

Structural studies and antimicrobial properties of norcembrane diterpenoid from an Indian soft coral *Sinularia ineleqans* Tixier–Durivault

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Abstract. Two metabolites featuring norcembranoid diterpene skeleton have been isolated from chloroform extract of soft coral of the genus *sinularia*, which was collected from the southern coast of India. The structures of the metabolites were determined by 1D, 2D NMR spectroscopic data and HRESIMS data analysis. The compounds have shown moderate-to-high antimicrobial activities. The structure and the relative stereochemistry of the metabolite **1** have been determined by X-ray crystallography.

Keywords. Diterpene; *sinularia*; stereochemistry; antimicrobial; crystallography.

1. Introduction

Soft corals have been recognized as a rich source of diterpenes belonging to cembranolides,^{1–5} norcembranoids,^{6,7} and amphilactane⁸ classes. Among the several diterpenes reported, cembranoids and their cyclised derivatives are the most abundant metabolites of soft corals and gorgonians.^{9–14} They are produced as defense against predators such as fishes, other corals and against settlement of microorganisms such as bacteria and fungi.^{15,16} These classes of diterpenoids are a large part of natural product compounds having variety of chemical structures. Many of these compounds were reported to possess cytotoxicity,¹⁷ anti-inflammatory^{18,19} and anti-microbial activities.²⁰

As a part of our effort to the discovery of bioactive metabolites from marine organisms,²¹ here, we wish to report the isolation of two norcembranoid diterpenes (**1** and **2**) that are epimer at one carbon atom (C-9) from the soft coral of the genus *sinularia*. Bioassay of the compounds against some microorganisms or clinical isolates viz. bacterium and fungus has been reported. The molecular structures of the compounds were determined by extensive NMR and ESIMS spectroscopic data analysis. The crystal

structure and relative stereochemistry of **1** have been established by single X-ray crystallography.

2. Experimental

2.1 General experimental procedures

Infrared spectra were obtained on a Shimadzu-8201PC spectrometer with sample prepared in KBr and NMR spectra were recorded on a Bruker-Avance 300 MHz spectrometer at 300.13 MHz for ¹H and 75.23 MHz for ¹³C, in CDCl₃ with SiMe₄ as internal reference and coupling constants are given in hertz. Proton chemical shifts were referenced to the residual CHCl₃ signal (δ , 7.2), ¹³C NMR spectra were referenced to the centre peak of CHCl₃ signal at δ 77.1. ESIMS data were performed on QSTAR XL (LC-MS) mass spectrometer. Column chromatography were performed on silica gel (230–400 mesh, 60–120 mesh, Merck, Darmstadt, Germany or SRL, Pvt. Ltd, India) and Shephadex LH-20 and spots were visualized by spraying with 5% H₂SO₄ solution followed by heating.

2.2 Animal materials

Specimens of the soft coral *Sinularia ineleqans* Tixier–durivault were collected by hand by divers

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equipped with SCUBA off the coast of Mandapanam, Tamil Nadu, India in July, 2006 at a depth of 5–10 m. The organism was identified by comparison with previous descriptions and a voucher specimen was deposited at the aquarium museum at the National Institute of Oceanography, Goa under Voucher No. NIO-878.

2.3 Extraction and isolation

Fridge dried and minced material of sinularia species (dry weight 180 g, dried using Lyophilizer) was extracted step-wise with petroleum ether, Chloroform and finally with *n*-butanol. The chloroform extract was concentrated using rotary evaporator and the residue was separated by flash chromatography on a silica gel (230–400 mesh) using petroleum ether and ethyl acetate solvent system as eluant in increasing polarity (step-wise 100:0, 10:90, petroleum ether/ethyl acetate and pure ethyl acetate) to yield 1–46 fractions. Fractions 38 and 39 gave a light colourless solid on evaporation at room temperature. Repeated crystallization of this solid with chloroform:petroleum ether gave a colourless rectangular crystals of compound 1 (50 mg) suitable for X-ray analysis. Further separation of fraction 34 to 37 on silica gel using increasing concentration MeOH in CHCl₃ yielded good quantity of compound 1 (130 mg) and 2 (110 mg).

The spectroscopic data of the compounds are as follows:

2.3a *Compound 1*: FTIR (KBr, cm⁻¹): ν_{\max} 3586, 3415, 2943, 1760, 1728, 1666, 1406, 1373, 1188, 1096. HREIMS: m/z 349.0734 [M + H]⁺ (Calcd for C₁₉H₂₅O₆, 349.1651).

¹HNMR (CDCl₃, δ): 6.44 (1H, *dd*, ³ $J_{\text{H-H}} = 4.8$, ³ $J_{\text{H-H}} = 6.3$, H-19), 4.88 (*s*, 1H, H-16), 4.81 (*s*, 1H, H-16), 4.61 (*d*, 1H, $J_{\text{H-H}} = 9$, H-4), 4.25 (*dd*, 1H, $J_{\text{H-H}} = 3$, $J_{\text{H-H}} = 5.4$, H-9), 3.70 (*ddd*, 2H, $J_{\text{H-H}} = 6.2$, $J_{\text{H-H}} = 9.9$, $J_{\text{H-H}} = 12.9$, H-18), 3.01 (*m*, 1H, H-14), 2.88 (*d*, 1H, $J_{\text{H-H}} = 3$, H-11), 2.82 (*d*, 1H, $J_{\text{H-H}} = 2.7$, H-11), 2.64 (*s*, 1H, H-7), 2.59 (*s*, 1H, H-7), 2.50 (*d*, 1H, $J_{\text{H-H}} = 3.3$, H-13), 2.47 (*d*, 1H, $J_{\text{H-H}} = 2.1$, H-13), 2.24 (*br*, 1H, H-18), 2.17 (*m*, 2H, H-5), 2.12 (*s*, 3H, H-10), 1.45 (*s*, 3H, H-17).

¹³CNMR (CDCl₃, δ): 214.61 (C-8), 205.52 (C-12), 168.55 (C-1), 147.14 (C-15), 145.16 (C-19), 132.36 (C-2), 110.46 (C-16), 83.26 (C-4), 79.16 (C-6), 75.06 (C-3), 74.86 (C-9), 51.33 (C-7), 46.17 (C-13), 43.19 (C-11), 42.25 (C-5), 39.69 (C-14), 28.51 (C-18), 26.51 (C-10), 21.62 (C-17).

2.3b *Compound 2*: IR and Mass analysis data are similar to that of 1.

¹HNMR (CDCl₃, δ): 6.88 (*dd*, 1H, $J_{\text{H-H}} = 5.2$, $J_{\text{H-H}} = 7.2$, H-19), 4.96 (*s*, 1H, H-16), 4.73 (*s*, 1H, H-16), 4.65 (*d*, 1H, $J_{\text{H-H}} = 6.6$, H-4), 4.31 (*dd*, 1H, $J_{\text{H-H}} = 4.2$, $J_{\text{H-H}} = 6.9$, H-9), 3.62 (*dd*, 2H, $J_{\text{H-H}} = 3.8$, $J_{\text{H-H}} = 6.4$, H-18), 3.04 (*m*, 1H, H-14), 2.83 (*d*, 1H, $J_{\text{H-H}} = 3.2$, H-11), 2.74 (*m*, 1H, H-11), 2.66 (*s*, 1H, H-7), 2.52 (*m*, 1H, H-7), 2.43 (*d*, 1H, $J_{\text{H-H}} = 3$, H-13) 2.27 (*br*, 2H-18), 2.19 (*m*, 2H, H-5), 2.14 (*m*, 3H, H-10), 1.49 (*s*, 3H, H-17).

¹³CNMR (CDCl₃, δ): 212.86 (C-8), 208.29 (C-12), 170.73 (C-1), 144.70 (C-15), 142.67 (C-19), 130.90 (C-2), 112.11 (C-16), 80.26 (C-4), 77.58 (C-6), 75.78 (C-3), 75.51 (C-9), 49.43 (C-7), 48.06 (C-13), 44.45 (C-11), 41.81 (C-5), 39.69 (C-14), 29.21 (C-18), 28.70 (C-10), 21.80 (C-17).

2.4 Antimicrobial assays

Antimicrobial properties of the compounds were tested by employing the Kirby-Bauer's Disc diffusion technique.²² Antifungal assay was carried out against the fungus *Aspergillus fumigatus*, *Rhodotorula*, *Candida albicans* and *Aspergillus niger*. The bacteria *Vibrio cholerae*, *Escherichia coli* and *Staphylococcus aureus* were used in the antibacterial assay. A 50–200 μg portion of the compounds were applied onto pre-sterilised disc. The disc was placed on agar plates that had been seeded with respective organisms. Diameters of inhibitory zones were measured after the plates were incubated at 28°C for 24–48 h. *Nystatin* and *Penicillium* were used as positive control for fungus and bacteria, respectively.

2.5 Crystallographic data analysis

Crystals of suitable for X-ray analysis were grown from chloroform and petroleum ether. X-ray structural data were measured at 130(2)°K on a Nonius-Kappa CCD diffractometer using Mo-K α radiation ($\lambda = 0.71073 \text{ \AA}$). A crystal of suitable size was mounted using epoxy or paraffin and the diffraction data were then collected. Data collection and cell refinement involved HKL-SCALEPACK²³ software packages. Molecular graphic was done using *maXus*²⁴ and *zortep*²⁵ softwares. The structure was solved by direct methods²⁶ and refined by full matrix least squares base on F^2 .²⁷ Weighting scheme calculated used was $w = 1/[s^2(Fo^2) + (0.0672P)^2 + 0.0000P]$,

Table 1. Selected bond lengths (Å) and angles (°) for the metabolite 1.

Bond lengths (Å)			
C15–C16	1.323 (4)	C14–C18	1.557 (3)
C5–C6	1.526 (4)	C9–H9	1.000
C1–O3	1.208 (3)	C15–C16	1.323 (4)
C3–O2	1.462 (3)	C18–C19	1.504 (4)
C1–O1	1.354 (3)	C14–C15	1.520 (3)
C8–O6	1.218 (3)	O2–H2	0.8400
Bond angles (°)			
C13–C14–C18	112.5 (2)	C4–O1–C1	112.08 (19)
C9–O4–C6	108.88 (18)	C4–C3–C2	104.2 (2)
C7–C8–C9	107.7 (2)	C16–C15–C17	121.9 (2)
C3–O2–H2	109.5	O1–C1–C2	109.7 (2)
O3–C1–O1	120.0 (2)	O3–C1–C2	130.2 (2)

Table 2. Summary of crystal structure determination and refinement of compound 1.

Empirical formula	C ₁₉ H ₂₄ O ₆
Formula weight	348.38
Temperature	130(2)K
Wavelength	0.71073 Å
Crystal description	Prism
Colour	Colourless
Crystal system	Monoclinic
Space group	<i>P</i> ₂₁
Unit cell dimensions	<i>a</i> = 11.5091(4) Å <i>α</i> = 90° <i>b</i> = 6.0530(2) Å <i>β</i> = 98.6837(14)° <i>c</i> = 12.5303(7) Å <i>γ</i> = 90°
Volume	862.91(6) Å ³
<i>Z</i>	2
Calculated density	1.341 Mg/m ³
Absorption coefficient	0.099 mm ⁻¹
<i>F</i> (000)	372
Crystal size	0.59 × 0.48 × 0.30 mm
Reflections for indexing	534
Theta range for data collection	2.24 to 28.31°
Index ranges	−15 ≤ <i>h</i> ≤ 14 −6 ≤ <i>k</i> ≤ 8 −16 ≤ <i>l</i> ≤ 16
Reflections collected/unique	3787/2256 [<i>R</i> (int) = 0.0406]
Completeness to <i>θ</i>	25.00–99.8%
Absorption correction	Semi-empirical from equivalents
Maximum and minimum transmission	0.9608 and 0.9537
Refinement method	Full-matrix least-squares on <i>F</i> ²
Data/restraints/parameters	2256/1/229
Goodness-of-fit on <i>F</i> ²	0.988
Final <i>R</i> indices	<i>R</i> ₁ = 0.0438, [<i>I</i> > 2σ(<i>I</i>)] <i>wR</i> ₂ = 0.1012
<i>R</i> indices (all data)	<i>R</i> ₁ = 0.0620, <i>wR</i> ₂ = 0.1086
Largest diff. peak and hole	0.202 and −0.221 eÅ ⁻³

where $P = (Fo^2 + 2Fc^2)/3$. Non hydrogen atoms were refined anisotropically and all hydrogen atoms were initially located in a difference Fourier map and were refined with a 'riding' model. Refinement

converged at a final $R_1 = 0.0438$ and $wR_2 = 0.1012$. Selected bond lengths and angles are presented in table 1. Summary of data collection parameters and refinement data are given in table 2.

3. Crystal structure

Crystals of compound **1** were obtained from chloroform and petroleum ether solvent system and crystallize in P_{21} space group in a monoclinic unit cell. The molecular structure of compound **1** with atoms labelling scheme is shown in figure 1. The structure consists of three cyclic chains, one five-member lactone, one five member ethereal ring and a thirteen-membered ring. In consistent with NMR data the structure showed the presence of a double bond between C2 and C19 (1.335(4) Å, which is within the usual range of C=C bond. All the three carbonyl bond lengths, C1–O3 (1.208), C8–O6 (1.218) and C12–O5 (1.218) Å are comparable and the values are within the reported range of C–O bond length.²⁸

4. Results and discussion

Chemical investigation of chloroform extract of soft coral of genus *sinularia* collected from the southern part of India leads to isolation of two known metabolites (**1** and **2**) featuring norcembranoids diterpene skeleton along with a few other metabolites, investigation of which is under progress. The norcembranoid **1** was C-9 epimer of **2** and the relative stereochemistry of **1** was established by X-ray crystallography and assigned the chiral centres as (3*S**, 4*R**, 6*S**, 9*R**, 14*S**). The compound **1** and **2** have been isolated from chloroform fraction with the help of chromatographic technique. The ¹H and ¹³C NMR spectrum of **2** is similar to that of **1** and initially they are difficult to distinguish by the NMR spectral data. However, a careful observation of their

¹³C NMR spectrum reveals that there is slight difference on their ¹³C chemical shift values suggesting that **2** is stereoisomer of **1**. Compound **1** was easily separated from the mixture as colourless crystals on crystallization in chloroform and petroleum ether or more conveniently using chloroform and ethyl acetate. While **2** was isolated from the mixture as light yellow compound upon repeated chromatography over silica gel followed by crystallization. It is noteworthy that **1** and **2** are isolated as a mixture by chromatography but on crystallization **1** can be readily isolated from **2**. The molecular formula of the compound **1** was found to be C₁₉H₂₄O₆ as determined by ESIMS (m/z 349.0734 [M + H]⁺ calculated for C₁₉H₂₅O₆, 349.1651). The presence of hydroxyl group was supported by the appearance of a broad absorption peak in the IR spectrum at 3452 cm⁻¹. The IR spectrum of compound **1** and **2** showed strong bands at 1760, 1728 cm⁻¹ consistent with the presence of lactone and carbonyl group.²⁹ The Proton NMR spectra of **1** and **2** showed the presence of *oxy-methylene* proton (see experimental section). The ¹³C NMR of the compounds displayed nineteen distinct carbon signals which were assigned by the assistance of DEPT as two methyls, six methylene, five methines including three oxygenated and one vinylic (δ_c 39.69, 145.16, 75.06, 83.26, 74.86), three quaternary carbons (147.14, 132.36, 79.16) and three carbonyl carbons. The carbon signals appearing at δ 214.6, 168.5 and 147.1 could be attributed to the presence of normal ketone and α, β conjugated ester in the compound **1**. The presence of hydroxyl group in compounds was recognised from ¹³C NMR resonance at δ_c 75.06 for **1** and 75.78 for **2**. Further MS/MS of the compound **1** gave peak at m/z 331.1096 (ESIMS, m/z 331.1096 [M-H₂O]⁺) corresponding to the loss of a water molecule indicating the presence of hydroxyl group in the molecule.

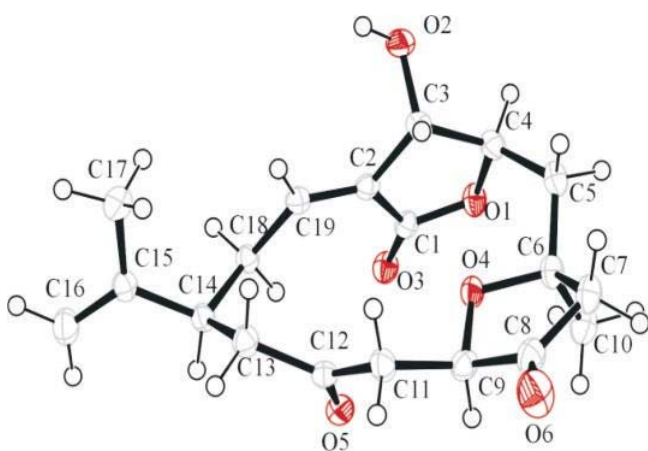
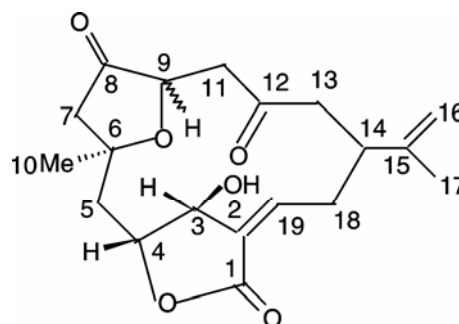
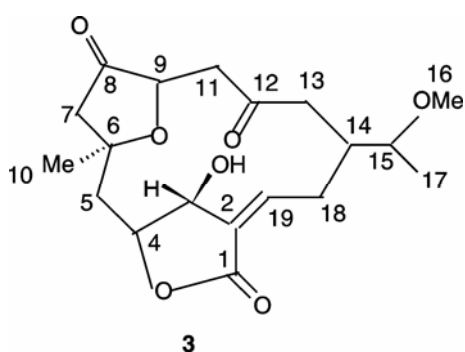


Figure 1. Molecular structure of compound **1** with atoms numbering scheme.



1 (9*R*) 2 (9*S*)

The presence of terminal alkene in **1** or **2** was supported by the appearance of singlet at 4.79 ppm in the proton NMR spectrum and is further confirmed by the following reaction.²⁸ Treatment of **1** or **2** with sodium in dry methanol resulted in hydrogenation at C-15 to give a methoxy derivative **3**. The formation of methoxy derivative **3** was confirmed by the absence of terminal olefinic proton signal at around δ 4.81 observed in **1** and appearance of a broad signal at δ 3.82 assignable to the proton of methoxy group. This observation leads to the conclusion that metabolite **1** contains a terminal alkene group and proposed the structure of the metabolite as **1**.



The solid state structure of **1** has been finally confirmed by crystallographic study. The metabolites **1** and **2** have been reported by earlier workers isolated from *sinularia leptocladus*³⁰ and other *sinularia* species.³¹ The spectroscopic data of **1** and **2** are well matched with the reported values. In this communication, we described the stereochemistry and antimicrobial properties of the metabolites.

Compounds were tested for antimicrobial activities against sixteen clinical isolates by following a procedure described previously.²² Bioassay studies of the compounds shown a moderate-to-high activities against certain pathogens such as *Vibrio cholerae*, *Aspergillus funigatus*, *Escherichia coli* and *Staphylococcus aureus*.

5. Conclusions

Two metabolites featuring norcembranoid diterpenes with antimicrobial properties have been isolated and fully characterized on the basis of FTIR, NMR (¹H, ¹³C, DEPT) and ESIMS data. The relative stereochemistry of the compound **1** was established with the help of X-ray analysis.

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Supplementary material

Crystallographic data for the structural analysis have been deposited at the Cambridge Crystallographic Data Centre (CCDC), with CCDC No. 673651 for **1**. Copies of this information may be obtained free of charge from the Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk or <http://www.ccdc.cam.ac.uk>).

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