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METABOLITES OF *SISTOTREMA RADULOIDES*

BY

ALBERT KUDZOVI AMEGADZIE ©

A THESIS SUBMITTED TO THE
FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE
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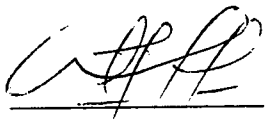

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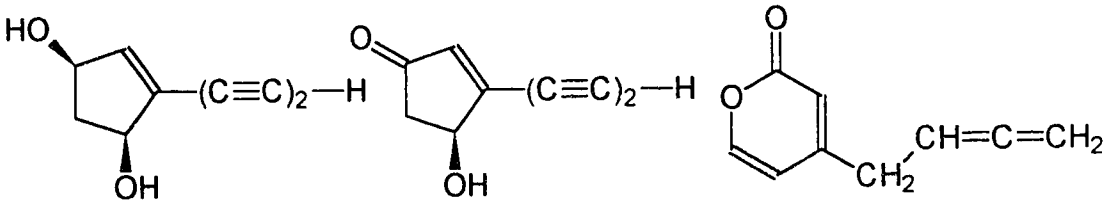
Date: Feb. 17, 1995

ABSTRACT

While investigating the flaky carbonaceous deposits produced by solid cultures of the wood-decay fungus *Sistotrema raduloides*, three new highly unstable natural products were uncovered. These three compounds, named **sistodiolynne**, **sistolynone**, and **sistopyrone**, were isolated from Sabourand dextrose liquid cultures of *S. raduloides*. Surprisingly, the flaky black substance was not observed in the liquid culture, although the crude organic extract underwent polymerization to give a black insoluble amorphous solid. To retard polymerization, the crude extract was kept in a small amount of solvent. Chemical analysis of the polymer revealed an empirical formula of C_3H_2O, which does not relate to the formula of any particular compound isolated.

A description of the isolation of these metabolites and their characterization by spectroscopic techniques is presented in this thesis. Chemical transformations were also used in some cases to verify structural assignments.

Biosynthetic studies were undertaken using singly and doubly ^{13}C-labelled acetate leading to the proposal of a possible biogenesis for the compounds isolated, and finally an explanation for the formation of the black polymer is proposed.



sistodiolynne **sistolynone** **sistopyrone**

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It was a great honour to work under the supervision of Prof. W. A. Ayer. I am very grateful for all his help, guidance, and support during my years as a graduate student.

I wish to thank L. Sigler for the cultures of *Sistotrema raduloides* and for bringing the problem to our attention.

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The technical staff of this department deserves to be mentioned for making my work possible. Dr. Tom Nakashima, Tom Brisbane, Glen Bigan, Gerdy Aarts, and L. Kong in the NMR Spectroscopy Laboratory and all the personnel in the Mass Spectrometry Laboratory, Electronics Department, Glass Blowing and Machine Shops.

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LIST OF ABBREVATIONS

$[\alpha]$	specific rotation
APT	attached proton test
b	broad (spectral)
CD	circular dichroism
COSY	correlated spectroscopy
δ	chemical shift in parts per million downfield from tetramethylsilane
d	doublet (spectral)
FTIR	Fourier transform infrared
g	gram(s)
HETCOR	heteronuclear correlation
HMBC	heteronuclear multiple bond coherence
HMQC	heteronuclear multiple quantum coherence
HREIMS	high resolution electron impact mass spectrometry
Hz	hertz
INADEQUATE	incredible natural abundance double quantum transfer experiment
J	coupling constant
m	multiplet (spectral), milli
MM2	molecular modelling program
m/z	mass to charge ratio (in mass spectrometry)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	nuclear magnetic resonance
nOe (NOE)	nuclear Overhauser enhancement
q	quartet (spectral)
R_f	retention factor (in chromatography)

s	singlet (spectral), second
t	triplet (spectral), time
TLC	thin layer chromatography
UAMH	University of Alberta Microfungus Herbarium
UV	ultraviolet
$\Rightarrow$	affects the signals at (in NMR)
$\Leftrightarrow$	correlates to (in NMR)

1. INTRODUCTION

The wood-decay fungus *Sistotrema raduloides* (P. Karst) Donk was recovered from indoor air during a study to evaluate air quality in a museum in San Juan, Puerto Rico. A member of the Corticiaceae (Basidiomycotina) subfamily with a widespread distribution in the temperate areas, *S. raduloides* is known to cause white rot decay of both angiospermous and gymnospermous wood (Nakasone, 1990).[1] It is among the 25 species of fungi considered the most important in decay of dead standing and fallen aspen.[2] There is no indication that *S. raduloides* causes decay in living aspen. Some of the known fungi which cause decay and stain in live aspen, including *Phellinus tremulae* (Bond), the most important in this group, were extensively studied by different groups including Ayer's.[3]

On agar media of various kinds, the Puerto Rican isolate was observed to form flaky carbonaceous deposits, especially where the mycelium came into contact with glass surfaces.* This study investigated the chemical structure and the biosynthesis of the compounds believed to be responsible for the black deposits.

While there have been no chemical investigations on cultures of *S. raduloides*, the liquid culture of a related species, *S. brinkmannii*, was found to regio- and stereoselectively reduce 18-methyl-4,15-estradien-3,17-dione to 17β-hydroxyl-18-methyl-4,15-estradien-3-one[4] as shown below.

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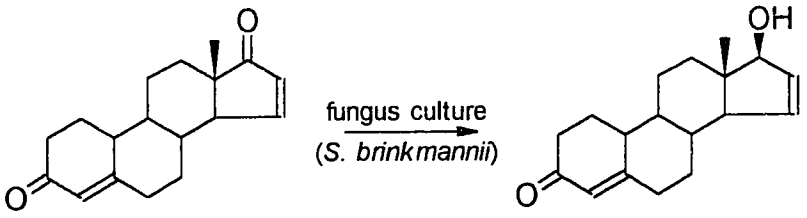
* L. Sigler. University of Alberta Microfungus Herbarium. Private communication.


O
O
fungus culture
(S. brinkmannii)
OH
O

2. RESULTS AND DISCUSSION

2.1. Liquid culturing of *Sistotrema raduloides*

Sistotrema raduloides was grown in still liquid culture on Sabourand dextrose broth, 15 g/L of water for 5 weeks. It is a slow growing fungus which is beige in colour, with a silk-like mycelium suspended on the surface of the liquid medium when fully grown.

2.2. Isolation of the metabolites from the fungus

The culture broth was separated from the mycelium by filtration and the volume of the culture filtrate (5 L) was reduced under vacuum to about 400 ml. The concentrated broth was first extracted with methylene chloride and then with ethyl acetate. The organic layers obtained were dried separately with anhydrous sodium sulphate, filtered and concentrated under vacuum to give a brown oil. On standing for about two minutes the crude extract, in each case, solidified, presumably by polymerization, into a totally insoluble hard black amorphous solid. In subsequent isolations the crude extract was not concentrated to dryness but was kept in a small amount of the extracting solvent. In this way, polymerization was avoided.

2.3. Structure elucidation

Sistodiolynne (1)

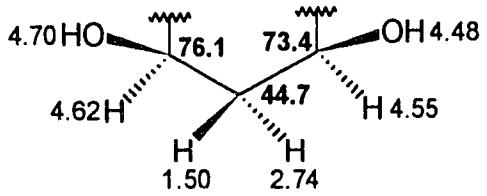
Sistodiolynne (**1**) was obtained by silica gel preparative TLC with 5% methanol in dichloromethane. To prevent polymerization the compound was eluted directly from the plate with deuterated acetone. The U.V. spectrum shows strong and sharp absorption bands at 310 and 252 nm. The former wavelength band is the stronger with a shoulder at 350 nm, which suggests an enyne system (–C=C–C≡C–).[5a]

The HREIMS spectrum obtained by placing a drop of the solution of the compound in deuterated acetone directly on the probe indicates a molecular ion peak at *m/z* 148 corresponding to a molecular formula of $C_9H_8O_2$. This accounts for six degrees of unsaturation. Loss of an H atom and H_2O account for peaks at *m/z* 147 and 130, respectively, suggesting the presence of hydroxyl group(s). This is apparent in the FTIR spectrum which shows an intense broad band at 3544 cm^{-1}. In the ^1H NMR spectrum signals at δ 4.70 (d, J = 6.5 Hz) and 4.48 (d, J = 6.5 Hz) disappeared when the sample was shaken with D_2O, indicating the presence of two hydroxyl groups.

An intense band at 3287 cm^{-1}, partially hidden by the hydroxyl absorption, and a weak band at 2230 cm^{-1} indicates an alkyne group. In addition, the ^{13}C NMR spectrum (Table 1) contains signals in the *sp* carbon region[6a] at δ_C 76.9, 72.0, 68.4, and 74.1 which account for two triple bonds.

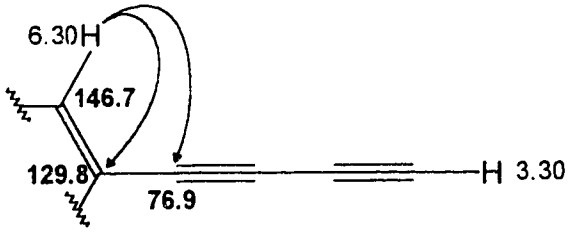
The ^1H NMR spectrum displays a total of eight signals which account for the eight hydrogens, including the two hydroxyl hydrogens. The ^{13}C NMR spectrum contains

nine signals, including two alkenic, four acetylenic, two methine, and a methylene carbon. All the data are summarized in Table 1. The sole methylene carbon at δ_C 44.7 was observed in an HETCOR[7] experiment to correlate to the protons at δ 2.74 (ddd, J =13.5, 7.0, 7.0 Hz) and 1.50 (ddd, J = 13.5, 6.2, 6.2 Hz). Each of these protons is coupled to the protons at δ 4.62 and 4.55, which are bonded to the methine carbons at δ_C 73.4 and 76.1, respectively. The chemical shifts for these methine groups suggests that they bear the hydroxyl groups. The chemical shift difference of 1.24 ppm[8,9] observed between the methylene protons agrees with a *cis* conformation for the hydroxyl groups. These data indicate the partial structure shown below.

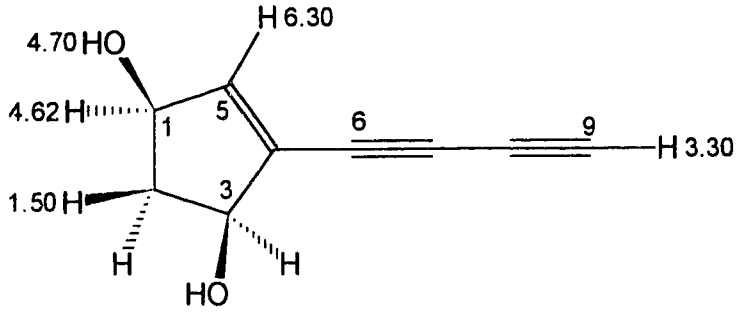


A singlet at δ 3.30 in the ^{1}H NMR spectrum could be assigned to the proton on the terminal alkyne, however due to the large coupling constant for ≡C–H ($J_{\equiv C-H} \approx$ 250 Hz) the ^{13}C NMR signal for the terminal alkyne carbon could not be deduced from the APT spectrum (τ = 7 ms) since its negative orientation indicated a fully substituted carbon. Using a J-Compensated APT[10] sequence (τ = 8 ms) however, it was shown that the carbon at δ_C 74.1 carried a hydrogen. The HMBC[11] spectrum, which shows correlation of protons and carbons separated by two to three bonds, and in certain cases four bonds, shows that the olefinic proton at δ 6.30 (dd, J = 1.0, 2.0 Hz) correlated with carbons at δ_C 129.8 and δ_C 76.9. This implied that the trisubstituted double bond is in conjugation with the carbon-carbon triple bonds. In combination with the unsaturation index the spectral data suggest the partial structure

shown below.

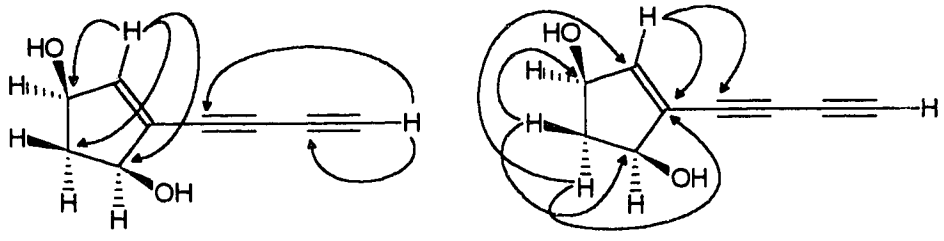


Connecting the two fragments results in the final structure **1**, 4-butadiyne-4-cyclopentene-1,3-diol (sistodiolynne). This structure is in good agreement with all spectral data. The mass spectral fragmentation pattern rationalized in scheme 1, supports the proposed structure.



1

Other correlations in the HMBC spectrum which are indicated, in Figure 1, confirm the structure as shown.

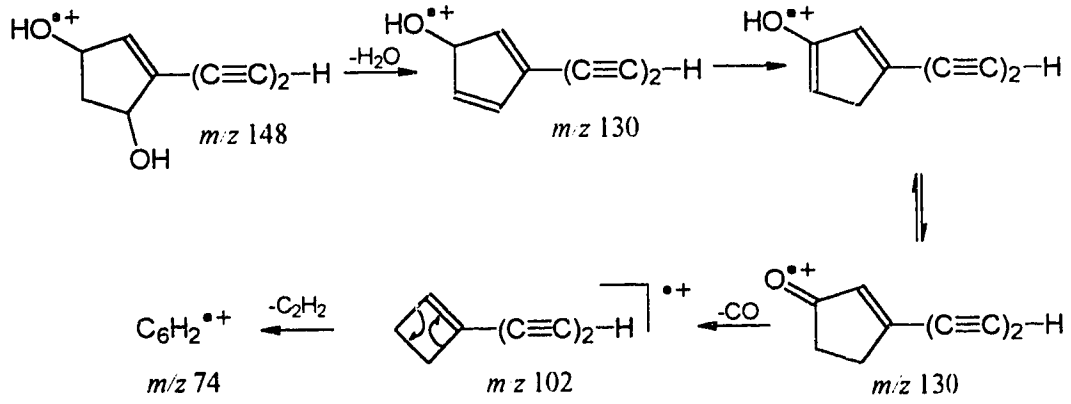


A **B**

Figure 1. Significant HMBC correlations of sistodiolynne (**1**).

Table 1. ^{1}H NMR of sistodiolynne (1) and 2, and ^{13}C NMR (CAPT) spectral data of 1.

	1	2	1	
Atom	δ,multi., *J* (Hz)	δ,multi., *J* (Hz)	Atom	δ_C,multi.
H-1α	4.62, bdd	5.60, m	C-1	76.1, d
H-2α	2.74, ddd, 13.5, 7.0, 7.0	2.95, ddd, 14.6, 6.7, 6.7	C-2	44.7, t
H-2β	1.50, ddd, 13.5, 6.2, 6.2	1.75, ddd, 14.6, 4.2, 4.2	C-3	73.4, d
H-3α	4.55, m	5.60, m	C-4	129.8, s
H-5	6.30, dd, 2.0, 1.0	6.44, bdd, 2.2	C-5	146.7, d
H-9	3.30, s	2.50, s	C-6	76.9, s
OH-1β	4.70, d, 6.5		C-7	72.0, s
OH-3β	4.48, d, 6.5		C-8	68.4, s
			C-9	74.1, d
-$COCH_3$		2.08, 2.02 (singlets)		



Scheme 1. Mass spectral fragmentation of sistodiolynne (**1**).

Stereochemistry of sistodiolynne

The relative stereochemistry at 1- and 3-positions is suggested by the chemical shift difference between the methylene protons at 2-position (1.24 ppm) mentioned earlier and nOe enhancements which indicate that the hydroxyl groups are in a *cis* arrangement. When the H-2α signal (δ 2.74) was irradiated in an nOe difference experiment, nOe enhancements (Figure 2) of 2-Hβ (21%), 1-Hα (7.5%) and 3-Hα (7.5%) were observed, which indicates that the hydrogens at 1- and 3-positions are on the same side of the molecule, and the hydroxyl groups at the opposite side.

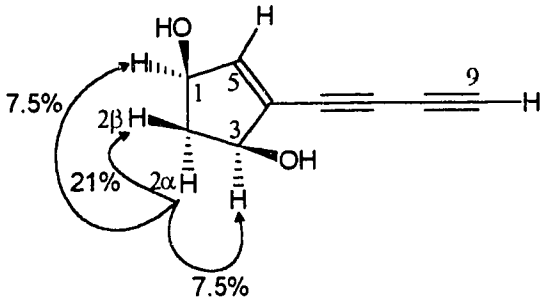


Figure 2. nOe enhancements of sistodiolynne (**1**).

Recent studies[8] have also shown that in cyclopent-4-ene-1,3-diol systems there is hydrogen bonding between the double bond in the ring and the hydrogens of the hydroxyl groups and the ring adopts an envelope conformation (Figure 3). In this conformation the lone pairs on the hydroxyl oxygens protrude in the direction of 2-Hβ (δ 1.50), explaining its high field shift relative to 2-Hα (δ 2.74), the result of a strong shielding effect by the lone pairs.

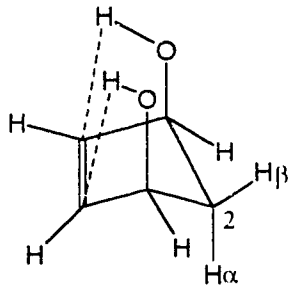
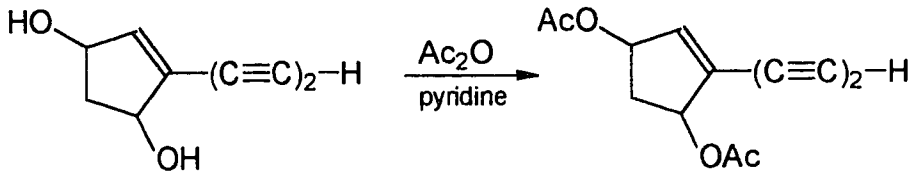


Figure 3. Proposed conformation of cyclopent-4-ene-1,3-diol systems.

Diacetate of sistodiolynne (1)

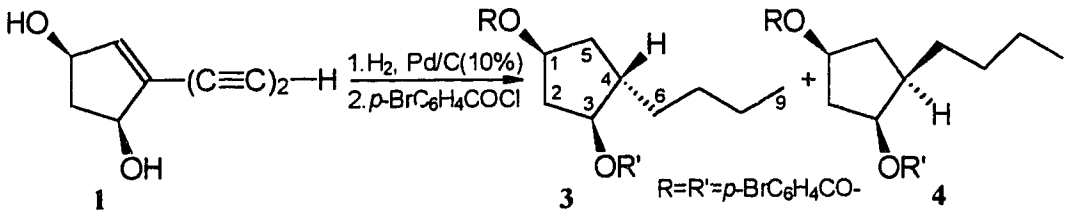
The reaction of **1** with acetic anhydride and pyridine in dichloromethane yielded the diacetate (**2**) as an unstable oil. The HREIMS shows $C_{13}H_{12}O_4$ as the molecular formula at *m/z* 232. A strong carbonyl absorption band at 1737 cm^{-1} in the FTIR spectrum corresponds to the ester functionality. The ^1H NMR signals for the hydroxyl groups in **1** had disappeared and were replaced by singlets at δ 2.08 and 2.02 representing the acetoxy methyls. The introduction of the acetyl groups causes an overall deshielding of the remaining signals summarized in Table 1.



1 **2**

Reduction and *p*-bromobenzoylation of sistodiolynne (1)

The hydrogenation of sistodiolynne (**1**) at 40 psi in the presence of palladium on carbon (10%) provided the saturated isomers. The resulting mixture of stereoisomers could not be separated and was treated with *p*-bromobenzoyl chloride in pyridine for 24 hours. The di-*p*-bromobenzoate derivatives were cleanly separated by preparative TLC with 5% ethyl acetate in hexane to give **3** (1*S*,3*S*-di-*p*-bromobenzoyloxy-4*S*-butylcyclopnetane) as the major component and **4** (1*S*,3*S*-di-*p*-bromobenzoyloxy-4*R*-butylcyclopentane) as the minor.



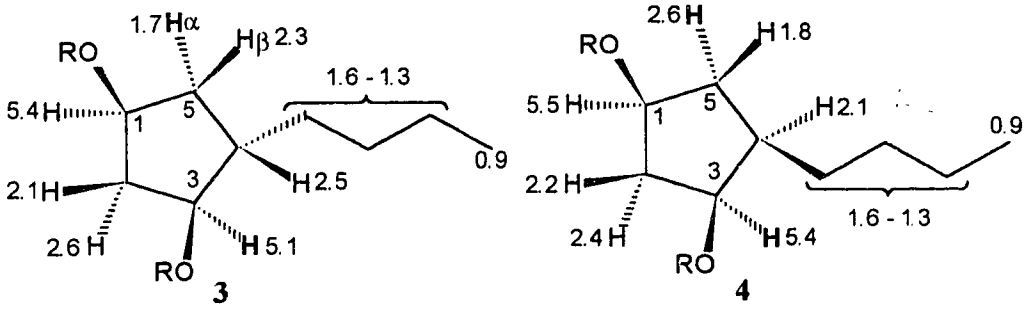
The HREIMS spectra of **3** and **4** are very similar with molecular ion peaks at *m/z* 522 corresponding to the molecular formula $C_{23}H_{24}O_4{}^{79}Br_2$. Loss of two molecules of *p*-bromobenzoic acid generates a C_9H_{14} fragment which accounts for the base peak at *m/z* 122.

The ^1H NMR spectra of **3** and **4** (Tables 2 and 3) are very similar with slight chemical shift differences for hydrogens at C-3, C-4, and C-5. The methylene hydrogens at 2- and 5-positions in both isomers were identified by their geminal couplings in their respective ^1H NMR spectra and by the ^1H-^1H COSY spectrum for **4**.

The ^{13}C NMR (APT) spectra shows three methines, two of which are oxygenated, five methylenes, and a methyl carbon; these results are consistent with the assignments

for the fully saturated isomers. The carbon resonances are summarized in Tables 2 and 3. The complete ^{13}C NMR signal assignments were made by comparing the results of an HMQC experiment (Table 4) to the ^{13}C NMR spectrum (Table 9) of the doubly labelled acetate compounds.

The position of the butyl group in **3** is *anti* with respect to the *p*-bromobenzoate groups and *syn* in **4**; this was assigned on the basis of the chemical shift differences between the following hydrogens. H-3α (δ 5.1) in **3**, absorbs at a slightly higher field than the same proton in **4** (δ 5.4) and H-5α (δ 2.3) in **3** absorbs at a higher field than H-5α (δ 2.6) in **4**. The presence of an adjacent butyl group in *syn* relationship to these hydrogens in **3** causes the upfield shift[12] shown below.



In addition, nOe experiments (Figure 4) with **4** in which signals of H-5α and [illegible] were irradiated confirms the positions as stated above.

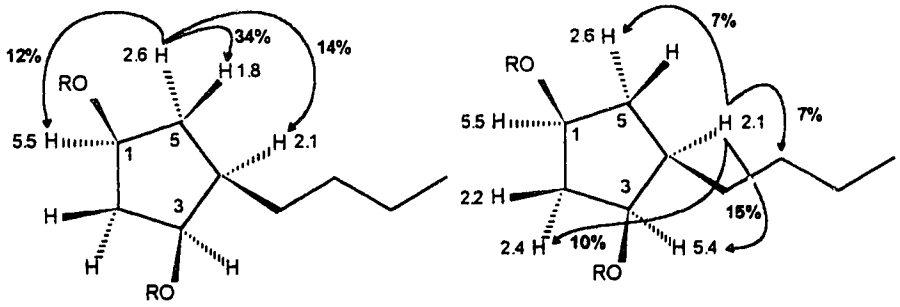


Figure 4. nOe enhancements of **4**.

Absolute configuration of 4

The di-*p*-bromobenzoate derivatives **3** and **4** were good candidates for the determination of the absolute configuration by CD techniques, since the natural metabolite sistodiolynne (**1**) was highly unstable.

The CD spectrum of **4** exhibited two Cotton effects opposite in sign, the first Cotton effect at longer wavelength (251 nm) has a value of $\Delta\varepsilon_1$ -1.20, while the second Cotton effect at shorter wavelength (231 nm) has a value of $\Delta\varepsilon_2$ +0.33. The summation of the amplitude of the two Cotton effects gave an A-value[13] of -1.53 ($A = \Delta\varepsilon_1 - \Delta\varepsilon_2$). A negative A-value implies that a left handed chirality exists between the chromophores. The ratio between the amplitudes was calculated to be -3.6.[14] The conclusion, therefore, can be drawn that sistodiolynne has a negative chirality and the absolute configuration shown in **4**.

Table 2: ^{1}H and ^{13}C NMR spectral data of **3**.

^{1}H NMR	δ, multi., *J* (Hz)	^{13}C NMR	δ, multi.
H-1α	5.4, m	C-1	75.6, d
H-2α	2.6, ddd, 15.5, 7.2	C-2	38.9, t
H-2β	2.1, bd, 15.5	C-3	80.5, d
H-3α	5.1, ddd, 7.2, 4.4, 3.8	C-4	44.0, d
H4β	2.5, m	C-5	37.5, t
H-5α	2.3, ddd, 15.0, 7.6, 2.1, 2.1	C-6	33.[illegible]
H-5β	1.7, ddd, [illegible], 5.8	C-7	30.1, t
H-6, 8, 7	1.6 - 1.3, m	C-8	22.8, t
H-9	0.9, t, 6.7	C-9	14.0, q
H-12, 12', 16, 16'	7.9, m	C-10, 10'	165.6, s
H-13, 13', 15, 15'	7.5, m	C-11, 11'	129.3, s
		C-12, 12', 16, 16'	131.7, d
		C-13, 13', 15, 15'	131.1, d
		C-14, 14'	128.1, s

Table 3: ^{1}H and ^{13}C NMR spectral data of **4**.

^{1}H NMR	δ, multi., *J* (Hz)	^{13}C NMR	δ, multi.
H-1α	5.5, dddd, 10.0, 7.5, 5.0, 2.5	C-1	75.0, d
H-2α	2.6, ddd, 15.5, 8.0, 5.0	C-2	39.8, t
H-2β	2.1, bd, 15.5	C-3	76.8, d
H-3α	5.4, bdd, 5.0, 4.1	C-4	43.6, d
H-4α	2.1, m	C-5	37.7, t
H-5α	2.6, ddd, 13.7, 7.5, 7.0	C-6	29.1, t
H-5β	1.8, ddd, 13.7, 10.0, 5.2	C-7	30.4, t
H-6	1.6 ,1.3, m	C-8	22.8, t
H-7, 8	1.5, 1.3, m	C-9	14.0, q
H-12, 16	7.9, d, 9	C-10	165.5, s
H-13, 15	7.6, d, 9	C-10'	165.4, s
H-12', 16'	7.8, d, 9	C-11	129.5, s
H-13', 15'	7.5, d, 9	C-11'	129.3, s
		C-12, 16	131.8, d
		C-12', 16'	131.6, d
		C-13, 15	131.1, d
		C- 13', 15'	131.0, d
		C-14, 14'	128.1, s

Table 4 HMQC data for **3** and **4**.

3		**4**	
C-atom	H-atom	C-atom	H-atom
80.5	5.1	76.8	5.4
75.6	5.4	75.0	5.5
44.0	2.5	43.6	2.1
38.9	2.6, 2.1	39.8	2.4, 2.2
37.5	2.3, 1.7	37.7	2.6, 1.8
33.1	1.6, 1.3	30.4	1.6, 1.3
30.1	1.3	29.1	1.5
22.8	1.3	22.8	1.3
14.0	0.9	14.0	0.9

Sistolynone (5)

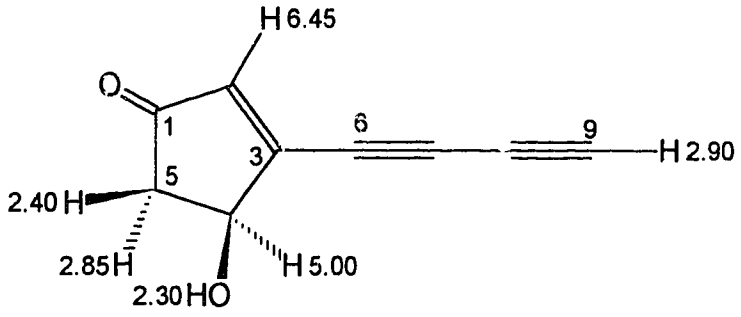
Sistolynone (**5**) was isolated as an unstable oil by preparative TLC using 5% methanol in dichloromethane as eluent. The compound was washed from the adsorbent with deuterated chloroform and was not taken to dryness in order to prevent decomposition. This compound was less polar (higher R_f) than sistodiolynne (**1**).

The molecular formula $C_9H_6O_2$ was determined by HREIMS which indicated a molecular ion peak at *m/z* 146. This formula corresponds to seven degrees of unsaturation. The peak at *m/z* 118 (Scheme 2) represents loss of CO suggesting the presence of a carbonyl group. A strong absorption band at 1710 cm^{-1} in the FTIR spectrum, and a signal in the ^{13}C NMR (APT) spectrum at δ_C 204.4, confirms a carbonyl. The position of this signal suggests an α,β unsaturated five membered ring ketone.[15] The band at 3389 cm^{-1} is a strong evidence for a hydroxyl group, and bands at 3284 and 2204 cm^{-1} indicate an alkyne group.

The ^1H NMR spectrum (Table 5) shows six signals which integrate for six hydrogens. A singlet at δ 2.9 was easily recognized as the CH on a terminal alkyne. A methylene pair is evident from signals at δ 2.8 and 2.4 (J_{gem} = 18.6 Hz), the chemical shifts and the magnitude of the coupling constant suggesting that they are attached to the carbon alpha to the carbonyl group.[16] The proton signal at δ 5.0 (dddd, J = 6.5, 5.0, 2.5, 1.0 Hz) is vicinally coupled to this methylene pair by 6.5 and 2.5 Hz, respectively[16,17] and by 5.0 and 1.0 Hz to protons at δ 2.3 and 6.4, respectively. The signal at δ 2.3 disappears when D_2O is added to the sample, suggesting that it is a hydroxyl hydrogen.

The ^{13}C NMR (APT) spectrum is very straightforward and contains nine signals, three of which appears in the region of C≡C at δ_C 89.8, 68.2 and 66.8. This suggests the presence of a dialkyne system by analogy to the spectra observed for **1**. The terminal alkyne CH at δ_C 78.3 was distinguished from the rest of the signals by *J*-Compensated APT.

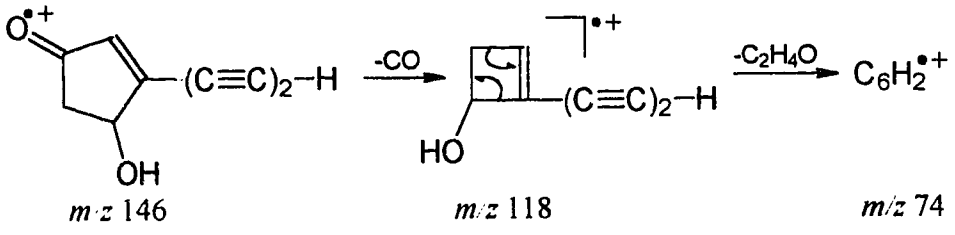
Based on all the spectral data discussed above and the fragmentation pattern (Scheme 2) the structure of sistolynone (**5**) below is proposed. The small quantity of sistolynone obtained prevented other correlation experiments and derivatization.



5

Table 5. ^{1}H and ^{13}C NMR spectral data of sistolynone (**5**).

^{1}H NMR	δ, mu'tiplicity, *J* (Hz)	^{13}C NMR	δ, multiplicity
H-2α	2.8, dd, 18.6, 6.5	C-1	204.4, s
H-2β	2.4, dd, 18.6, 2.5	C-2	139.3, d
H-3α	5.0, dddd, 6.5, 5.0, 2.5, 1.0	C-3	154.7, s
H-5	6.4, d, 1.0	C-4	72.1, d
H-9	2.9, s	C-5	44.0, t
OH-3β	2.3, d, 5.0	C-6	68.2, s
		C-7	89.9, s
		C-8	66.8, s
		C-9	78.3, d

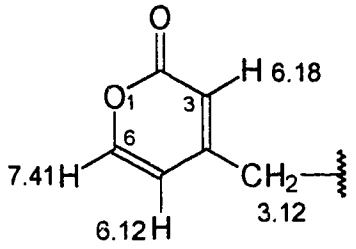


Scheme 2. Mass spectral fragmentation pattern of sistolynone (**5**).

Sistopyrone (6)

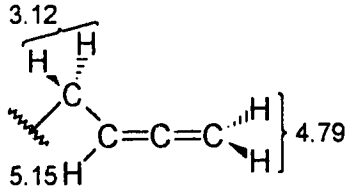
Sistopyrone (**6**) was also obtained as an unstable oil by preparative TLC with 5% methanol in dichloromethane. The HREIMS spectrum displays a molecular ion peak at *m/z* 148 to establish $C_9H_8O_2$ as the molecular formula. The strong absorption at 1725 cm^{-1} in the FTIR spectrum suggests a carbonyl.

The ^1H NMR spectrum (Table 6) displays a total of six signals, three of which appear in the olefinic region between δ 7.41 and 6.18. The proton at δ 6.18 (ddd, *J* = 2.0, 1.0, 1.0 Hz) is coupled to protons at 7.41 (dd, *J* = 5.2, 1.0 Hz), 6.12 (dd, *J* = 5.2, 1.0 Hz) and 3.12 (dtd, *J* = 7.0, 2.9, 1.0 Hz). The chemical shifts and coupling constants displayed by these olefinic protons in addition to signals at δ_C 162.2 (s), 156.8 (s), 150.7 (d), 113.7 (d) and 108.0 (d) in the ^{13}C NMR spectrum (Table 6) suggests a pyrone ring substituted at 4-position.[18,19] HMQC[20] correlations of the spectral data mentioned above confirm the assignments for the partial structure shown below.

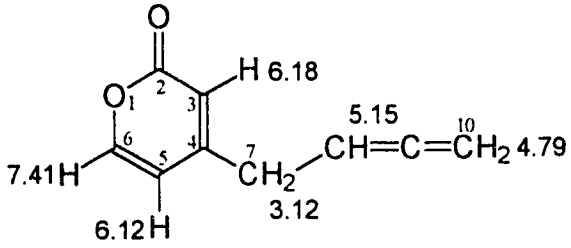


Infrared bands at 1955 and 853 cm^{-1} are indicative of an allene group[21] (–HC=C=CH_2), which is evident in the ^{13}C NMR spectrum as signals at δ_C 209.7 (s), 85.5 (d), and

76.3 (t).[22] The remaining signals in the ^{1}H NMR spectrum are a one proton signal at δ 5.15 (qn., J = 7.0 Hz) and a two proton signal at δ 4.79 (dt, J =6.7, 2.9 Hz). Decoupling experiments provide complete coupling information about the allene group as summarized in Table 7. In particular, the irradiation of the signal at δ 3.12 (dtd, 7.0, 2.9, 1.0 Hz) results in the collapse of signals at δ 6.18, 5.15 and 4.79. The signal at δ 5.15 becomes a triplet with J = 6.7 Hz, coupling only to the signal at δ 4.79. It became evident at this point that the signal at δ 5.15 is in fact an overlapping triplet of triplets rather than a quintet. The partial structure below is consistent with these data.

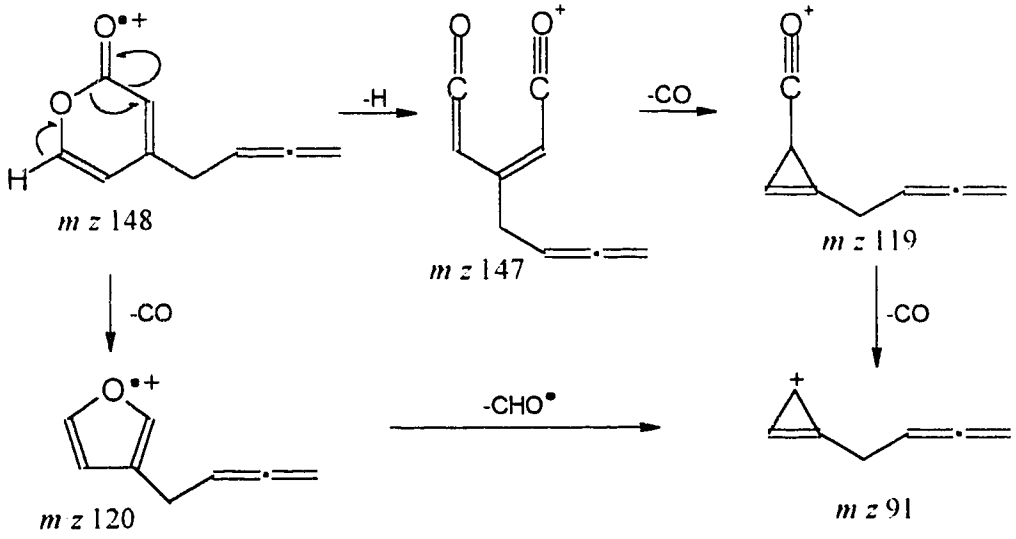


Linking the two fragments results in the final structure **6** which we have named sistopyrone.



6

The fragmentation pattern supports the structure assignment, with the peak at m/z 91 due to the loss of 2 CO and an H atom which is typical for pyrones,[23] as shown in Scheme 3.



Scheme 3. Possible mass spectral fragmentation of sistopyrone (**6**).

Table 6. ^{1}H and ^{13}C NMR spectra of sistopyrone (**6**).

^{1}H NMR	δ, multiplicity, *J* (Hz)	^{13}C NMR	δ, multiplicity
H-3	6.18, ddd, 2.0, 1.0, 1.0	C-1	204.4, s
H-5	6.12, dd, 5.2, 2.0	C-2	139.3, d
H-6	7.41, dd, 5.2, 1.0	C-3	154.7, s
H_2-7	3.12, dtd, 7.0, 2.9, 1.0	C-4	72.1, d
H-8	5.15, tt, 7.0, 6.7	C-5	44.0, t
H_2-10	4.79, dt, 6.7, 2.9	C-6	68.2, s
		C-7	89.9, s
		C-8	66.8, s
		C-9	78.3, d

Table 7. Homonuclear decoupling data of sistopyrone (**6**).

Signal irradiated	Signals affected	
	δ, multiplicity, *J* (Hz)	Observed change
6.18, ddd, 2.0, 1.0, 1.0 Hz	7.41, dd, 5.2, 1.0 (H-6)	d, 5.2 Hz
	6.12, dd, 5.2, 2.0 (H-5)	d, 5.2
	3.12, dtd, 7.0, 2.9, 1.0 (H_2-7)	dt, 7.0, 2.9
4.79, dt, 6.7, 2.9 Hz	5.15, tt, 7.0, 6.7 (H-8)	t, 7.0
	3.12, dtd, 7.0, 2.9, 1.0 (H_2-7)	dd, 7.0, 1.0
3.12, dtd, 7.0, 2.9, 1.0 Hz	6.18, ddd, 2.0, 1.0 1.0 (H-3)	dd, 2.0, 1.0
	5.15, tt, 7.0, 6.7 (H-8)	t, 6.7
	4.79, dt, 6.7, 2.9 (H_2-10)	d, 6.7

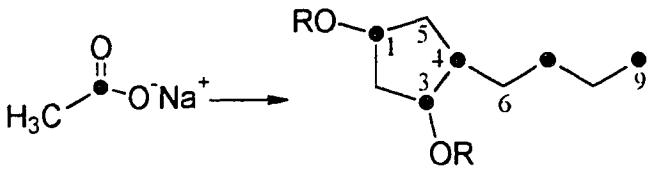
3. BIOSYNTHESIS

3.1. Labelled sistodiolynne

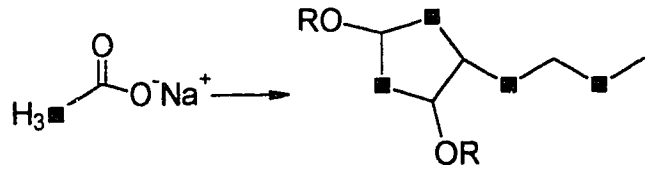
Sistotrema raduloides was cultured as before in liquid still culture of Sabourand dextrose broth and was periodically supplemented with injections of a sterile solution of C-labelled sodium acetate [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]. The culture was grown for five weeks after which it was harvested. Silica gel preparative TLC of the ethyl acetate extract from the broth gave sistodiolynne (**1**), but due to its unstable nature, it was reduced and benzoylated to give the di-*p*-bromobenzoate derivatives **3** and **4**, which were sufficiently stable for analysis.

The ^{13}C NMR spectra were recorded using identical NMR instrument parameters and similar concentrations for the unlabelled, [2-^{13}C]-acetate, and [1,2-$^{13}C_2$]-acetate labelled **3**. The percentage enrichment[24] was calculated for **3** derived from [2-^{13}C]-acetate by subtracting 1.1 from the ratio of the labelled signal intensity and the natural abundance intensity shown in Table 8. A lesser quantity was obtained for the [1-^{13}C] labelled compound, so the percentage enrichment was not calculated, but the enhanced signals were obvious in the ^{13}C NMR spectrum (Table 8).

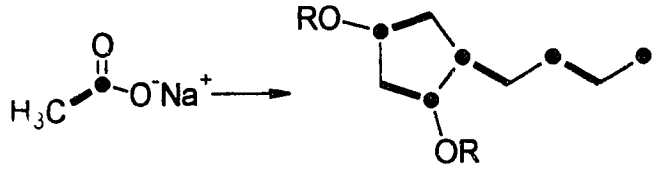
In the ^{13}C NMR spectrum of **3** derived from [1-^{13}C]-acetate, the signals from C-1 (δ_C 76.7), C-3 (80.5), C-4 (44.0), C-7 (30.1) and C-9 (14.0) are significantly enhanced and C-3 and C-4 are spin coupled, to appear as apparent triplets (J_{C-C} = 34 Hz). From these data, the labelling pattern shown below was deduced.



In the ^{13}C NMR spectrum of the derivative **3** incorporating [2-^{13}C]-acetate, the signals from C-2 (δ_C 38.9), C-5 (37.5), C-6 (33.1) and C-8 (22.8) were significantly enhanced, which leads to the labelling pattern shown below.



The ^{13}C NMR spectrum of the derivative **3** enriched with [1,2-$^{13}C_2$]-acetate shows all signals as doublets except C-9 which appears as an enhanced singlet implying that it does not arise from an intact acetate unit. The carbon-carbon coupling constants ($^1J_{C\text{-}C}$) (Table 9) from the ^{13}C NMR spectrum helped identify the carbons which originated from the incorporation of four intact acetate units. The carbon in the 9-position has been determined to come from C-1 of an acetate in singly labelled experiments incorporating [1-^{13}C] and [2-^{13}C]-acetates. These results give rise to the pattern shown below.

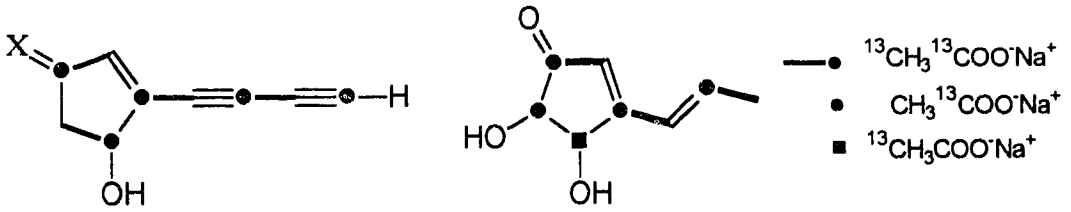


The results of a 2D-INADEQUATE[25] experiment of **3** obtained from [1,2-$^{13}C_2$]-

acetate (SEE EXPERIMENTAL) confirms the spin-coupled carbons and identifies carbon pairs from intact acetate units as C-1,C-5; C-2,C-3; C-4,C-6; and C-7,C-8. This proved the presence of four intact acetate units, and agreed with the assignments on the basis of $^{1}J_{C\text{-}C}$ values in Table 10.

3.2. Biosynthesis of some five membered rings

The only mono-alkylated five membered ring similar to sistodiolynne (**1**) and sistolynone (**5**) which was found to be derived from polyketide is terrein[26,27] (7) isolated from *Aspergillus terreus*. Despite the structural similarities between terrein, sistodiolynne, and sistolynone, the biogenesis must follow a different path, since the labelling pattern observed for terrein (Figure 5) differs from that of **1** and **5**.



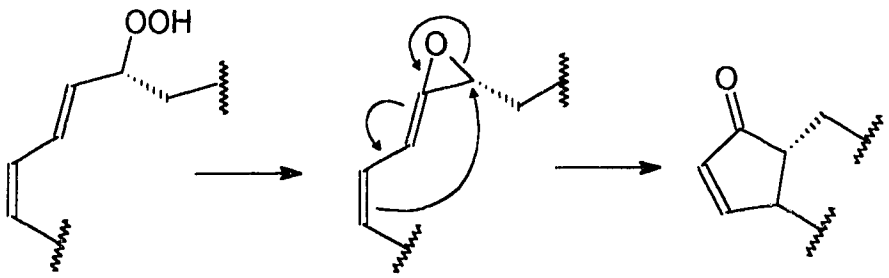
1; X = H, OH
5; X = O

7

Figure 5. Labelling pattern observed in 7, **1**, and **5**.

Also allene oxide intermediates are very reactive epoxides that are prone to nucleophilic substitution and intramolecular rearrangements, leading to the formation of prostaglandin-like molecules (Scheme 4). However, an allene oxide could not be involved in the present case, since the products of this intermediate are generally di-

alkyl substituted five membered rings[28,29,30] (Scheme 4)

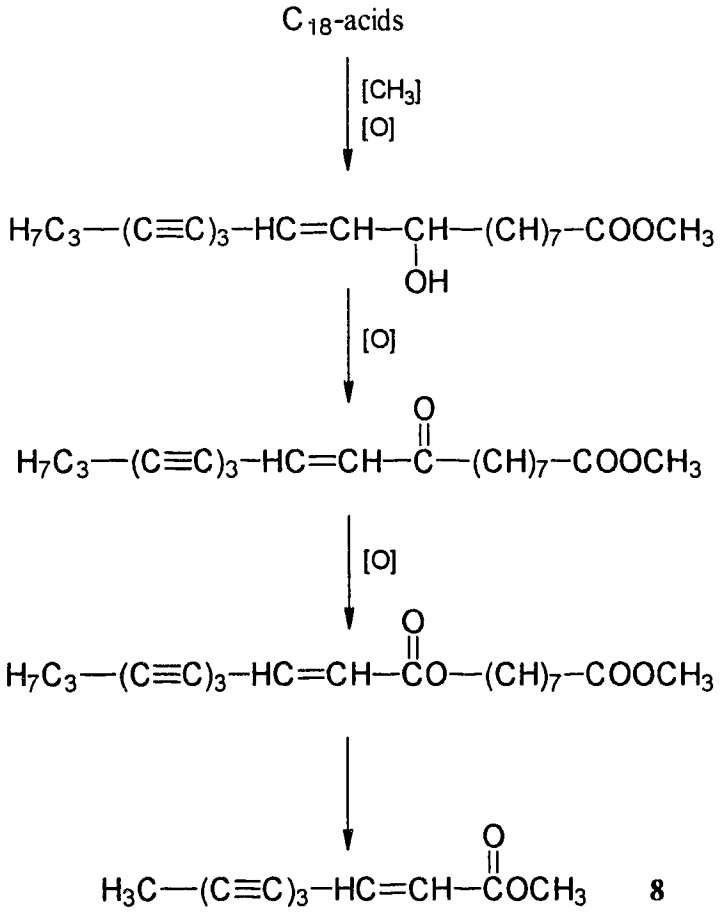


Scheme 4. Allene oxide intermediate.

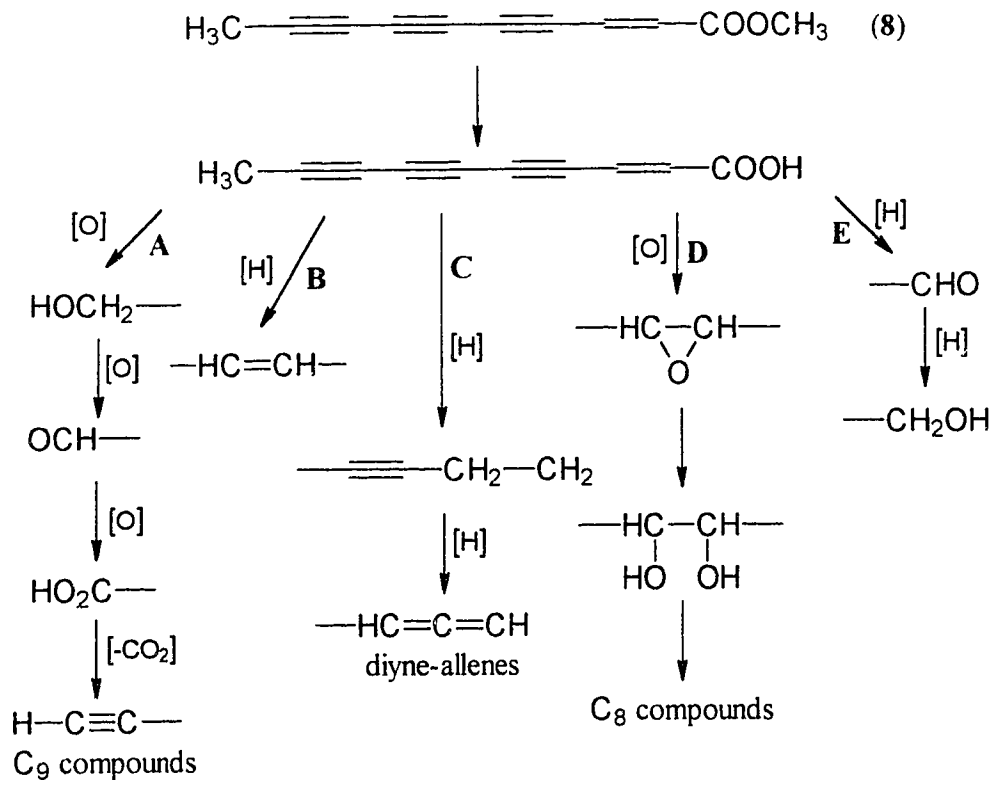
3.3. Biosynthesis of polyenynes

It was reported by Bu'Lock and Smith[31] that oleic acid is the most important precursor in the biosynthesis of many acetylenes isolated from fungal cultures. Degradation of this long chain acid ester leads to the formation of most short chain (C_{10}) acetylenes. Scheme 5 shows the formation of a typical straight chain C_{10} acetylene by Baeyer-Villiger like oxidation.[5b]

A considerable number of C_{10} acetylenes have been isolated from mic· ·· ganisms; most of them are probably derived from dehydromatriacariaester (**8**). A number of reactions can be hypothesized in the formation of some known acetylenes from intermediate **8**,[5c] as shown in Scheme 6.



Scheme 5. Proposed biogenesis of C_{10} acetylenes.



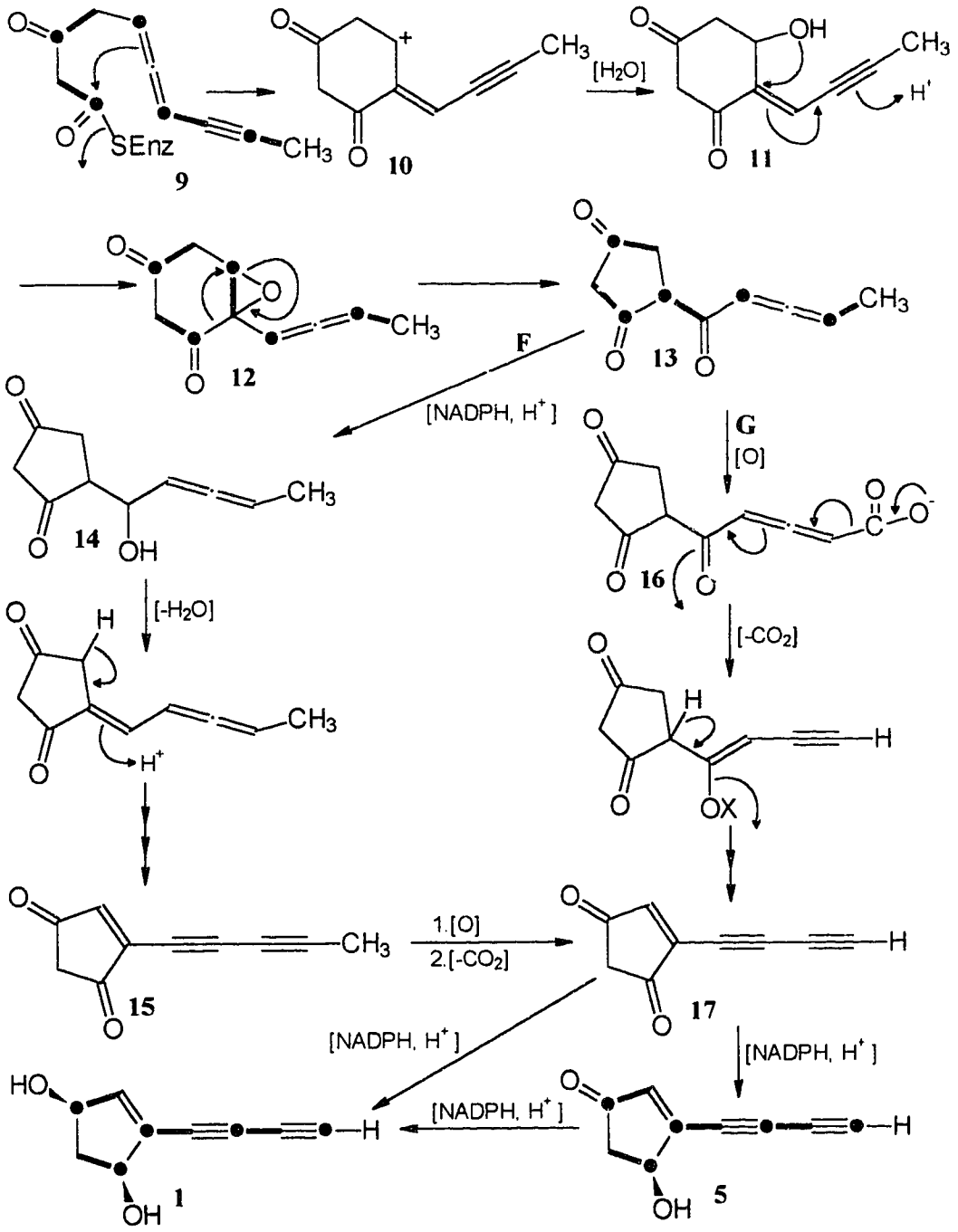
Scheme 6. Some biochemical pathways of acetylenes.

3.4. Possible biogenesis of sistodiolynne (1) and sistolynone (5)

The absolute configuration and the labelling pattern observed for **1** causes difficulty in correlating with known natural occurring five membered ring compounds. This has led to the proposal of the following two mechanisms based on the results described above.

1. Starting with **9**, a C_{10} intermediate (Scheme 7a) which is probably derived from **8**, an intramolecular addition reaction in **9** would give rise to a six membered ring carbocation intermediate **10**. This can be captured by water to give allylic alcohol **11**.

Acid catalyzed attack of the hydroxyl group on the enyne system in **11** could give the epoxide **12**.



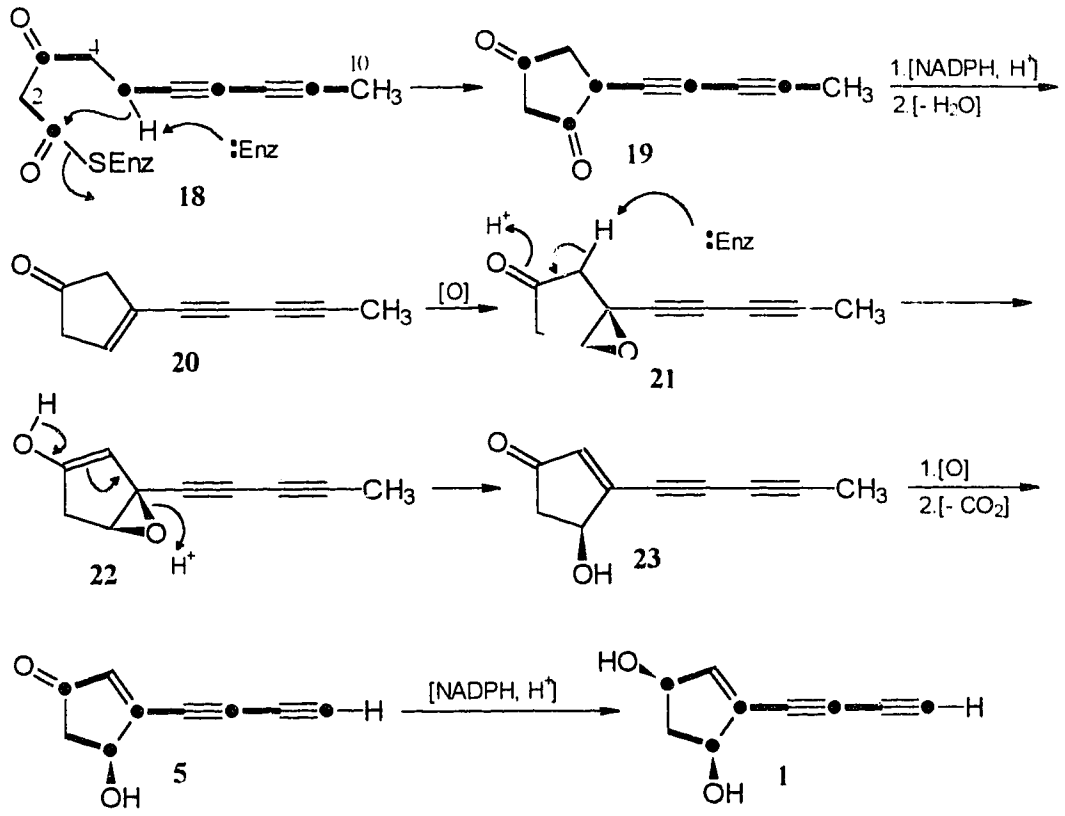
Scheme 7a. Proposed biosynthesis of **1** and **5**.

The opening of the epoxide ring, triggered by a 1,2-shift[32a] results in the formation of the five membered ring **13** with the correct labelling pattern. Intermediate **13** can then undergo a series of reactions depicted in Scheme 7a, pathway **F**, to give **15**. The degradation of the side chain from C_5 to C_4 by oxidation of the terminal methyl to a carboxylic acid followed by decarboxylation is analogous to the known pathway **A** in Scheme 6 and gives rise to the terminal alkyne intermediate, **17**. This decarboxylation step can occur earlier in the sequence from intermediate **13** through pathway **G** (Scheme 7a) to produce **17**.

Enzymatic reduction of one of the carbonyl groups would give sistolynone (**5**) and subsequent reduction of the second carbonyl would give sistodiolynne (**1**).

2. The formation of the five membered ring could also occur via enzymatic hydrogen abstraction from the 5-position in **18**, giving a carbanion which could condense directly onto the carbonyl at the 1-position to produce **19** (Scheme 7b). The reduction of one ketone followed by elimination of water would give **20**. The opening of the epoxide formed from intermediate **20** would give the keto-hydroxyl intermediate **23**. The oxidation of the terminal methyl in **23** to a carboxylic acid followed by decarboxylation would give **5**, and reduction of the ketone in **5** to form **1**.

Incorporation experiments using ^{2}H and ^{18}O could be undertaken to distinguish between the above pathways. These would be expensive and time consuming and were not carried out. The ^{13}C-labelling experiments, however, do indicate that these compounds are unique natural products.



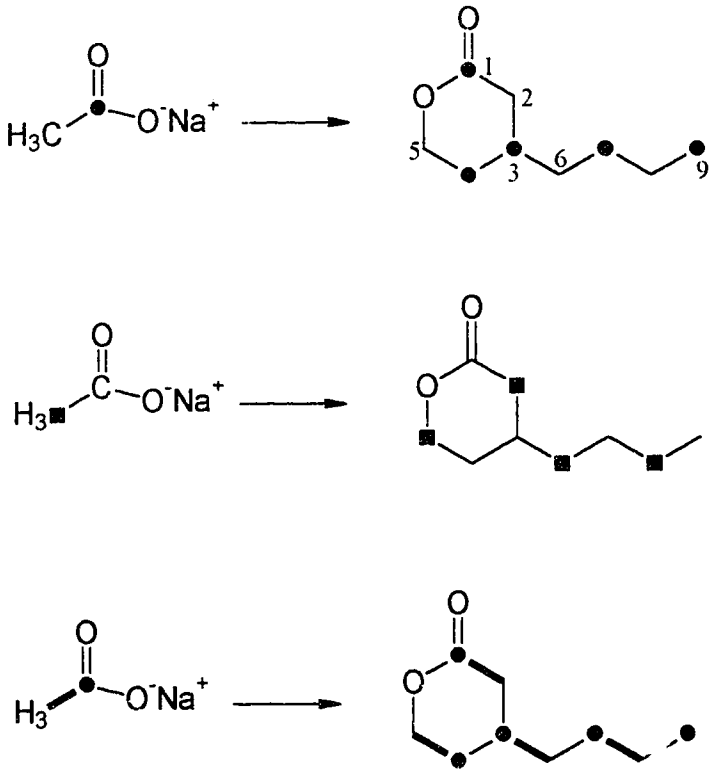
Scheme 7b. Proposed biosynthesis of **1** and **5**.

3.5. Labelled sistopyrone (6)

Sistopyrone, ^{13}C-labelled by the incorporation of [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]-sodium acetate, was obtained in the same manner as described for sistodiolynne and sistolynone. The percentage enrichment was not calculated in each case since differing quantities were obtained. Nonetheless, the enhanced signals were easily identified in the ^{13}C NMR spectra (Tables 8-9). The ^{13}C NMR spectrum of labelled **6** derived from [1,2-$^{13}C_2$]-sodium acetate displays signals with ^{13}C–^{13}C coupling constants

characteristic of the hybridizations[6b] of the atoms.

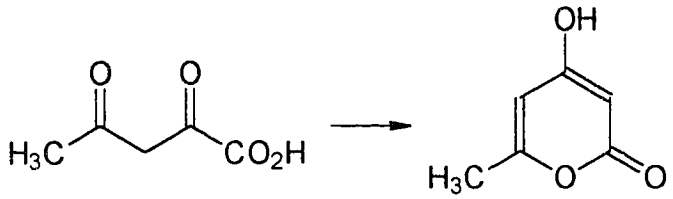
The skeletal labelling patterns observed for **6** incorporating [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]-sodium acetate are represented below.



3.6. Biosynthesis of pyrones

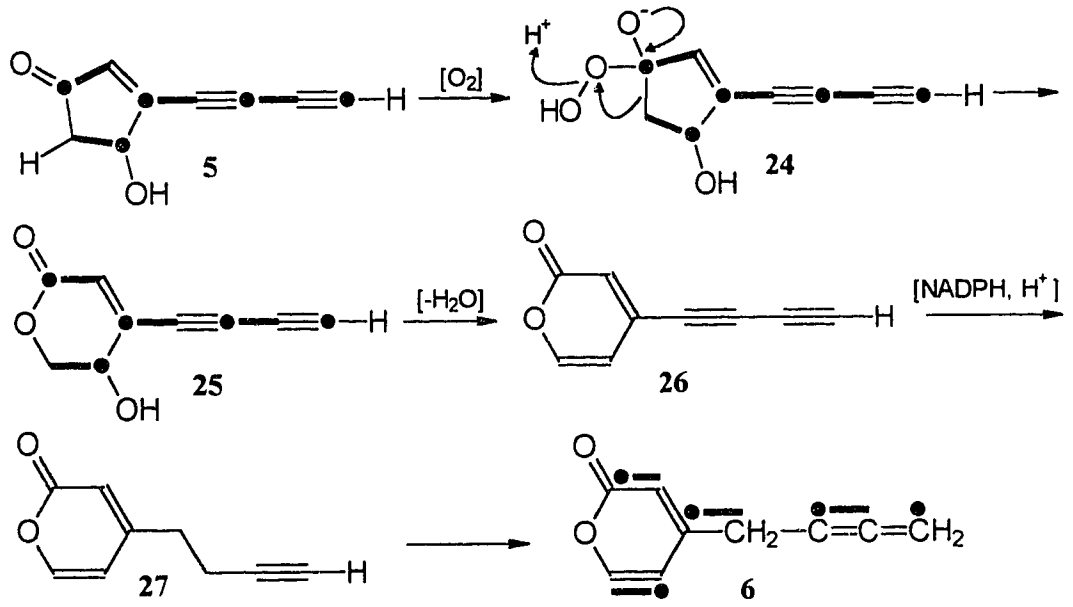
The pyran-2-one moiety is well represented in nature, from both plants and fungal sources, and some of these compounds are responsible for a wide range of biological effects, e.g., antibiotics, antifungals, etc. The labelling pattern and the position of the side chain in **6** is unique, and does not follow a known pathway to the pyran-2-one

moiety. The known pathway involves β-polyketomethylene intermediates of the acetate-methylmalonate pathway[32b] shown below.



3.7. Possible biosynthesis of sistopyrone (6)

The results of the labelling experiments indicate a possible biogenesis starting with sistolynone (**5**), (Scheme 8). Addition of oxygen in an enzymatic Baeyer-Villiger[33] type oxidation could give the hydroperoxide intermediate **24**, which could then ring expand to form **25**.



Scheme 8. Proposed biosynthesis of **6**.

Dehydration of the lactone ring in **25**, and the reduction of one triple bond followed by

the isomerization of the second would lead to the formation of **6**.

Table 8. ^{13}C NMR spectral data of **3** and **6** labelled with [1-^{13}C] and [2-^{13}C]-acetates.

	3			**6**		
		[1-^{13}C]	**[2-^{13}C]**		**[1-^{13}C]**	**[2-^{13}C]**
C-atom	δ_C	Enhanced signals	Atom % enrichment•	δ_C	Enhanced signals	Enhanced signals
C-1	76.7	enhanced	–	162.2	enhanced	–
C-2	38.9	–	13.9	113.7	–	enhanced
C-3	80.5	enhanced*	–	156.8	enhanced*	–
C-4	44.0	enhanced*	–	108.0	enhanced*	–
C-5	37.5	–	14.9	150.7	–	enhanced
C-6	33.1	–	13.9	34.3	–	enhanced
C-7	30.1	enhanced	–	85.5	enhanced	–
C-8	22.8	–	12.4	209.7	–	enhanced
C-9	14.0	enhanced	–	76.3	enhanced	–

• Atom % enrichment = $\frac{\text{acetate labelled signal intensity}}{\text{natural intensity}}$ - 1.1

* Signals shows coupling appearing as triplets.

Table 9. ^{13}C NMR data of **3** and **6** labelled with [1,2-$^{13}C_2$]-acetate.

	3		**6**	
C-atom	δ_C	multiplicity, $J_{C,C}$(Hz)	δ_C	multiplicity, $J_{C,C}$(Hz)
C-1	76.1	d, $J_{1,5}$ = 36.0	162.2	d, $J_{1,2}$ = 74.1
C-2	39.3	d, $J_{2,3}$ = 37.6	113.7	d, $J_{2,1}$ = 74.1
C-3	80.9	d, $J_{3,2}$ = 37.6	156.8	d, $J_{3,6}$ = 42.1
C-4	44.3	d, $J_{4,6}$ = 35.6	108.0	d, $J_{4,5}$ = 70.5
C-5	37.8	d, $J_{5,1}$ = 36.0	150.7	d, $J_{5,4}$ = 70.4
C-6	33.5	d, $J_{6,4}$ = 35.7	34.3	d, $J_{6,3}$ = 42.1
C-7	30.5	d, $J_{7,8}$ = 34.5	85.5	d, $J_{7,8}$ = 102.1
C-8	23.1	d, $J_{8,7}$ = 34.5	209.7	d, $J_{8,7}$ = 102.1
C-9	14.0	s	76.3	s

4. ELEMENTAL ANALYSIS RESULTS ON BLACK SOLID

In an attempt to classify the kind of polymer formed from the crude extract and also to verify if it results from only one compound, elemental analysis was done on the insoluble carbonaceous substance (2.5 mg) after the crude extract was taken to complete dryness. The black solid gave the following percentage compositions of atoms, %N=0.51; %C=63.58; %H=4.98; %O=32.86. The calculated empirical formula of $C_3H_2ON_{0.02}$ is approximated to C_3H_2O. Unfortunately this formula is not consistent with the molecular formula of any individual compound isolated. However this result may show that the polymerization is not as a result of any one particular compound but might be due to all three compounds isolated. In addition, there are several compounds which were not isolated because of the low yield but were evident by TLC of the crude extract. It is well known that polyacetylenes are easily polymerized.[5d]

5. CONCLUSIONS

The major goal of this project was to identify the constituents of the graphite-like particles observed by Sigler on solid agar media of the growing fungus *S. radulodes*. This led to the isolation of three new highly unstable natural products from the liquid culture namely, sistodiolynne (**1**), sistolynone (**5**), and sistopyrone (**6**). In the absence of solvent, both the crude organic extract and the individual compounds readily polymerized into a black substance. The polymerization was minimized if the crude extract was kept in a small amount of organic solvent such as ethyl acetate or dichloromethane. The black substance formed was insoluble in most organic solvents.

Generally the polymers of acetylenes are black and insoluble,[34] and from our experience with these compounds it can be concluded that the graphite-like particles observed on solid agar media are the result of the compounds produced by the fungus, which undergo rapid polymerization, three of which we were able to isolate with some difficulty. They were obtained in their natural state, despite the instability, by silica gel preparative TLC, eluting the individual compounds from the silica gel with deuterated solvents for analysis. The yields of compounds obtained from this procedure were fairly low. In the case of sistodiolynne (**1**), reduction and benzoylation gave the more stable derivative **3** and **4**.

The elemental analysis results on the polymer gave an empirical formula C_3H_2O which does not agree with the formula any one particular compound isolated; thus, the mode of formation of this polymer is not known.

The structural elucidation of the compounds was mainly based on spectroscopic data.

The absolute stereochemistry for sistodiolynne (**1**), based on the CD results with the di-*p*-bromobenzoate derivative (**4**), indicate that C-1 and C-3 are in the *S* configuration. The absolute stereochemistry at the C-4 position for sistolynone (**5**), may be assigned as *S* on the basis of the biogenesis, although the CD spectrum was not recorded owing to the small quantity of sistolynone (**5**) obtained.

The ^{13}C-labelling observed for these compounds is unique, and two possible biogenetical pathways are proposed for sistodiolynne and sistolynone, one involving ring contraction from 6 to 5 membered ring, and the other by direct cyclization to 5 membered ring. The proposed pathway for sistopyrone is rationalized by ring expansion with oxygen insertion. To date, the enzymes responsible for the biogenesis of carbon carbon triple bonds have not been isolated and little is known about the process.[35]

The naturally occurring acetylenes and allenes have been isolated from fungi belonging to the Basidiomycetes,[5e] with a few exceptions. *S. raduloides* belongs to this class and confirms the chemotaxonomical trend. Some of the naturally occurring acetylenes and allenes are powerful antifungal agents. The activities of some were not determined because of their instability, which is the case with the compounds we have isolated from the liquid culture of *S. raduloides*.

6. EXPERIMENTAL

High resolution electron impact mass spectra (HREIMS) were recorded on a Kratos AEI MS-50 mass spectrometer. Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 7199 FTIR and Nicolet MX-1 FTIR. Nuclear magnetic resonance (NMR) spectra (^{1}H and ^{13}C) were obtained on Varian Unity-500 spectrometer and Bruker WH-300, WM-360, and WH-400 spectrometers with either Aspect 2000 or 3000 computer systems. Carbon-13 multiplicities were determined using spin echo *J*-modulated experiments APT (Attached proton test) and CAPT (Compensated attached proton test).[10] Methyl and methine groups are shown as signals possessing opposite phase with respect to the deuteriochloroform signal, whereas methylene, carbonyl, and fully substituted carbons appear in phase. Nuclear Overhauser Enhancement (NOE) experiments were determined in the difference mode in which a control (unsaturated) spectrum was computer-subtracted from the irradiated spectrum after Fourier transformation. Positive enhancements appear as signals possessing opposite phase with respect to the irradiated signal. The following 2D experiments, Correlated Spectroscopy (COSY), Heteronuclear Multiple Quantum Coherence (HMQC)[20], and Heteronuclear Multiple Bond Coherence (HMBC)[11] were obtained on a Varian unity-500 spectrometer. Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE)[25] was performed using a WH-300 spectrometer. All NMR spectra were recorded in $CDCl_3$, CD_2Cl_2, or $(CD_3)_2CO$ solution. ^{1}H chemical shifts and coupling constants are reported as if they are first order. $CDCl_3$, CD_2Cl_2, and $(CD_3)_2CO$ chemical shifts were used as reference for ^{1}H and ^{13}C chemical shifts having tetramethylsilane as an internal standard. Ultraviolet (UV) spectrum was obtained on a Hewlett Packard 8450A diode array

spectrophotometer and optical rotation in a Perkin Elmer 241 polarimeter. Circular dichroism (CD) spectrum was measured using Jasco SS-20-2 spectropolarimeter. Elemental analysis was performed using CARLO ERBA EA-1180 CHNS-O analyzer. The analyzer analyzes carbon, hydrogen, nitrogen, and sulphur simultaneously and oxygen separately. Preparative thin layer chromatography (PTLC) was performed on E. Merck precoated 20 x 20 glass plates of silica gel 60 F-254. Analytical thin layer chromatography (TLC) was carried out on cut pieces (sections) of E. Merck precoated aluminium sheets of silica gel 60 F-254. A UV lamp (254 nm) was used to identify UV active materials. Liquid media were prepared using Sabourand Dextrose Broth (SDB). Labelling experiments were performed using [1-^{13}C]-sodium acetate, [2-^{13}C]-sodium acetate, and[1,2-^{13}C]-sodium acetate purchased from ICON Isotopes, ICON Services. All solvents were distilled prior to use.

6.1. Growth of the fungus

Solid medium and storage of the fungus

Sistotrema raduloides strain UAMH 7326 was isolated from ex indoor air in archives in San Juan, Puerto Rico by Bolanos, B. The strain was obtained from UAMH on agar plates of Sabourand Dextrose Agar (20 g/L), and was recultured and stored as a stock culture on the same medium in slant tubes stored at 5 °C.

Liquid medium and culturing of the fungus

Sistotrema raduloides was cultured in 5 or 10 L in Sabourand Dextrose Broth (15 g in 1 L of double distilled water) with final pH of 5.7. The solution was sterilized in an autoclave, and inoculated with 10 ml portions of blended fungus from a prepared slant tube in sterilized double distilled water (100 ml). The cultures were grown as still cultures at room temperature for 5 weeks.

6.2. Isolation of the metabolites

The culture broth obtained after 5 weeks was separated from the mycelium by filtration and the volume of the culture filtrate (5 L) was reduced under vacuum to about 400 ml. The concentrated broth was first extracted with methylene chloride and then with ethyl acetate. The organic layers obtained were dried separately with anhydrous sodium sulphate, filtered and concentrated to small volume under reduced pressure. Polymerization occured if the solvent was completely removed. The methylene chloride extract after preparative TLC gave compounds **1**, **5**, and **6**. The ethyl acetate extract gave mainly compound **1**.

Sistodiolynne (1)

Sistodiolynne, (-)1*R*,3*S*-4-butadiyne-4-cyclopentene-1,3-diol, was isolated as an unstable white oil, **TLC**: R_f 0.18 (dichloromethane:methanol, 19:1); **UV** (MeOH) 310, 252 nm;

FTIR: ($CDCl_3$, cast) 3544 (O–H), 3287 (≡C–H), 2230 (C≡C) cm^{-1}; **HREIMS**: (probe 100°C) *m z* cacld. for $C_9H_8O_2$ (M^+): 148.0524; found: 148.0509 (11%), 147 ($C_9H_7O_2$, 25%), 130 (C_9H_6O,10%), 119, (C_8H_7O, 49%), 102 (C_8H_6, 62%), 76 (C_6H_4, 89%) 74 (C_6H_2, 53%);**^{1}H NMR** [$(CD_3)_2CO$, 400 MHz]δ: 6.30 (1H, dd, *J* = 2.0, 1.0 Hz), 4.70 (1H, d, *J* = 6.5 Hz), 4.62 (1H, bdd), 4.55 (1H, m), 4.48 (1H, d, *J* = 6.5 Hz), 3.30 (1H, s), 2.74 (1H, ddd, *J* = 13.5, 7.0, 7.0, Hz), 1.50 (1H, ddd, *J* = 13.5, 6.2, 6.2, Hz); [**$(CD_3)_2CO/D_2O$**, 300 MHz]δ: 6.30 (1H, dd, *J* = 2.0, 1.0 Hz), 4.60 (1H, m), 4.50 (1H, dd, *J* = 7.0, 6.0 Hz), 3.85 (**HOD**, bs), 3.30 (1H, s), 2.74 (1H, ddd, *J* = 13.5, 7.0, 7.0, Hz), 1.50 (1H, ddd, *J* = 13.5, 6.0, 6.0, Hz); **^{13}C NMR** [$(CD_3)_2CO$, 100 MHz]δ: 146.7 (C-5, d), 129.8 (C-4, s), 76.9 (C-6, s), 76.1 (C-1, d), 74.1 (C-9, d), 73.4 (C-3, d), 72.0 (C-7, s), 68.4 (C-8, s), 44.7 (C-2, t); **HETCOR**. [$(CD_3)_2CO$; 100, 400 MHz]: 146.7,6.30; 76.1, 4.62; 74.1, 3.30, 73.4, 4.55; 44.7, 2.74 & 1.50: **HMBC:** (125, 500 MHz): δ_H 6.30 ⇔ δ_C 76.9, 76.1, 73.4, 72.0, 44.7; δ_H 2.74 ⇔ δ_C 76.1, 73.4;δ_H 1.50 ⇔ δ_C 146.7, 129.8; δ_H 3.30 ⇔ δ_C 76.9, 68.4.

Sistodiolynne diacetate (2)

Pyridine (1.0 ml) and acetic anhydride (0.2 ml) were added to a solution of sistodiolynne (unknown amount) in methylene chloride (2.0 ml). After stirring for 12 hours at room temperature, the solution was concentrated under vacuum. The diacetate (**2**) was isolated as an unstable yellow oil from the resulting residue by preparative TLC in 5% MeOH in CH_2Cl_2 and was eluted with deuterated chloroform; **TLC**: R_f 0.72; **FTIR**: ($CDCl_3$ cast) 3251 (≡C–H), 2924, 2851 (C–H), 1737 (–COO–), 1230 (–C–O–) cm^{-1}; **HREIMS**: (probe 100 °C) *m z* cacld. for $C_{13}H_{12}O_4$ (M^+): 232.0736; found: 232.0730

(5%), 172 ($C_{11}H_8O_2$, 11%), 130 (C_9H_6O, 100%), 102 (C_8H_6, 34%); **^{1}H NMR** ($CDCl_3$, 300 MHz)δ: 6.44 (1H, bd, *J* = 2.2 Hz), 5.60 (2H, m), 2.95 (1H, ddd, *J* = 14.6, 6.7, 6.7, Hz), 2.50 (1H, s), 2.08 (3H, s), 2.02 (3H, s)1.75(1H, ddd, *J* = 14.6, 4.2, 4.2 Hz).

Reduction and *p*-bromobenzoylation of sistodiolynne

Reduction and *p*-bromobenzoylation of sistodiolynne (**1**) to 1*S*,3*S*-di-*p*-bromobenzoyloxy-4*R*(*S*)-butyl-cyclopentane (**3** and **4**).

Pd/C (10%) catalyst (100 mg) was added to solution of **1** in ethyl acetate (5 ml) and shaken under hydrogen at 40 psi for 18 hours. The mixture was filtered and concentrated under vacuum to give an oil (14 mg). The mixture was benzoylated by adding *p*-BrC_6H_4COCl (40 mg) and pyridine (2 ml) with stirring at room temperature for 24 hours, and the resulting solution was evaporated under vacuum to dryness. Preparative TLC of the resulting oil with 5% ethyl acetate in hexane gave compounds **3** (5.0 mg) and **4** (4.4 mg).

di-*p*-bromobenzoate derivative of 1 (4)

Compound **4**, (-) 1*S*,3*S*-di-*p*-bromobenzoyloxy-4*R*-butyl-cyclopentane, was recrystallized from hexane to give fine white needles, **TLC**: R_f 0.27(hexane:ethyl acetate, 9:1) **mp** (110 - 111°C); $[\alpha]_D$ -14° (c 0.004, hexane) **CD**: $\Delta\varepsilon_{241}$ -3.6[14] (c 8.8×10^{-4},

hexane); **FTIR**: (CH_2Cl_2 cast) 2957, 2955, 2856 (C–H), 1717 (–COO–), 1590 (C=C) cm^{-1}; **HREIMS**: (probe 150°C) *m z* cacld. for $C_{23}H_{24}O_4{}^{79}Br^{81}Br$ (M^++2): 524.0021; found: 524.0001 (2%), 252 (M^+,$C_{23}H_{24}O_4{}^{79}Br_2$, 1%), 322 ($C_{16}H_{19}O_2{}^{79}Br$, 3%), 122 ($C_9H_{14}$, 100%), 80 ($C_6H_8$, 48%); **^{1}H NMR** ($CDCl_3$, 400 MHz)δ: 7.9 (2H, *J* = 9.0 Hz), 7.8 (2H, d, *J* = 9.0 Hz), 7.6 (2H, *J* = 9.0 Hz), 7.5 (2H, *J* = 9.0 Hz), 5.5 (1H, dddd, 10.0, 7.5, 5.0, 2.5 Hz), 5.4 (1H, bdd, 5.0, 4.1 Hz), 2.6 (1H, ddd, *J* = 13.7, 7.5, 7.0 Hz), 2.4 (1H, ddd, *J* = 15.5, 8.0, 5.0 Hz), 2.2 (1H, bd, *J* = 15.5 Hz), 2.0-2.1(1H, m), 1.8 (1H, ddd, *J* = 13.7, 10.0, 5.2 Hz), 1.6-1.3 (6H, m), 0.9 (3H, m); **^{1}H-^{1}H COSY** ($CDCl_3$,500 MHz):δ 5.5 (H-1α) ⇒ 2.6 (H-5α), 2.4 (H-2α), 2.2 (H-2β),1.8 (H-5β); 5.4 (H-3α) ⇒ 2.4 (H-2α), 2.2 (H-2β), 2.1 (H-4α); 2.6 (H-5α) ⇒ 5.5 (H-1α), 2.1 (H-4α), 1.8 (H-5β); 2.4 (H-2α) ⇒ 5.5 (H-1α), 5.4 (H-3α), 2.2 (H-2β); 2.2 (H-2β) ⇒ 5.5 (H-1α), 5.4 (H-3α), 2.4 (H-2α), 1.8 (H-5β); 2.1 (H-4α) ⇒ 5.4 (H-3α), 2.6 (H-5α), 1.8 (H-5β), 1.6-1.3 (H-6); 1.8 (H-5β) ⇒ 5.5 (H-1α), 2.6 (H-5α), 2.1 (H-4α); 1.6 (H-6a) ⇒ 2.1 (H-4α), 1.3 (H-6b), 1.5-1.3 (H-6,7); **differential nOe:** H-4α to H-3α 15%, H-2α 10%, H-5α 7%, H-6a 7%; H-5α to H-5β 34%, H-4α 14%, H-1α 12%; **^{13}C NMR** ($CDCl_3$, 100 MHz)δ: 165.5 (1C, s),165.4 (1C, s), 131.8 (2C, d), 131.6 (2C, d), 131.1 (2C, d), 131.0 (2C, d), 129.5 (1C, s), 129.3 (1C, s),128.1 (2C, s), 76.8 (C-3, d), 75.0 (C-1, d), 43.6 (C-4, d), 39.8 (C-2, t), 37.7 (C-5, t), 30.4 (C-7, t), 29.1 (C-6, t), 22.8 (C-8, t), 14.0 (C-9, q); **HMQC** (125, 500 MHz)δ: 76.8⇔5.4; 75.0⇔5.5; 43.6⇔2.1; 39.8⇔2.4, 2.2; 37.7⇔2.6, 1.8; 29.1⇔1.5; 22.8⇔1.3; 14.0⇔0.9.

di-*p*-bromobenzoate derivative of 1 (3)

Compound **3**, (-)1*S*,3*S*-di-*p*-bromobenzoyloxy-4*S*-butyl-cyclopentane, isolated as a

yellowish sticky solid which could not be crystallized. **TLC**: R_f 0.31(hexane:ethyl acetate, 9:1); **FTIR**: (CH_2Cl_2 cast) same as **4**; **HREIMS**: same as **4**; **^{1}H NMR** ($CDCl_3$, 400 MHz)δ: 7.9 (4H, m), 7.5 (4H, m), 5.4 (1H, m), 5.1 (1H, ddd, *J* = 7.2, 4.4, 3.8 Hz), 2.6 (1H, ddd, *J* = 15.5, 7.5, 7.2 Hz), 2.5 (1H, m), 2.3 (1H, dddd, *J* = 14.0, 7.6, 2.1, 2.1 Hz), 2.1 (1H, bd, *J*=15.5 Hz), 1.7 (1H, ddd, *J* = 14.0, 9.3, 5.8 Hz), 1.6-1.3 (6H, m), 0.9 (3H, t, *J* = 6.7 Hz); **^{13}C NMR** ($CDCl_3$, 100 MHz)δ:165.6 (2C, s), 131.7 (4C, d), 131.1 (4C, d), 129.3 (2C, s), 128.1 (2C, s), 80.5 (C-3, d), 75.6 (C-1, d), 44.0 (C-4, d), 38.9 (C-2, t), 37.5 (C-5, t), 33.1 (C-6, t), 30.1 (C-7, t), 22.8 (C-8, t), 14.0 (C-9, q); **HMQC** (125, 500 MHz)δ: 80.5⇔5.1; 75.6⇔5.4; 44.0⇔2.5; 38.9⇔ 2.6, 2.1; 37.5⇔2.3, 1.7; 33.1⇔1.6, 1.3; 30.1⇔1.3; 22.8⇔1.3; 14.0⇔0.9.

Sistolynone (5)

Sistolynone, 3-butadiyne-4-hydroxyl-2-cyclopenten-1-one, was isolated as an unstable oil. **TLC**: R_f 0.33 (dichloromethane:methanol, 19:1); **FTIR**: ($CDCl_3$ cast) 3387 (O–H), 3284 (≡C–H), 2204 (C≡C), 1711, 1683 (αβ-unsat. –CO–), 1583 (C=C) cm^{-1}; **HREIMS**: (probe 100°C) *m/z* cacld. for $C_9H_6O_2$ (M^+): 146.0368; found: 146.0363 (51%), 118 (C_8H_6O, 31%), 74 (C_6H_2, 100%); **^{1}H NMR** ($CDCl_3$, 400 MHz)δ: 6.4 (1H, d, *J* = 1.0 Hz), 5.0 (1H, dddd, *J* = 6.5, 5.0, 2.5, 1.0 Hz), 2.9 (1H, s), 2.8 (1H, dd, *J* = 18.6, 6.5 Hz), 2.4 (1H, dd, *J* = 18.6, 2.5 Hz), 2.3 (OH, d, *J* = 5.0 Hz); **^{13}C NMR** ($CDCl_3$, 100 MHz)δ: 204.4(C-1, s), 154.7 (C-3, s), 139.3 (C-2, d), 89.9 (C-7, s), 78.3 (C-9, d), 72.1 (C-4, d), 68.2 (C-6, s), 66.8 (C-8, s), 44.0 (C-5, t).

Sistopyrone (6)

[4-(2-dien-1-ye)-pyran-2-one] was obtained in deuterated chloroform as an oil after preparative **TLC** R_f 0.73 (dichloromethane:methanol, 19:1); **FTiR**: ($CDCl_3$, cast) 1955 (C=C=C), 1725 (αβδγ unsat.–COO–), 1651, 1637 (C=C), 853 (=CH_2) cm^{-1}; **HREIMS**: (probe 100 °C) *m/z* calcd. for $C_9H_8O_2$: 148.0524 (M^+); found: 148.0522 (8%), 120 (C_8H_8O, 75%), 91 (C_7H_7, 100%), 65 (C_5H_5, 23%), 39 (C_3H_3, 34%); **^{1}H NMR** ($CDCl_3$, 400 MHz)δ: 7.41 (1H, dd, *J* = 5.2, 1.0 Hz), 6.18 (1H, ddd, *J* = 2.0, 1.0, 1.0 Hz), 6.12 (1H, dd, *J* = 5.2, 2.0 Hz), 5.15 (1H, tt, *J* = 7.0, 6.7 Hz), 4.79 (2H, dt, *J* = 6.7, 2.9 Hz), 3.12 (2H, dtd, *J* = 7.0, 2.9, 1.0 Hz); **Homonuclear decoupling** (irradiation ⇒ effect): δ 6.18 ⇒7.41 (dd to d, *J* = 5.2 Hz), 6.12 (dd to d, *J* = 5.2 Hz), 3.12 (dtd to dt, *J* = 7.0, 2.9 Hz); δ 4.79 ⇒5.15 (tt to t, *J* = 7.0 Hz), 3.12 (dtd to dd, *J* = 7.0, 1.0 Hz); δ 3.12 ⇒6.18 (ddd to dd, *J* = 2.0, 1.0 Hz), 5.15 (tt to t, *J* = 6.7 Hz), 4.79 (dt to d, *J* = 6.7 Hz); **^{13}C NMR** ($CDCl_3$, 125 MHz)δ: 209.7 (C-9, s), 162.2 (C-2, s), 156.8 (C-4, s), 150.7 (C-6, d), 113.7(C-3, d), 108.0 (C-5, d), 85.5 (C-8, d), 76.3 (C-10, t), 34.3 (C-7, t). **HMQC** (125, 500 MHz)δ: 113.7⇔6.18; 108.0⇔6.12; 76.3⇔4.79; 34.3⇔3.12.

6.3. Labelling experiments

Administration of ^{13}C-labelled sodium acetate

Labelled ^{13}C-sodium acetate (0.5 g, 0.2 mol) was dissolved in redistilled water (30 ml) and sterilized prior to use. The sterilized solution (5 ml) was injected into the culture

of *S. raduloides* (1 L) starting at day 3 after inoculation and every other day for 10 days. The culture was then allowed to grow for 5 weeks and harvested. The workup procedure was the same as described previously.

[1,2-$^{13}C_2$]-acetate labelled metabolites

[1,2-$^{13}C_2$]-di-*p*-bromobenzoate derivative (**3**), **^{13}C NMR** (CD_2Cl_2, 100 MHz) spin-coupled carbons: δ 80.9 (C-3, d, $J_{3,2}$ = 37.6 Hz), 76.1 (C-1, d, $J_{1,5}$ = 36.0 Hz), 44.3 (C-4, d, $J_{4,6}$ = 35.6 Hz), 39.3 (C-2, d, $J_{2,3}$ = 37.6 Hz), 37.8 (C-5, d, $J_{5,1}$ = 36.0 Hz), 33.5 (C-6, d, $J_{6,4}$ = 35.7 Hz), 30.5 (C-7, d, $J_{7,8}$ = 34.5 Hz), 23.1 (C-8, d, $J_{8,7}$ = 34.5 Hz); singlet carbon: 14.0 (C-9); **INADEQUATE** (C_2DCl_2, 75 MHz) cross peaks (two double quantum); δ: 80.9 (C-3) ⇔ 39.3 (C-2), 76.1 (C-1) ⇔ 37.8 (C-5), 44.3 (C-4) ⇔ 33.5 (C-6), 30.5 (C-7) ⇔ 23.1 (C-8).

[1,2-$^{13}C_2$]-sistopyrone (**6**), **^{13}C NMR** ($CDCl_3$, 75 MHz) spin-coupled carbons: δ 209.7 (C-8, d, $J_{8,7}$ = 102.1 Hz), 162.2 (C-1, d, $J_{1,2}$ = 74.1 Hz), 156.8 (C-3, d, $J_{3,6}$ = 42.1 Hz), 150.7 (C-5, d, $J_{5,4}$ = 70.4 Hz), 113.7 (C-2, d, $J_{2,1}$ = 74.1 Hz), 108.0 (C-4, d, $J_{4,5}$ = 70.5 Hz), 85.5 (C-7, d, $J_{7,8}$ = 102.1 Hz), 34.3 (C-6, d, $J_{6,3}$ = 42.1 Hz); singlet carbon : 76.3 (C-9).

[1-^{13}C] acetate labelled metabolites

[1-^{13}C]-di-*p*-bromobenzoate derivative (**3**), **^{13}C NMR** ($CDCl_3$, 100 MHz) enhanced

signals: δ 80.5 (C-3), 76.7 (C-1), 44.0 (C-4), 30.1 (C-7), 14.0 (C-9); natural abundance signals: δ 38.9 (C-2), 37.5 (C-5), 33.1 (C-6), 22.8 (C-8).

[1-^{13}C]-sistopyrone (**6**), **^{13}C NMR** ($CDCl_3$, 100 MHz) enhanced signals: δ 162.2 (C-1), 156.8 (C-3), 108.8 (C-4), 85.5 (C-7), 76.3 (C-9); natural abundance signals: δ 209.7 (C-8), 150.7 (C-5), 113.7 (C-2), 34.3 (C-6).

[2-^{13}C] acetate labelled metabolites

[2-^{13}C]-di-*p*-bromobenzoate derivative (**3**), **^{13}C NMR** ($CDCl_3$, 100 MHz) enhanced signals: δ 38.9 (C-2), 37.5 (C-5), 33.1 (C-6), 22.8 (C-8); natural abundance signals: δ 80.5 (C-3), 76.7 (C-1), 44.0 (C-4), 30.1 (C-7), 14.0 (C-9).

[2-^{13}C]-sitopyrone (**6**), **^{13}C NMR** ($CDCl_3$, 100 MHz) enhanced signals: δ 209.7 (C-8), 150.7 (C-5), 113.7 (C-2), 34.3 (C-6); natural abundance signals: δ 162.2 (C-1), 156.8 (C-3), 108.8 (C-4), 85.5 (C-7), 76.3 (C-9).

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