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Bisdioxycalamenene: A Bis-Sesquiterpene from the Soft Coral *Rhytisma fulvum fulvum*

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Abstract: A dichloromethane extract of the soft coral *Rhytisma fulvum fulvum* collected in Madagascar afforded a novel compound possessing an unprecedented pentacyclic skeleton, bisdioxycalamenene (1), as well as seven known sesquiterpenes. The structures of the compounds were elucidated using 1D and 2D NMR techniques, as well as high-resolution mass spectrometry. The absolute configuration of 1 was determined using X-ray diffraction analysis and anomalous dispersion effects. The structure elucidation and a possible biogenesis of the compound are discussed.

Keywords: *Rhytisma fulvum fulvum*; soft coral; bisdioxycalamenene; bis-sesquiterpene

1. Introduction

The soft coral *Rhytisma fulvum fulvum* (Forskål) (*R. fulvum fulvum*) (previously *Parerythropodium*), family Alcyoniidae, was originally described from the Red Sea [1,2]. Its present zoogeographical distribution extends south to the reefs of Madagascar and eastward to Indonesia [3,4]. The Genera *Rhytisma* is a rich source of sesquiterpenoids and norsesquiterpenoids containing a variety of skeletons as well as sterols [5–9]. Dozens of compounds have thus far been reported with different ring systems [5–9]. In previous research, our group compared the yellow and gray morphs that inhabit the reefs of the Gulf of Eilat and found differences in the compounds they produce [6]. *Inter alia*, we isolated a volatile yellow pigment, fulfulvene, from the yellow morph. This pigment is responsible for the soft coral’s yellow color [6]. The pigment and other volatile compounds, mainly sesquiterpenes, are lost during freeze drying and/or evaporation [6].

The present study describes the isolation and structure elucidation of a novel bis-sesquiterpene designated bisdioxycalamenene (1) and of seven known sesquiterpenes, 5-hydroxy-8-methoxycalamenene (2) [10], 8-methoxycalamenene (3) [10], 5-hydroxy-8-methoxycalamenene-15-al (4) [10], (1S,4S,10S,12S,Z)-1,3,12-trimethyl-5-oxo-1,4,5,6,7,10,11,12-octahydrobenzo[8]annulene-4,10-diyldi-acetate (5) [11], 2-oxolemnacarnol (6) [5], lemnacarnol (7) [5], and 6α-acetyl-4β,5β-dimethyl-1(10)-α-epoxy-2α-hydroxy-7-oxodecalin (8) [12], from a yellow morph specimen of *Rhytisma fulvum fulvum* collected on December 2012 at Banc du Castor, Mitsios Archipelago, Madagascar (Figure 1). Bisdioxycalamenene (1) is a bis-sesquiterpene possessing an unprecedented pentacyclic skeleton that is probably derived from dimerization of 5-hydroxy-8-methoxycalamenene (2) [10].
2. Results and Discussion

Bisdioxycalamenene (1) was isolated from the crude extract using solvent partition, followed by separations on Sephadex LH-20 and Silica gel-H. The positive atmospheric pressure photoionization high-resolution mass spectrometry (APPI HRMS) of 1 exhibited a molecular ion [M]+ at m/z 478.3080 consistent with the molecular formula C_{31}H_{42}O_{4} and eleven double bond equivalents. The structure elucidation of 1 was based on its mass-spectrometric and NMR data (Table ??, CDCl_3).

The $^1$H NMR exhibited two singlet signals in the lower and mid-field of the spectrum ($\delta_H$ 6.36 and 4.39), a methoxyl ($\delta_H$ 3.74), six protons resonating between 3.40 and 2.50 ppm, ten $sp^3$ protons resonating between 2.00 and 1.40 ppm, a singlet methyl ($\delta_H$ 1.33) and six doublet methyl signals resonating between 1.10 and 0.75 ppm. The $^{13}$C NMR presented two ketone carbonyls ($\delta_C$ 199.4 s and 195.3 s), eight $sp^2$ carbon atoms ($\delta_C$ 151.7, 150.3, 147.3, 144.0, 130.6, 129.1 and 115.4, all quaternary carbons, and 107.5, CH), a oxymethine carbon ($\delta_C$ 83.9, CH), and a methoxyl ($\delta_C$ 55.2, CH3). In the high field, one quaternary $sp^3$ carbon ($\delta_C$ 48.0), six methine carbons ($\delta_C$ 37.0, 36.9, 32.3, 31.1, 27.2 and 26.5), four methylene carbons ($\delta_C$ 25.6, 25.5, 19.5 and 18.5) and seven methyl carbons ($\delta_C$ 21.7, 21.4, 21.3, 20.2, 21.9, 20.0 and 21.8) were observed. The above proton and carbon signals counted to 31 carbons and 42 protons, agreeing with the molecular formula of 1.

Table 1. NMR Data of Bisdioxycalamenene (1) in CDCl_3.

| Position | $\delta_C$, Type b | $\delta_H$, Mult (f in Hz) | HMBC Correlations c | COSY Correlations |
|----------|-------------------|----------------------------|---------------------|-------------------|
| 1        | 27.2, CH          | 2.99, ddq (1.3, 6.8, 7.0)  | 2, 3, 5, 8, 9, 10, 11| 2a, 11            |
| 2        | 25.5, CH_2        | 1.84, m                    | 10                  | 1                 |
|          |                   | 1.46, m                    |                     | 3a, 3b, 12        |
| 3        | 18.5, CH_2        | 1.74, m                    | 10                  | 2b, 3b, 4, 12     |
|          |                   | 1.61, m                    | 4, 10               | 2b, 3a, 4         |
| 4        | 36.9, CH          | 2.66, dt (2.3, 6.0)        | 2, 3, 5, 9, 10, 12, 14| 3a, 3b, 12       |
| 5        | 199.4, qC         |                            |                     |                   |
| 6        | 48.0, qC          |                            |                     |                   |
| 7        | 83.9, CH          | 4.39, s                    | 5, 6, 8, 9, 15, 5', 15'|                   |
| 8        | 195.3, qC         |                            |                     |                   |
The C-H correlations from a heteronuclear single quantum coherence (HSQC) experiment established the one bond connectivity between the carbons and protons (Table ??). The H-H correlations from a correlation spectroscopy (COSY) experiment furnished two similar segments; a and b, shown in Figure 2, leaving an additional isolated strong coupled methylene group (δH 3.35 and 2.53 ppm) and four isolated proton signals (δH 6.36, s, 1H; 4.39, s, 1H; 3.74, s, 3H; 1.33, s, 3H) uncorrelated. Segments a and b are similar each other and both to the aliphatic portion of the calamenene sesquiterpenes (2–4) [10].

Using long-range C-H correlations from a heteronuclear multiple bond correlation (HMBC) experiment enabled the connection of all the proton and carbon signals to the final gross planar structure of 1 (Figure 3). H-1 and -4 exhibited correlations with C-8 and -5, respectively, and both with C-9 and -10, extending fragment a to a 1-isopropyl-4-methyl-2-cyclohexene moiety substituted at positions 2 and 3 by two ketone groups. Oxymethine-7 presented HMBC correlations with C-5, -6, -8 and -9, while

|   |   |   |   |   |
|---|---|---|---|---|
| 9 | 150.3, qC |   |   |   |
| 10 | 147.3, qC |   |   |   |
| 11 | 20.1, CH₃ | 1.10, d (7.0) | 1, 2, 9, 10 | 1 |
| 12 | 31.1, CH | 1.82, m | 3, 4 | 2b, 3a, 4, 13, 14 |
| 13 | 21.3, CH₃ | 0.87, d (7.0) | 4, 12, 14 | 12 |
| 14 | 20.2, CH₃ | 0.85, d (7.0) | 4, 12 | 12 |
| 15 | 21.8, CH₃ | 1.33, s | 5, 6, 7, 6', 15' |   |
| 1' | 26.5, CH | 3.09, dq (6.5,7.0) | 2', 3', 8', 9', 10', 11' | 2'a, 11' |
| 2' | 25.6, CH₂ | 1.93, m | 10' | 1', 2'b, 3' |
| 3' | 19.5, CH₂ | 1.70, m | 1', 4', 10', 12' | 2'a, 2'b, 4' |
| 4' | 37.0, CH | 2.72, dt (2.3, 5.9) | 2', 5', 9', 10', 12', 14' | 1', 3', 12' |
| 5' | 144.0, qC |   |   |   |
| 6' | 115.4, qC |   |   |   |
| 7' | 107.5, CH | 6.36, s | 5', 8', 9', 15' |   |
| 8' | 151.7, qC |   |   |   |
| 9' | 130.6, qC |   |   |   |
| 10' | 129.1, qC |   |   |   |
| 11' | 21.9, CH₃ | 1.07, d (7.0) | 1', 2', 9' | 1' |
| 12' | 32.3, CH | 1.86, m | 3', 10', 14' | 4', 13', 14' |
| 13' | 21.7, CH₃ | 0.86, d (7.0) | 4', 12' | 12' |
| 14' | 21.4, CH₃ | 0.77, d (7.0) | 4', 12' | 12' |
| 15' | 33.1, CH₂ | 3.35, d (16.5) | 5, 6, 7, 8, 15, 5', 6', 7', 8', 9' |   |
|    |   | 2.53, d (16.5) | 5, 6, 7, 15, 5', 6', 7', 8', 9', 10' |   |
| 16' | 55.2, CH₃ | 3.74, s | 7', 8' |   |

*500 MHz for ¹H, 125 MHz for ¹³C; b Multiplicity and assignment from HSQC experiment; c HMBC correlations, optimized for 8 Hz, are from the proton(s) stated to the indicated carbon.

**Figure 2.** COSY (bold line) correlations of 1.
methyl-15-protons exhibited correlations with C-5, -6, and -7, closing a second six-membered ring. H-1' and -4' demonstrated a similar pattern of correlations (Figure 3), extending fragment b to a 1,4-disubstituted cyclohexene ring. H-7' exhibited HMBC correlations with C-1', -5', -6', -8', -9' and -15', while methylene-15'-protons presented correlations with C-5', -6', -7' and -8', and the methoxy-protons exhibited correlations with C-8', supporting a substituted 15-methylene-8-methoxy-5-oxy-calamenene substructure. The HMBC correlations of H-7 with C-5' and -15', of methyl-15-protons with C-15' and of both protons of methylene-15' with C-5, -6 and -7 suggested the connection of C-6 with C-15' and C-7 through the oxygen to C-5', establishing the planar structure of 1.

Figure 3. COSY (bold line) and key HMBC correlations (arrows) of 1.

The relative trans relationships of the 1,4- and 1',4'-substituents of the two cyclohexene rings were suggested when comparing the chemical shifts of the corresponding protons and carbons with those of the known calamenes (2–4). The cis 6–7 junction was established based on the NOE between H-7 and methyl-15. The complete structure including absolute configuration was confirmed by the X-ray diffraction analysis.

Crystallization of bisdioxycalamenene (1) from CHCl₃/MeOH solution furnished suitable crystals for an X-ray diffraction analysis. Bisdioxycalamenene (1) comprises of a pentacyclic structure derived from coupling of two sesquiterpenes. The asymmetric unit contains assemblages of two molecules of 1, thus in one monoclinic cell there are four molecules of 1. Anomalous dispersion effects and relation of the two enantiomeric forms of the structural model to the diffracted intensities established the absolute configuration. The preferred fit is indicated by the Flack and Parsons parameter (Flack parameter, x = 0.01 (9)) [13]. The X-ray diffraction analysis established the complete structure of 1 including the absolute configuration of the six chiral centers (1S,1'S,4R,4'R,6S,7R) shown in Figure 4.

Figure 4. Oak Ridge Thermal-Ellipsoid Plot Program (ORTEP) presentation of 1.
To the best of our knowledge, there are no earlier reports of such a skeleton. A possible biogenetic route to 1 is a hetero Diels Alder condensation of the para-quinone c with the ortho-quinonemethide, d [14,15] (Figure 5).

![Figure 5. A possible biogenetic route for 1.](image_url)

Compound 1 was evaluated for lethal toxicity in a brine shrimp toxicity assay (Artemia salina) [16] and displayed mild toxicity (LD$_{50}$ 15 µg/mL). Compound 1 was assayed for antibacterial activity against Pseudomonas aeruginosa and Escherichia coli and found to be inactive at 10 µg/mL.

3. Experimental Section

3.1. General Experimental Procedures

Optical rotations were determined on a JASCO P-1010 polarimeter. UV spectra were recorded on an Agilent 8453 spectrophotometer. NMR spectra were recorded on a Bruker DMX-500 spectrometer at 500.13 MHz for $^1$H and 125.76 MHz for $^{13}$C and a Bruker Avance 400 spectrometer at 400.13 MHz for $^1$H and 100.62 MHz for $^{13}$C. DEPT, COSY-45, gTOCSY (mixing time 60 ms), gROESY (spinlock pulse 0.2 s), gHSQC, and gHMBC spectra were recorded using standard Bruker pulse sequences. Mass spectra were recorded on a Synapt High Definition Mass Spectrometry (Waters Inc., Milford, MA, USA) instrument. For the GC MS analysis, separation of the crude samples was performed on an Agilent-7890-GC equipped with Agilent-5977A-MSD with an HP-5MS UI column (30 meter $\times$ 0.25 mm $\times$ 0.25 µm).

The X-ray diffraction patterns were obtained with CuK$_\alpha$ radiation from an Imus microsource, on an ApexDuo Bruker-AXS diffractometer.

3.2. Biological Material

A sample of the soft coral R fulvum fulvum (yellow morph, Phylum Cnidaria, Class Anthozoa, Order Alcyonacea, Family Alcyoniidae) was collected in December 2012 at a depth of 15 m at Banc du Castor, Mitsios Archipelago, Madagascar. It was identified by Professor Yehuda Benayahu. A voucher specimen (MAD12-IM052) was deposited at the Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments (LCSNSA) at the University of Reunion Island, France. The soft coral sample was frozen immediately after collection and kept at $-20^\circ$C until processed.

3.3. Isolation Procedure

The wet sample (0.3 kg wet weight) of the soft coral was extracted with CH$_2$Cl$_2$–MeOH (1:1, 300 mL) at room temperature overnight. The aqueous phase was separated from the organic layer, which was dried and evaporated to afford an oily crude extract (9 g, 3% of dry weight). The crude organic extract was separated in 2 g portions using Kupchan solvent partition [17]. The petroleum ether fraction (970 mg) was chromatographed on a Sephadex LH-20 column (petroleum ether/CH$_2$Cl$_2$/MeOH, 2:1:1) followed by several separations on Silica gel 60 H (Merck) columns (VLC and gravitational columns), eluting with EtOAc-petroleum ether mixtures to afford bisdioxycalamenene (I) (eluted with 2% EtOAc in petroleum ether, 15 mg, 0.023% yield from wet sample) and seven known
Toxicity of the compound was evaluated in brine shrimp toxicity assay and displayed mild toxicity (LD₅₀ > 10 µg/mL).
the measurements of the mass spectra; and Sigal Shefer from the Department of Zoology, for guidance with the brine shrimp toxicity assay.

Author Contributions: Y.J.T. contributed to the isolation and structure elucidation and writing of the manuscript with the supervision of S.C. and Y.K. M.A. and A.Gauvin.-B. contributed to the collection and extraction of the sample of the soft coral; Y.B. contributed to the identification the soft coral. S.C. and Y.K. contributed to the structure elucidation, writing and editing of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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