49.1	1.60 (1H, m)
C-9	55.0	2.33 (1H, m)
C-10	27.6	1.50 (H-10, 1H, m), 1.86 (H-10', 1H, m)
C-11	43.5	1.46 (2H, m)
C-12	44.3*	—
C-13	43.3	1.48 (H-13, 1H, m), 1.74 (H-13', 1H, dd, $J=3$ Hz, 14 Hz),
C-14	46.8	2.00 (1H, m)
C-15	110.0	4.70 (H-15, 1H, s, br), 4.87 (H-15', 1H, s, br)
C-16	18.7	0.97 (3H, s)
C-17	147.5	—
C-18	18.7	1.65 (3H, s)
C-19	109.8	4.64 (2H, m)
C-20	10.7	0.73 (3H, s)

* ^{13}C assignments may be interchanged

The ^{13}C and DEPT NMR spectral data of **1** (table 1) indicated twenty carbons with the following functionalities: two olefinic methylene carbons at δ 109.8 and 110.0; two non-protonated olefinic carbons at δ 147.2 and 147.5; five methine (two of which are oxygenated at δ 82.4 and 71.5), three methyl and two quaternary carbons. The ^{13}C and ^1H assignments for **1** were verified by ^{13}C - ^1H short-range correlation experiment (XHCORR).

Three isolated spin systems were deduced from the COSY spectrum of **1**. The first spin system was delineated as follows. The carbonyl proton at δ 3.02 was coupled to another carbonyl hydrogen at δ 3.46 by 9 Hz. The latter proton was in turn coupled to the methylene protons at δ 2.58 and 2.07. The proton at δ 2.07 was also correlated to the methine proton at δ 2.00 (1H, m) and to the exomethylene protons at δ 4.87 (s, br) and 4.70 (s, br) which were correlated to each. The methine proton at δ 2.00 was in turn coupled to methylene protons at δ 1.74 and 1.48. No other correlations were observed, thus giving fragment A (Figure 2).

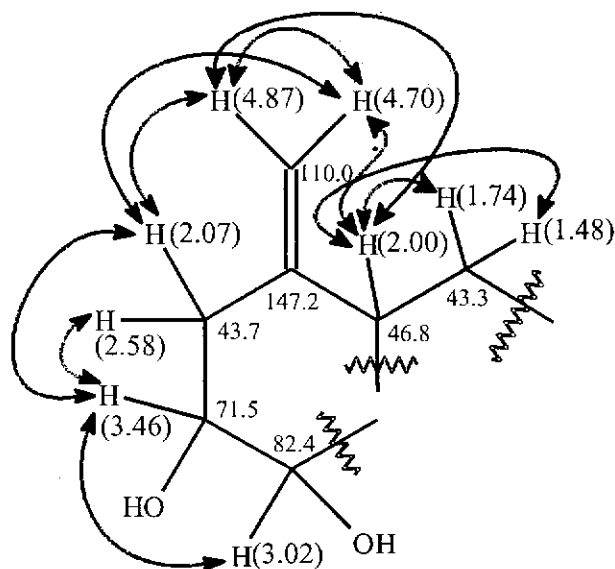


Figure 2. Fragment A deduced from the COSY spectrum

The exocyclic methylene protons at δ 4.87 and 4.70 were broad singlets due to very small coupling between these protons and the two allylic protons at δ 2.07 and 2.00. Allylic coupling and coupling between geminal olefinic protons are from 0-3 Hz. Typical allylic coupling is 1.5 Hz, while typical geminal olefinic coupling is 0-2 Hz⁸. W coupling was observed between the methylene proton at δ 2.07 and the methine proton at δ 2.00. W coupling occur in strained ring systems (bridged rings)⁸.

The second spin system was deduced as follows. The methylene protons at δ 2.10 and δ 1.58 were both correlated to the other methylene hydrogens at δ 1.29 and δ 1.62. The proton at δ 1.29 was also correlated to the methine proton at δ 1.60 which was in turn correlated to the proton resonating at δ 2.33. The latter proton was also correlated to the methylene protons at δ 1.50 and 1.86. A correlation between the proton at δ 1.86 and the methylene protons at δ 1.46 (2H, m) was also observed. This gives rise to fragment B (Figure 3).

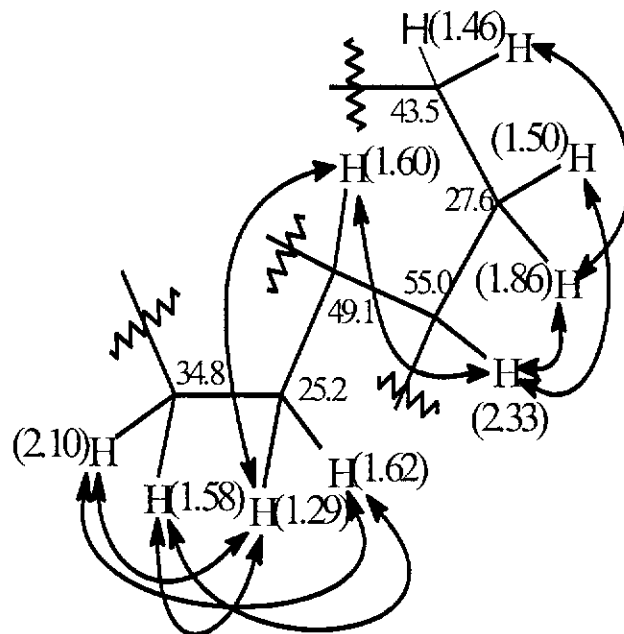


Figure 3. Fragment B deduced from the COSY spectrum

The third spin system, fragment C (Figure 4) was delineated as follows. The methyl protons at δ 1.65 (3H, s, br) were correlated only to the olefinic methylene protons at δ 4.64 (2H, m). This methyl must be bonded to a non-protonated carbon and is allylic to the methylene. This indicates an isopropylene group as follows:

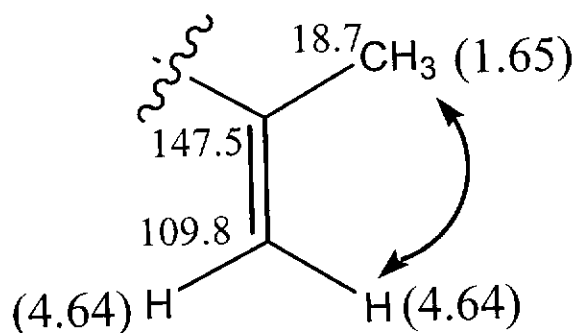


Figure 4. Fragment C deduced from the COSY spectrum

The geminal olefinic protons at δ 4.64 appear as closely spaced multiplets due to the second order splitting of the almost overlapping protons which are also coupled to the allylic methyl at δ 1.65. As mentioned earlier, olefinic geminal protons and allylic protons have small J values (0-3Hz)⁸. The resonances of the geminal protons of isopropylene and the allylic methyl have been reported as broad singlet⁹.

The two other methyls at δ 0.73 and 0.97 are singlets indicating that they are attached to non-protonated carbons.

The ^1H and ^{13}C assignments of **1** were deduced from a ^{13}C - ^1H short-range correlation experiment, XHCORR. This is a 2D NMR spectrum, which shows cross-peaks between carbons and protons directly bonded to each other. Thus, the resonances of the carbon atoms in the fragments deduced from COSY have already been assigned in the following discussion as COLOC.

The two-dimensional COLOC experiment

(Table 2) established the connectivity of **1**. The structure of Fragment A was supported by the observed long-range correlation between the proton at δ 4.87 and the carbon at δ 46.8. Fragment A was attached to fragment B through a long range correlation between the proton at δ 1.74 and the carbon at δ 49.1 and between the proton at δ 0.97 and the carbon at δ 49.1. The terminal carbons at δ 43.3 and 43.5 of fragments A and B respectively were bonded to a non-protonated carbon. There were two non-protonated carbons at δ 44.3 and 43.8 deduced from the ^{13}C /DEPT spectra. Either one of these carbons could connect fragments A and B. The methyl singlet at δ 0.97 is long range correlated to the carbon at δ 49.1 which was part of fragment B. This methyl singlet was attached to the carbon at δ 44.3 or 43.8 which joins fragments A and B. Thus the following is a partial structure of **1** (Figure 5).

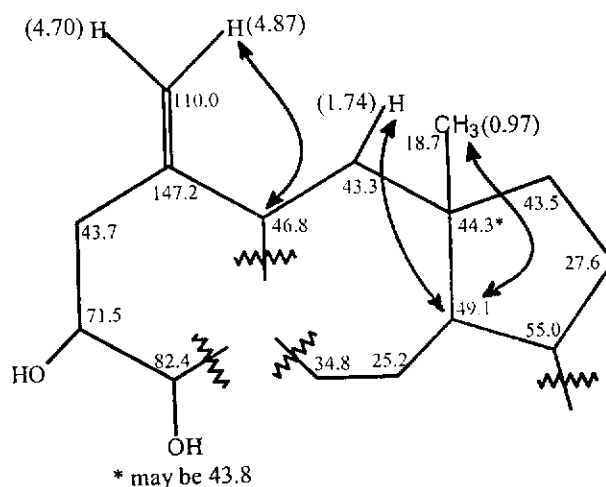


Figure 5. Partial Structure A deduced from the COLOC spectrum

Fragment C was attached to the partial structure above through long range correlations between the carbon at δ 55.0 and the methyl protons at δ 1.65 and the olefinic protons at δ 4.64. A correlation between the methyl protons at δ 1.65 and the non-protonated olefinic carbon at δ 147.5 was also

observed giving partial structure B below (Figure 6).

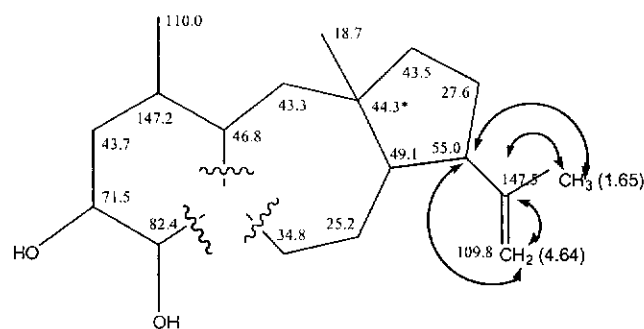
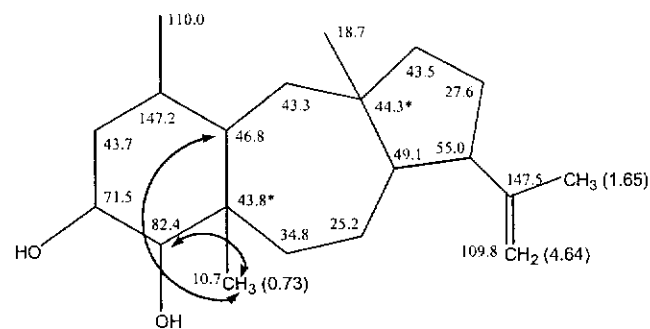


Figure 6. Partial Structure B deduced from the COLOC spectrum

The remaining methyl singlet at δ 0.73 was long-range correlated to the carbons at δ 82.4 and 46.8. There was only one remaining unattached quaternary carbon at δ 44.3 or 43.8 which could be

bonded to this methyl group and the carbons at δ 34.8, 46.8 and 82.4. This gave the complete structure of **1** (Figure 7) shown below.



*may be interchanged

Figure 7. Structure of **1** deduced from the COLOC spectrum

Table 2. COLOC Correlations of Compound **1** in CDCl_3

Carbon No.	^{13}C , δ	^1H , δ of Correlated Protons
C-1	147.2	2.00 (H-14), 2.58 (H-2')
C-2	43.7	-
C-3	71.5	2.07 (H-2)
C-4	82.4	2.58 (H-2')
C-5	43.8*	-
C-6	34.8	-
C-7	25.2	-
C-8	49.1	0.97 (H-16), 1.74 (H-13')
C-9	55.0	1.65 (H-18), 4.64 (H-19)
C-10	27.6	-
C-11	43.5	-
C-12	44.3*	-
C-13	43.3	-
C-14	46.8	0.73 (H-20), 4.87 (H-15')
C-15	110.0	-
C-16	18.7	-
C-17	147.5	1.65 (H-18)
C-18	18.7	-
C-19	109.8	-
C-20	10.7	-

* ^{13}C assignments may be interchanged

Compound **1** was previously isolated from the same species of soft coral collected from Laing Island, Papua New Guinea.⁴ The structure of **1** was determined by chemical intercorrelation with **3** whose structure and relative chemistry was determined by X-ray crystallography. Another study reported the absolute configuration of **1** by correlating it with the absolute configuration of **2** and **3** determined by modified Horeau method.⁵ Table 3 compares the ¹H NMR spectral data of **1** and (3S,4S)-clavulara-1(15),17-diene-3,4-diol.⁴ The differences in the resonances for the olefinic protons of (3S,4S)-clavulara-1(15),17-diene-3,4-diol obtained from a 60 MHz NMR and **1** obtained from a 300 MHz NMR may be explained by the low resolution NMR used in the previously reported compound. This resulted in the overlapping of resonances at around δ 4.70 (4H, m) in the low resolution NMR, while the spectrum is better resolved in the high resolution NMR.

There are seven chiral carbons in **1**. The relative stereochemistry of these chiral carbons could not be established by coupling constants since H-3, H-8, H-9, and H-14 were multiplets, while C-5 and C-12

were quaternary carbons. Only H-4 was deduced as axial due to a larger coupling constant ($J = 9\text{ Hz}$) for this proton. Axial-axial coupling is typically between 8-10 Hz while axial-equatorial coupling is typically between 2-3 Hz respectively.⁸ The relative stereochemistry of **1** was no longer determined by NOE since its relative stereochemistry has already been established through correlations with closely related compounds which were also isolated from *C. inflata*.⁵

The ¹H, ¹³C and DEPT NMR analysis of **2** indicated structural similarity to **1**. The only difference is that the ¹H NMR data of **2** gave only one signal, a doublet of doublet, at δ 3.28 instead of the two carbonyl resonances at δ 3.02 and δ 3.46. This suggests that there is only one hydroxyl group in **2** and that this hydroxymethine proton is adjacent to two non-magnetically equivalent protons. This is consistent with **2** having a hydroxyl group at C-4 but not C-3. Compound **2** also indicated overlapping resonances from δ 1.29 to δ 2.05. It is expected that resonances for protons from H-6 to H-14 (δ 1.29- 2.33) are similar for compounds **1** and **2**. However, the exact signals for the protons

Table 3. Comparison of 300 MHz ¹H NMR Spectral Data of **1 and 60 MHz ¹H NMR Spectral Data of (3S,4S)-clavulara-1(15),17-diene-3,4-diol ⁴.**

Carbon No.	¹ H, δ (1)	¹ H, δ 3S,4S-clavulara-1(15),17-diene-3,4-diol
C-3	3.46 (1H, m)	3.41 (1H, m)
C-4	3.02 (1H, d, $J = 9\text{ Hz}$)	2.99 (1H, d, $J = 9\text{ Hz}$)
C-15	4.70 (1H, s, br) 4.87 (1H, s, br)	4.70 (2H, m)
C-16	0.97 (3H, s)	0.98 (3H, s)
C-18	1.65 (3H, s)	1.67 (3H, bs)
C-19	4.64 (1H, s, br)	4.70 (2H, m)
	4.69 (1H, s, br)	
C-20	0.73 (3H, s)	0.73 (3H, s)

Table 4. Comparison of 300 MHz ^1H NMR Spectral Data of 1 and , (4R)-clavulara-1(15),17-diene-4-ol 2.

Carbon No.	^1H , δ (1)	^1H , δ (2)
C-3	3.46 (1H, m)	1.80 (1H, m), 2.10 (1H, m)
C-4	3.02 (1H, d, J = 9 Hz)	3.28 (1H, dd, J = 9 Hz, 3 Hz)
C-15	4.70 (H-15, 1H, s, br), 4.87 (H-15', 1H, s,br)	4.70 (1H, s, br), 4.87 (1H, s,br)
C-16	0.97 (3H, s)	0.97 (3H, s)
C-18	1.65 (3H, s)	1.70 (3H, s)
C-19	4.64 (2H, m)	4.64 (1H, s, br), 4.69 (1H, s, br)
C-20	0.73 (3H, s)	0.73 (3H, s)

of **2** could not be assigned due to overlapping signals. Furthermore, HMQC, which is essential in determining the exact location of protons, was not obtained for **2**. A comparison of the ^1H NMR spectral data of **1** and **2** is presented in Table 4.

The loss of one of the hydroxyl groups in **2** was confirmed by the DEPT and ^{13}C spectra showing only one methine oxygenated carbon at δ 77.9. Instead of the carbinyl carbon at δ 71.5 in **1**, an additional methylene carbon at δ 32.3 was found in **2**. The loss of the hydroxyl group in **2** also resulted in the shielding of C-2 from δ 43.7 in **1** to 35.7 in **2** and C-4 from δ 82.4 in **1** to 77.9 in **2**. The carbons at δ 147.2 (C-1) and 43.8 were deshielded to δ 150.3 and 46.6, respectively in **3**. Comparison of the ^{13}C NMR spectral data of **1** and **2** is given in Table 5.

The structure of **2** is identical with (4R)-clavulara-1(15),17-diene-4-ol isolated from *clavulara inflata* collected from Papua New Guinea. A comparison of their ^1H NMR spectral data is given in Table 6.

The ^1H NMR spectrum of **3** indicated a closely related structure to **2**. It is expected that resonances for protons H-6 to H-14

Table 5. 75 MHz ^{13}C NMR Spectral Data of 1 and 15 MHz ^{13}C NMR Spectral Data 2 in CDCl_3

Carbon No.	^{13}C , δ (1)	^{13}C , δ (2)
C-1	147.2	150.3
C-2	43.7	35.7
C-3	71.5	32.3
C-4	82.4	77.9
C-5	43.8*	46.6
C-6	34.8	34.6
C-7	25.2	25.7
C-8	49.1	49.2
C-9	55.0	54.9
C-10	27.6	28.0
C-11	43.5	43.6
C-12	44.3*	44.3
C-13	43.3	43.6
C-14	46.8	46.6
C-15	110.0	109.6
C-16	18.7	18.7
C-17	147.5	147.7
C-18	18.7	18.7
C-19	109.8	108.8
C-20	10.7	9.6

* ^{13}C assignments may be interchanged

Table 6. Comparison of 300 MHz ^1H NMR Spectral Data of 2 and 60 MHz ^1H NMR Spectral Data (4R)-clavulara-1(15),17-diene-4-ol ⁴.

Carbon No.	^1H , δ (2)	^1H , δ 4R-clavulara-1(15),17-diene-4-ol
C-4	3.28 (1H, dd, J = 4.5, 11 Hz)	3.28 (1H, dd, J = 4.5, 11 Hz)
C-15	4.70 (1H, s, br) 4.87 (1H, s, br)	4.70 (2H, m)
C-16	0.97 (3H, s)	0.97 (3H, s)
C-18	1.65 (3H, s)	1.65 (3H, bs)
C-19	4.60 (1H, s, br), 4.65 (1H, s, br)	4.70 (2H, m)
C-20	0.73 (3H, s)	0.72 (3H, s)

Table 7. Comparison of 300 MHz ^1H NMR Spectral Data of 2 and 3

Carbon No.	^1H , δ (2)	^1H , δ (3)
C-4	3.28 (1H, dd, J = 4.5, 11 Hz)	3.21 (1H, m),
C-15	4.70 (1H, s, br) 4.80 (1H, s, br)	1.14 (3H, s)
C-16	0.97 (3H, s)	0.95 (3H, s)
C-18	1.65 (3H, s)	1.65 (3H, s)
C-19	4.60 (1H, s, br), 4.65 (1H, s, br)	4.64 (1H, s, br), 4.69 (1H, s, br)
C-20	0.73 (3H, s)	0.90 (3H, s)

Table 8. Comparison of 300 MHz ^1H NMR Spectral Data of 3 and 60 MHz ^1H NMR Spectral Data of (1R,4R)-clavulara-17-ene-1,4-diol ⁴.

Carbon No.	^1H , δ (3)	^1H , δ 1R,4R-clavulara-17-ene-1,4-diol
C-4	3.21 (1H, m)	3.20 (1H, m)
C-15	1.14 (3H, s)	1.14 (3H, s)
C-16	0.95 (3H, s)	0.94 (3H, s)
C-18	1.65 (3H, s)	1.67 (3H, bs)
C-19	4.67 (2H, m)	4.68 (2H, bs)
C-20	0.90 (3H, s)	0.90 (3H, s)

(δ 1.29-2.05) are similar for compounds **2** and **3**. However, the signals in the shielded region are overlapping and HMQC was not obtained. Thus, only the differences in the spectra were compared in Table 7. The only difference is that the exomethylene protons at δ 4.70 (H-15) and δ 4.87 (H-15') in **2** were no longer found in **3**. Instead, an additional methyl singlet at δ 1.14 was found in **3**. Since the methyl group is a singlet, compound **3** could not have resulted from reduction of the double bond as this would give rise to a methyl doublet. Thus, **3** may have resulted from the hydration of **2**.

The structure of **3** is identical with (1R,4R)-clavulara-17-ene-1,4-diol which was isolated from the same species of soft coral collected from Papua New Guinea. A

comparison of their ^1H NMR spectral data is given in Table 8.

EXPERIMENTAL

General

The ^1H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra at 75.5 MHz. NMR spectra were recorded in deuterated chloroform (CDCl_3) solutions on a Bruker AM-300 spectrometer with CDCl_3 (d 7.26, 77.0ppm) as reference. Two dimensional XHCORR, COLOC and COSY were obtained by using the standard Bruker pulse sequences. Column chromatography was performed with Merck silica gel (5-20 μm) was used for column chromatography. TLC was performed with aluminum-backed plates coated with silica gel F₂₅₄; plates were visualized by spraying with vanillin- H_2SO_4 and warming. All solvents were freshly distilled.

Sample Collection and Extraction

The soft coral, *Clavularia inflata* was collected by SCUBA diving at Thetford Reef, North Queensland, Australia and a voucher specimen C34 is held at James Cook University, Townsville, Australia. The freeze-dried sample (94 g) was repeatedly extracted with dichloromethane affording 5.3 g. of crude extract.

Isolation of the Diterpenes

A portion of the crude extract (1.5 g) was chromatographed on a vacuum liquid chromatography (VLC) column—a sintered glass filter funnel with dry silica gel (tlc grade, 5-20 mm) with a stepped gradient from dichloromethane to ethyl acetate. Fractions eluted with dichloromethane/ethyl acetate (7:3) were rechromatographed with petroleum ether/ethyl acetate (7:3) then recrystallized in petroleum ether/dichloromethane yielding **1** (500 mg, 33%). Fractions eluted with pure ethyl acetate were

rechromatographed using petroleum ether/ethyl acetate (6:4) yielding **3** (20 mg, 1.3%). The fractions eluted with pure dichloromethane was rechromatographed with petroleum ether/ethyl acetate (8.8:1.2) yielding **2** (15 mg, 1.0%)

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LS 100Q, 200, 230 – These laser diffraction particle size analyzers are fully automated and have an extremely wide dynamic range: 0.04 μm to 2000 μm in particle diameter. The entire range can be measured in a single run without changing optics or instrument setting. Exceptionally high resolution (up to 116 size channels) provides a detailed picture of sample's true particle size distribution.

N4 PLUS – This sub-micron particle size analyzer is a Photon Correlation Spectroscopy (PCS) based instrument providing unmatched performance and accuracy with the patented multiangle capability and multi-TAU correlator. It includes a dedicated computer complete with a Microsoft® Windows based operating environment. The N4 Plus also incorporates a more powerful laser to ensure unparallel accuracy.

SA 3100 – This very high speed surface area and pore size analyzer features a specially designed user interface. Detailed, fully equilibrated physisorption data may be measured on powders and solid piece samples using nitrogen, krypton and other non-corrosive adsorbates. Results are suitable for QC or research. Sample preparation is fully automatic. The SA 3100 utilizes an integral outgassing system and includes a vacuum pump.

Z1 – A compact, highly accurate system which counts particles from 1 μm to 120 μm . Thresholds are entered by keypad and results are displayed on an integral LCD display. The Z1 eliminates calibration drift and determines particle counts above the lower threshold, above the upper threshold and between thresholds. The Z1 incorporates a unique, oil displacement pump and requires no mercury.



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