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Secondary metabolite chemistry of the marine-derived fungus *Massarina* sp., strain CNT-016

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Abstract

Chemical investigation of the culture broth extracts of the marine-derived fungus *Massarina* sp. (strain CNT-016) has yielded two secondary metabolites, spiromassaritone (**1**) and massariphenone (**2**), as well as the previously reported fungal metabolites 6-epi-5′-hydroxy-mycosporulone (**3**) and enalin A (**4**). The structures of these compounds were established by a variety of one- and two-dimensional NMR experiments, while the relative configuration of spiromassaritone (**1**) was determined by X-ray crystallographic methods. The fungal strain was isolated as a sterile mycelium from an ocean mud sample and identified using ITS sequence analysis.

Keywords: *Massarina*; Marine-derived fungi; Secondary metabolites; Spiromassaritone; Spirolactones; X-ray structure analysis

1. Introduction

Terrestrial fungi have been a rich source of biologically-active secondary metabolites. More recently, these studies have expanded to include marine-derived species (e.g. Shin and Fenical, 1987; Adam et al., 1996; Smith et al., 2000; Cueto et al., 2001; Wu et al., 2005). As part of our ongoing studies to develop the biomedical potential of marine microorganisms (e.g. Fenical, 1993; Liu et al., 2003; Oh et al., 2005), we examined marine fungi isolated from ocean muds collected at various depths. Here, we report the structures of two new metabolites, spiromassaritone (**1**) and massariphenone (**2**), and two known fungal metabolites, 6-epi-5′-hydroxy-mycosporulone (**3**) and enalin A (**4**), from cultures of a sterile fungal strain isolated from a mud sample collected at 5 m depth adjacent to the Palau Islands in the Pacific Ocean. Phylogenetic analysis demonstrated that the isolated fungus belongs to the genus *Massarina* (Ascomycota).

The genus *Massarina* (Lophiostomataceae, Pleosporales, Ascomycetes), which had previously been placed under the genus *Massaria* De Not, was established by Saccardo in 1883 to segregate fungi with hyaline ascospores. Species of *Massarina* are saprophytes and endophytes of woody plants, with a few species that are plant pathogens (Shoemaker et al., 1991; Hyde, 1995). Aptroot (1998) published a world revision of *Massarina* providing a list of 160 species of which he accepted 43 species. Since then, 19 new species have been described bringing the total number of the species in the genus to 62 (Tanaka and Harada, 2003; Tanaka et al., 2005). The known aquatic *Massarina* are composed of 18 species, 10 of which have been described from fresh water environments and 8 are from coastal saline habitats. Six of the marine *Massarina* species were described from mangroves (Hyde et al., 1992), while two were described from the salt marsh plant *Juncus roemerianus* (Kohlmeyer and Kohlmeyer, 1979; Kohlmeyer et al.,

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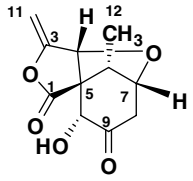
1995). The secondary metabolite chemistry of *Massarina* species is virtually unexplored; only one freshwater species, *M. tunicata* Shearer and Fallah, has been investigated and 11 new bioactive compounds have been recorded (Oh et al., 1999, 2001, 2003). Although limited data are available, these studies indicate that *Massarina* species are chemically productive.

2. Results and discussion

2.1. Chemistry

Chemical studies of extract from the culture afforded spiromassaritone (**1**), massariphenone (**2**) and the known compounds **3** and **4**. Here, we report the structure elucidation and biological activities of compounds **1** and **2**.



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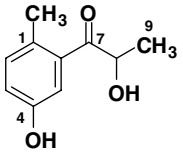

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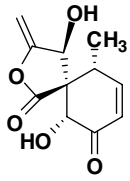

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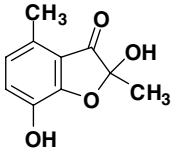

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Spiromassaritone (**1**) analyzed for the molecular formula $C_{11}H_{12}O_5$ (six degrees of unsaturation), on the basis of ^1H, ^{13}C NMR and HR-ESI-TOF-MS data. IR absorbance bands recorded for **1** at 3440, 3405 and 1140 cm^{-1} indicated the presence of hydroxyl and ether functionalities, while other bands at 1805 and 1720 cm^{-1} suggested the presence of lactone and saturated ketone carbonyl groups in the molecule. Interpretation of ^1H, ^{13}C, and DEPT NMR spectroscopic data (Table 1) indicated the presence of a $–CHCH_3$ moiety, a ketone, an ester carbonyl, an sp^3 methylene unit, three oxymethine protons, a non-oxygenated quaternary carbon, a hydroxyl group, and an oxygenated terminal olefin unit. These data indicated that spiromassaritone (**1**) is a tricyclic metabolite of likely polyketide origin.

Analysis of ^1H–^1H COSY NMR spectroscopic data allowed $–CH_2–CHO–$ and $CH_3–CH–$ subunits to be defined, and showed that the *exo*-methylene signals at δ 4.81, 4.57 were allylically coupled to the oxymethine proton signal at δ 4.51 (H-4). The connectivity between these subunits in **1** was demonstrated by interpretation of the HMBC correlation data (Table 1). Proton H-10 (δ 4.65) showed correlations to the quaternary carbon C-5 (δ 62.5), C-1 (δ 169.8), C-6 (δ 45.4), and to the carbonyl at C-9 (δ 207.3). In addition, HMBC correlations were observed from H_2-8 (δ 2.81, 2.67) to the ketone carbonyl at C-9, to C-10 (δ 76.5), C-7 (δ 78.7), and to C-6 (δ 45.4). Proton H-6 (δ 2.86) showed correlations to C-4, C-5, C-7, C-10 and C-12, while the methyl group at δ 1.21 showed correlations to C-5, C-6 and C-7, thus allowing a cyclohexanone ring to be constructed. The exomethylene protons, H_2-11 (δ 4.81, 4.57) showed correlations to C-3 (δ 156.9), but in addition H-11_d (δ 4.81) also showed a correlation to C-4 (86.0). The oxygenated methine H-4 showed correlations to the lactone carbonyl C-1 (δ 169.8) and also to C-7, revealing an ether linkage between C-4 and C-7. These data allowed the tetrahydrofuran ring to be assigned and established the structure of compound **1** as shown.

The relative configuration of spiromassaritone (**1**) was assigned by analysis of NOE NMR data. These data suggested that the five-membered rings in **1** are slightly puckered, while the cyclohexanone ring has a chair conformation. The observation of NOE correlations between H-4 and H-8_α, from H-7 to H-8_β, and from

Table 1
NMR spectroscopic data (500 MHz) for spiromassaritone (**1**) in acetone-d_6

C/H #	δ_H (*J* Hz)	δ_C		COSY	HMBC	NOESY
1		169.8	C			
2						
3		156.9	C			
4	4.51 t (2.9)	86.0	CH	H-11_u, H-11_d	C-1, C-7, C-5	H-8_α
5		62.5	C			
6	2.86 q (7.3)	45.4	CH	H-12	C-5, C-7, C-10, C-12	
7	4.97 t (2.5)	78.7	CH	H-8_β, H-8_α	C-5, C-10	H-8_β
8_β	2.67 dd (17.4, 2.5)	48.3	CH_2	H-7, H-8_α	C-6, C-7, C-9, C-10	H-7
8_α	2.81 dt (17.4, 2.5)			H-7, H-8_β	C-7, C-9	H-4
9		207.3	C			
10	4.65 br s	76.5	CH		C-1, C-5, C-6, C-9	H-6
11_u	4.57 dd (2.9, 2.0)	90.1	CH_2	H-4, H-11_d	C-3	
11_d	4.81 t (2.0)			H-11_u	C-3, C-4	
12	1.21 d (7.3)	14.2	CH_3	H-6	C-5, C-6, C-7	

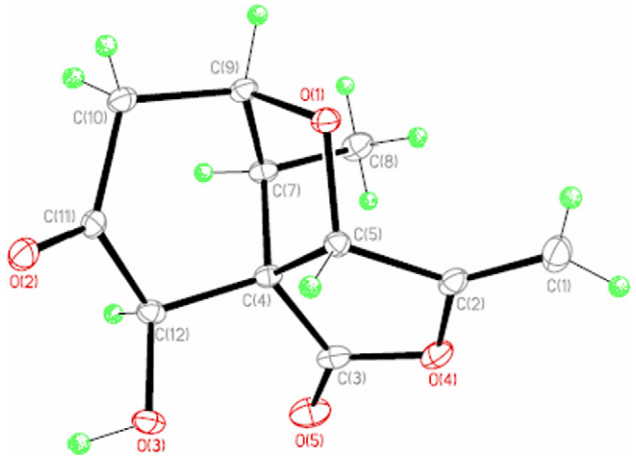


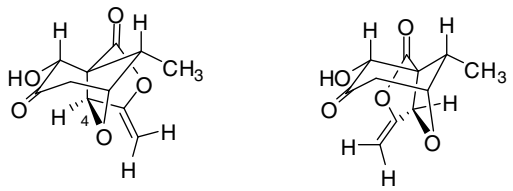
Fig. 1. X-ray structure of spiromassaritone (**1**).

H-10 to H-6 indicated that the H-7 and H-12 methyl groups are in equatorial positions, while H-6, H-8_β and H-10 occupy axial orientations.

The assigned relative configuration of **1** was confirmed by X-ray diffraction analysis. Crystals of **1** suitable for X-ray diffraction were obtained by slow evaporation of a solution of **1** in acetonitrile–water (30:70). The final X-ray crystallographic model of **1** (Fig. 1) confirmed the structure and relative configuration of spiromassaritone as shown.

The rare spiro-5,6-lactone ring skeleton found in compound **1** has been reported for fungal secondary metabolites from *Massarina tunicata* (Oh et al., 2001), *Mycosphaerella rosigena* (Albinati et al., 1980), *Microsphaeropsis* sp. (Fukami et al., 1999), and *Arthropsis truncate* (Ayer et al., 1992). A literature search revealed that spiromassaritone (**1**) is closely related to V214w (**5**), previously reported from an unidentified fungal strain (Roll et al., 2002). The compounds differ in their configuration at C-4 (Fig. 2). In compound **1**, a NOE correlation was observed from H-4 to H-8_α, while this NOE correlation was missing in the case of compound **5**.

Massariphenone (**2**) was obtained as an optically active oil ($[\alpha]_d$ −18.5, CH_3OH). The molecular formula of **2** was established as $C_{10}H_{12}O_3$ by high-resolution mass spectrometric data that showed a pseudo-molecular ion peak at 203.0675 ($[M+Na]^+$, error 2.0 ppm). The molecular formula indicated that massariphenone contained 5 degrees of unsaturation. The ^1H NMR spectrum of **2** showed signals of a 1,2,4- tri-substituted benzene ring, an aryl methyl group, and an $OCHCH_3$ unit. The ^{13}C NMR spectrum showed the presence of one carbonyl carbon, three sp^2 quaternary carbons, three sp^3 carbons, one methine carbon, and two methyl carbons. The gross structure was then assembled from HMBC correlations between these subunits. The key HMBC correlations were from H-3 to C-7 and H-8 to C-7 to provide structure **2**. The configuration of the hydroxyl-bearing carbon was not determined.



1 **5**

Fig. 2. Stereostructures of spiromassaritone (**1**) and V214w (**5**).

The molecular formula of compound **3** was assigned as $C_{11}H_{12}O_5$ on the basis of HRESIMS and ^1H and ^{13}C NMR spectroscopic data. Examination of related compounds tabulated in "AntiBase" (Laatsch, 2005) led to three compounds with the same molecular formula, massarigenin C, massarigenin D (Oh et al., 2003), and 6-epi-5′-hydroxy-mycosporulone (Fukami et al., 1999), each possessing stereochemical differences. An observed NOE correlation between H-6 with H-10 indicated a *cis*-1,3-diaxial-type orientation of these protons, while an NOE correlation between H-4 and H_3-12 showed that **3** is the known compound, 6-epi-5′-hydroxy-mycosporulone.

The structure of compound **4** was assigned as enalin A by detailed analysis of spectroscopic data and comparison with those reported for enalin A produced by the marine-derived fungus *Verruculina enalia* (Lin et al., 2002).

2.2. Biology

Massarina sp., strain CNT-016, was isolated as a sterile mycelium, which failed to form spores that could have been useful for identification of the species. To identify this fungal strain, we used phylogenetic analyses based on DNA sequence analysis. Phylogenetic analyses of the ribosomal intergenic spacer region (ITS) showed that strain CNT 016 belongs to clade 1 of the genus *Massarina* (Liew et al., 2002), and is most closely related to *M. corticola* and an uncultured ascomycete with good statistical support in all analyses (Fig. 3). However, *Massarina* sp., strain CNT-016, differs from both its closest relatives by more than 25 substitutions in the ITS region alone, and is thus possibly a new species.

The fungi in clade 1 of the *Massarina* are from different geographic locations and substrates. The uncultured ascomycete relative of this *Massarina* sp., strain CNT-016, was the only one identified from a terrestrial soil habitat (O'Brien et al., 2005); the other strains in this clade were isolated from plant material (Aptroot, 1998) collected from locations including Europe, North America, Asia and Papua New Guinea. No secondary metabolites are known from species in the *Massarina* clade 1 except for the study reported here.

The Massarina CNT-016 metabolites (**1**–**4**) were subjected to cytotoxicity evaluation against the human colon carcinoma cell line, HCT-116, and also for antimicrobial activity. The crude extract as well as the fractions from which the compounds were isolated showed weak activity against HCT-116, however none of the compounds isolated

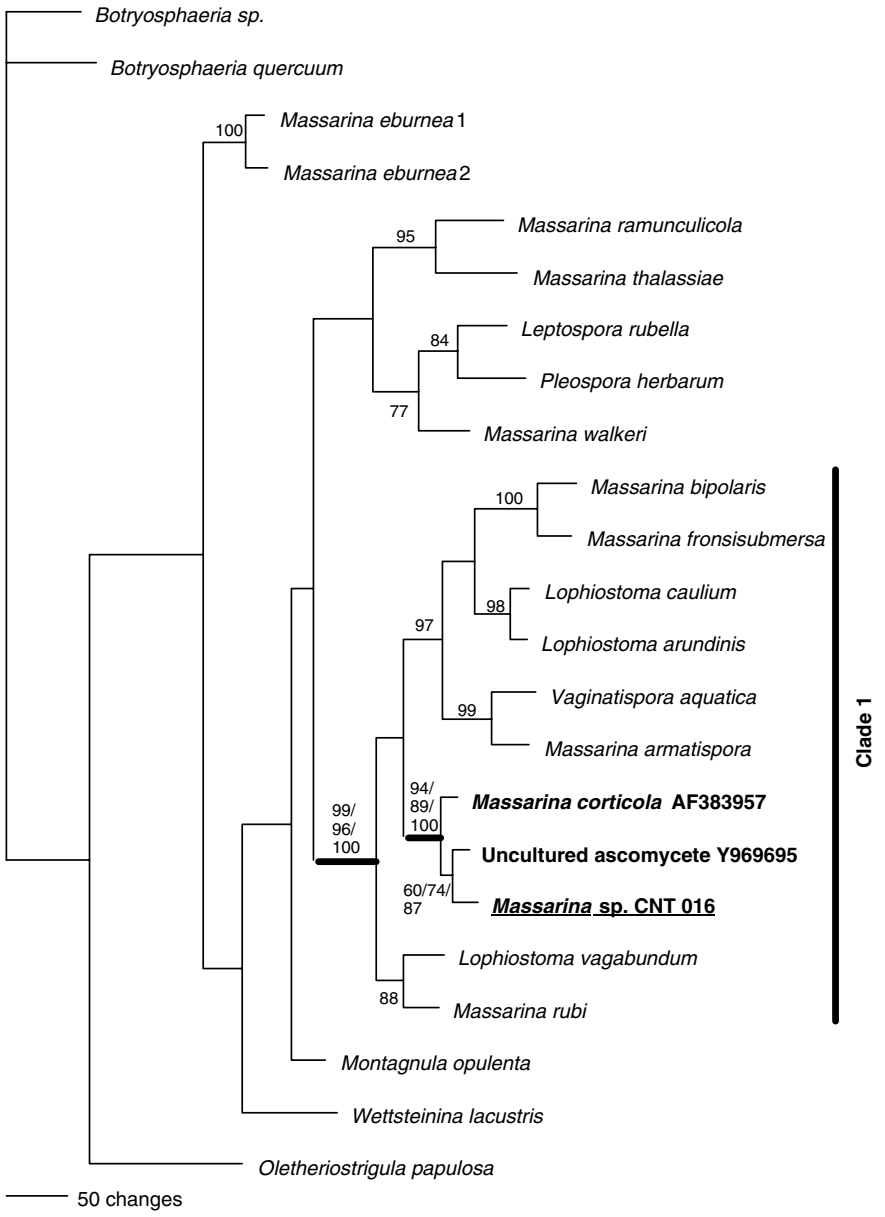


Fig. 3. *Massarina corticola* and an unknown ascomycete are closest relatives of *Massarina* sp. CNT-016. One of three most parsimonious trees is illustrated (tree length: 1707 steps). Support values by the branches are from parsimony, likelihood and Bayesian analyses in that order for branches of interest in this study. Otherwise parsimony support percentages above 70% are given. Well-supported branches and taxa of interest in this study are in bold, *Massarina* sp. CNT-016 is underlined. *Massarina* sp. CNT-016 groups in clade 1 (Liew et al., 2002) with high support in all analyses, and is the closest relative of *M. corticola* and an uncultured ascomycete with 94%, 89% and 100% support. See Liew et al. (2002) for GenBank accession numbers not included here.

showed significant activity against HCT-116, *Candida albicans* or *Staphylococcus aureus*.

3. Concluding remarks

Compounds **1** and **3** are closely related and appear to be possibly derived from the same type of precursor. These compounds possess similar structures to several other fungal metabolites, such as rosigenin, massarilactones, and the spirostaphylotrichins. The possible biosynthesis of such type of compounds has been reported in the literature to be originating from a mixed origin involving polyketide precursor and malic acid (Ayer et al., 1992).

4. Experimental

4.1. General

Melting points were determined on a Mel-Temp apparatus and are uncorrected. Optical rotations were measured

on an Autopol automatic polarimeter (Rudolph research, Flanders, NJ). UV spectra were recorded on a Varian Cary UV/vis spectrophotometer, and IR spectra were obtained with a Perkin–Elmer 1600 series FTIR spectrometer. NMR spectra were acquired on a Varian INOVA spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. HRESITOFMS data were obtained at The Scripps Research Institute, La Jolla, CA. Electrospray mass spectra were obtained on a Hewlett-Packard HP1100 integrated LC-MS system. Reversed-phase HPLC separations were performed using a semi-preparative C18 Altima 5 μm, 60 Å, (250 × 10) mm columns using a Waters R401 refractive index detector. Preparative HPLC was performed on a Waters 4000 system, monitoring at 210 nm, using a C18 Nova-Pak 6 μm 60 Å, (300 × 40) mm column.

4.2. Isolation

Massarina sp. (strain CNT-016, deposited at the Center for Marine Biotechnology and Biomedicine fungal strain repository) was isolated from a marine mud sample collected (−5 m) in the Palau Islands 7°20.4′N, 134°27.2′ E. The mud sample was stored in a sterile plastic bag and transported to the laboratory where it was kept frozen until processed. The sample was diluted 10 times using sterile seawater. One ml of the diluted sample was processed utilizing the pour plate method in GYA medium (1 g glucose, 0.1 g yeast extract, 18 g agar and 1 L seawater). Plates were incubated at 25 °C under white light and examined daily by stereomicroscopy to record fungal growth. The fungal strain used in this study appeared after six days. Hyphal tips were transferred to new GYA plates until enough new growth had been established.

4.3. DNA extraction and sequencing

DNA was extracted from the freeze–dried mycelium using the standard phenol–chloroform extraction method (Lee and Taylor, 1990). The ribosomal internal transcribed spacer region (ITS) was amplified using primers ITS4 and ITS5 (White et al., 1990), and sequenced in both directions and assembled using SeqMan 6.1 (DNAStar Inc.).

4.4. Phylogenetic analyses

A blastn search (Altschul et al., 1997) with the *Massarina* sp., CNT-016 (PC48) ITS sequence at GenBank returned a DNA sequence of an 'uncultured ascomycete' (GenBank AY969695) and *Massarina corticola* (AF383957) as the closest matches. Alignment M958 (Liew et al., 2002) containing 21 taxa including *M. corticola* was retrieved from TreeBase and the ITS sequences of *Massarina* sp., CNT-016 and the unknown ascomycete were added. An overall alignment was produced with ClustalX 1.8 (Thompson et al., 1997) and manually optimized. Phylogenetic analyses were performed with PAUP v.4.0b 10 (Swofford, 2002) for parsimony and likelihood, and MrBayes v3.0b4 for Bayesian analyses of phylogeny (Huelsenbeck and Ronquist, 2001) with settings described in Inderbitzin et al. (2005). Bootstrap support percentages for parsimony and likelihood were based on 500 and 107 replicates, respectively. Parsimony, likelihood and Bayesian inference of phylogeny all showed that *Massarina* sp., CNT-016 belonged to clade 1 (Liew et al., 2002) with respectively 99%, 96% and 100% support (Fig. 3). *Massarina* sp., CNT-016, was most closely related to *M. corticola* and the unknown ascomycete, with 94%, 89% and 100% support, respectively (see Fig. 3). A closest relative of *Massarina* sp., CNT-016, could not be determined, as *Massarina* sp. CNT-016 and the unknown ascomycete received only moderate support of 60%, 74% and 87 %. The *Massarina* sp., CNT-016 ITS sequence was submitted to GenBank (No. DQ863675).

4.5. Cultivation and extraction

Plugs of agar supporting mycelial growth were cut and transferred aseptically to 11 2.8-L Fernbach flasks each containing 1.25 L of the medium YMG (4 g yeast extract, 10 g malt extract, 10 g glucose per 1 L seawater). Flasks were incubated at 27 °C at 280 rpm shaking speed for 16 days. The combined culture broth was extracted twice with ethyl acetate and then dried by rotary evaporation to give 4.8 g of a yellow brown crude extract.

4.6. Isolation of metabolites

The crude extract from the 11 L cultivation (*Massarina* sp., strain CNT-016) was subjected to C18 vacuum liquid column chromatography (H_2O/CH_3OH; gradient 90:10 to 0:100 %) to give 7 fractions. HPLC purification of fraction 3 (25% CH_3CN/H_2O isocratic) using a reversed-phase (Prep Nova-Pak® HR C18, 6 μm, 300 × 40 mm) column afforded spiromassaritone (**1**; 10.5 mg), and **3** (8.9 mg). Fraction 4 was subjected to preparative HPLC using a reversed-phase column (Prep Nova-Pak® HR C18, 6 μm, 300 × 40 mm) with CH_3CN/H_2O gradient system (20:80 to 30:70 over 30 min) as eluent, followed by semi-preparative HPLC isocratic (20% CH_3CN/H_2O with 0.1% TFA) to give massariphenone (**2**; 3.7 mg) and enalin A (**4**) (9.8 mg).

4.6.1. Spiromassaritone (*1*)

White crystals, obtained by slow evaporation of a solution of **1** in $CH_3CN–H_2O$ (30:70), showed m.p. 131–132 °C; $[\alpha]_D$ +15 (MeOH, *c* 0.13); UV (MeOH) λ_{max} nm ($\log\varepsilon$): 220 (4.78). IR (NaCl) ν_{max} cm^{-1} 3440, 3405, 2950, 1805, 1720, 1675, 1140, 890; For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HRESITOFMS $[M+H]^+$ *m/z* 225.0748 ($C_{11}H_{13}O_5$, calcd. 225.0757).

4.6.2. Massariphenone (*2*)

Colorless oil; $[\alpha]_D$ −18.5 (MeOH, *c* 0.08); UV (MeOH) λ_{max} nm ($\log\varepsilon$): 220 (4.78), 250 (4.19), 310 (4.10); IR (NaCl)

ν_{max} cm^{-1} 3400, 3045, 2965, 1680, 1615, 1575, 1460, 1310, 1160, 890; ^1H NMR (500 MHz, CD_3OD): δ 1.29 (3H, d, $J = 7.2$ Hz, 9-CH_3), 2.29 (3H, s, 10-CH_3), 4.96 (1H, dq, $J = 14.2$, 7.2 Hz, 8-H), 6.83 (1H, dd, $J = 8.4$, 2.6 Hz, 5-H), 7.00 (1H, d, $J = 2.6$ Hz, 3-H), 7.09 (1H, d, $J = 8.4$ Hz, 6-H); ^{13}C NMR (125 MHz, CD_3OD): δ 19.1 (C-9), 19.3 (C-10), 70.8 (C-8), 115.0 (C-3), 118.4 (C-5), 129.3 (C-2), 132.6 (C-1), 155.9 (C-4), 206.9 (C-7); HRESI-TOFMS $[M+Na]^+$ m/z 203.0675 ($C_{10}H_{12}O_3Na$, calcd. 203.0679).

4.7. Crystal data for Spiromassaritone (*1*)

A crystal of **1** (0.404×0.304 × 0.03 mm) was tetragonal, space group *P4(1)2(1)2* with cell dimensions $a = 6.5306$ (7), $b = 6.5306$ (7), $c = 49.270$ (10) Å. Data were collected on BRUKER SMART diffractometer (graphite-monochromated Mo Kα radiation, $\lambda = 0.71073$ Å) equipped with a low-temperature device in omega-scan model at 100(2) K. The data were integrated with BRUKER SAINT. The structure was solved by direct methods (SHELXS-97, Sheldrick, 1990) and refined by full-matrix least squares methods against F^2 (SHELXL-97, Sheldrick, 1997). All non-hydrogen atoms were refined with anisotropic displacement parameters. The final refinement gave $R_1 = 0.0412$, $wR_2 = 0.1074$. Crystallographic data for spiromassaritone (**1**) have been deposited with the Cambridge Crystallographic Data Centre as supplementary Publication No. CCDC-616226. Copies of the data can be obtained free of charge via www.ccdc.cam.ac.uk/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

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