New Bioactive Rosigenin Analogues and Aromatic Polyketide Metabolites from the Freshwater Aquatic Fungus *Massarina tunicata*

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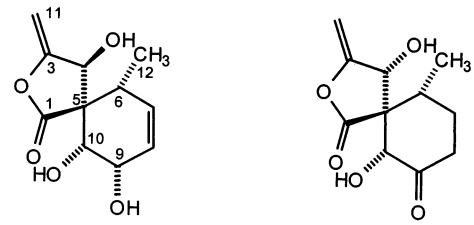
Four new rosigenin analogues (massarigenins A−D; **1**−**4**) and two new aromatic polyketide-derived secondary metabolites (massarinins A and B; **6**, **7**) and have been isolated from the freshwater aquatic fungus *Massarina tunicata*. The structures of these compounds were determined primarily by analysis of NMR data, and that of compound **1** was verified by X-ray crystallography. The known compound 4-(2-hydroxybutynoxy)benzoic acid (**11**) was also obtained, and its absolute stereochemistry was assigned. Several of these metabolites showed antibiotic activity against Gram-positive bacteria.

Freshwater aquatic fungi remain relatively unexplored as sources of biologically active natural products. Our prior chemical investigations of the freshwater aquatic fungus *Massarina tunicata* Shearer & Fallah (A-25-1; Lophiostomataceae) have resulted in the isolation of three new sequiterpenoids[1] and two polyketide-derived lactones with novel ring systems.[2] Studies of *M. tunicata* scale-up cultures have afforded several additional new compounds, including four new rosigenin analogues (massarigenins A−D; **1**−**4**) and two new aromatic polyketide metabolites (massarinins A and B; **6** and **7**). Details of the isolation and characterization of these metabolites are presented here.

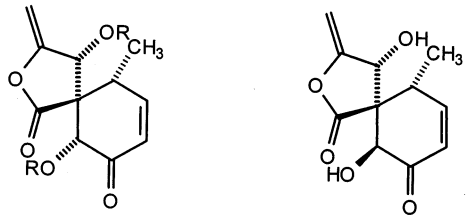
Results and Discussion

The molecular formula of massarigenin A (**1**) was established as $C_{11}H_{14}O_5$ on the basis of HRFABMS and ^{13}C NMR data. The ^1H, ^{13}C, and DEPT NMR spectra for **1** (Tables 1 and 2) revealed the presence of a $-CHCH_3$ moiety, an ester carbonyl, three hydroxy groups, three sp^3 oxymethine units, a non-oxygenated quaternary carbon, a 1,2-disubstituted olefin, and an oxygenated terminal olefin unit. The remaining two degrees of unsaturation required by the molecular formula indicated that **1** must be bicyclic. Analysis of the results from a series of $^1H-^1H$ homonuclear decoupling experiments permitted the establishment of two isolated spin-systems corresponding to the C3/C4/C11 and C6−C10/C12 portions of structure **1**.

HMBC results (Table 3) revealed connections of these subunits to the ester carbonyl and the quaternary carbon, and identified the location of one hydroxy group. HMBC correlations of H-9 and H_3-12 to the quaternary carbon C-5, together with correlations of H-6 and H-10 to C-1 and C-4, suggested the connection of C-1, C-4, C-6, and C-10 to C-5, although the observation of a (selective INEPT) correlation of H-9 with the carboxy carbon C-1 initially complicated the unambiguous positioning of this group. The chemical shift of C-4 (δ 68.5), along with HMBC correlations of the hydroxy proton signal at δ 4.02 to C-3 and C-5, was consistent with attachment of a hydroxy group at this position. Although the other hydroxy proton signals were too broad to show HMBC correlations, structure **1** appeared most likely based on chemical shift and structural considerations. Fortunately, suitable crystals were obtained, and its complete structure and relative stereochemistry were assigned as shown in **1** by single-crystal X-ray diffraction analysis. The four-bond heteronuclear correlation between H-9 and C-1 could be ascribed to a *W*-type conformational relationship between these nuclei, as evident in the final X-ray model (Figure 1). Compound **1** is closely related to the known fungal metabolite rosigenin (**8**)[3] and other previously reported compounds.[4−10] Although the relative stereochemistry of **1** was provided by the X-ray structure, NOESY data were acquired to assist in stereochemical comparisons with compounds **2**−**4** (see below). As might be expected for the 3D structure depicted in Figure 1, a strong correlation was observed between H-6 and H-10. Weak correlations were also observed between H_3-12 and both of the C-11 protons.

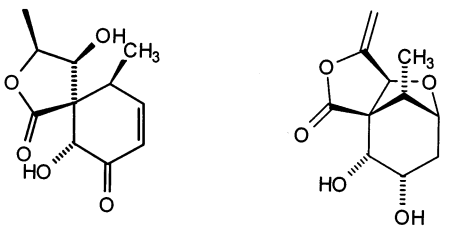


1 **2**



3 R = H
5 R = Ac

4



8 **9**

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Table 1. ^{1}H NMR Data [δ (mult.; J_{H-H} in Hz)] for Massarigenins A−D (**1**−**4**)

C#	**1**[a]	**2**[b]	**3**[a]	**4**[a]
4	5.51 (dt; 3.9, 3.0)	4.82 (br t; 2.1)	5.07 (br t; 2)	5.06 (br t; 2.4)
6	2.58 (m)	2.59 (m)	3.34 (m)	3.01 (m)
7	5.67 (dd; 9.9, 1.7)	1.90 (dq; 4.7, 13) 2.03 (m)	6.74 (dd; 10, 2.1)	6.98 (dd; 10, 5.1)
8	5.77 (ddd; 9.9, 5.1, 3.2)	2.54 (m) 2.66 (ddd; 16, 4.5, 2.1)	6.03 (dd; 10, 3.3)	6.00 (dd; 10, 0.9)
9	4.29 (m)			
10	4.11 (dd; 3.3, 3.6)	4.28 (d; 1.2)	4.47 (s)	4.70 (s)
11	4.67 (dd; 2.5, 2.4) 4.51 (t; 2.3)	4.86 (dd; 2.8, 2.7) 4.63 (dd; 2.8, 2.1)	4.75 (t; 2.4) 4.55 (dd; 2.4, 1.8)	4.62 (dd; 2.7, 2.4) 4.55 (dd; 2.4, 2.1)
12	1.21 (d; 7.5)	0.98 (d; 6.8)	1.16 (d; 7.5)	1.38 (d; 7.2)
4-OH	4.02 (d; 3.9)[c]	not observed	not observed	not observed
9-OH	4.72 (d; 5.8)[c]			
10-OH	4.86 (br d; 3.3)[c]	not observed	not observed	not observed

[a] Acetone-d_6; 300 MHz. [b] $CDCl_3$; 300 MHz. [c] These OH signals and their coupling effects were variable in appearance and were sometimes not observed.

Table 2. ^{13}C NMR Data for Massarigenins A−D (**1**−**4**)

position	**1**[a]	**2**[b]	**3**[a]	**4**[a]
1	δ 176.2	δ 173.3	δ 174.4	δ 174.2
3	159.1	158.2	159.8	160.0
4	68.5	69.2	69.0	70.5
5	55.8	58.6	61.1	57.1
6	39.6	34.4	37.1	37.1
7	135.2	28.9	151.4	153.3
8	125.9	37.6	127.8	125.6
9	66.7	207.2	196.1	197.4
10	70.1	77.8	77.2	73.1
11	86.6	88.8	88.7	86.5
12	16.3	15.7	15.7	15.5

[a] Acetone-d_6. [b] $CDCl_3$.

Table 3. HMBC and Selective INEPT Data for Massarigenins A−C (**1**−**3**)

	1[a]	**2**[b]	**3**[c]
^{1}H signal	HMBC correlations (C#)	selective INEPT correlations (C#)	selective INEPT correlations (C#)
4	3, 6, 10, 11	3, 5, 6, 10	3, 5, 6, 10, 11
6	1, 4, 5, 7, 8, 12	*d*	1, 4, 5, 7, 8, 10, 12
7	5, 6, 8, 9, 12	5, 6, 8, 9, 12	*d*
8	6, 7, 9, 10	6, 7, 9, 10	6, 10
9	1,[e] 5, 7, 8, 10	*d*	*d*
10	1, 4, 5, 6	1, 4, 5, 9	1, 4, 5, 6, 9
11	3, 4	3, 4	3, 4
12	5, 6, 7	5, 6, 7	5, 6, 7

[a] Data recorded at 600 MHz (^{1}H dimension) in acetone-d_6 solution. [b] Data recorded at 300 MHz in $CDCl_3$ solution. [c] Data recorded at 300 MHz in acetone-d_6 solution. [d] Experiments were not conducted for these proton signals. [e] This four-bond correlation was not present in the HMBC spectrum, but was observed in a selective INEPT experiment.

Compound **2** (massarigenin B) was determined to be an isomer of **1** on the basis of HRFABMS and ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectra of **2** (Tables 1 and 2) were very similar to those of **1**, except that the data for **2** contain signals for two mutually coupled methylene units and a ketone carbonyl in place of the signals for the 1,2-disubstituted olefin and one of the sp^3-hybridized oxymethines observed in the spectra of **1**. ^{1}H−^{1}H homonuclear decoupling experiments conducted in DMSO-d_6 solution confirmed the coupling of the two exchangeable hydroxy proton signals with H-4 and H-10, respectively, and placed the hydroxy groups at these positions. Analysis of selective INEPT data (Table 3) allowed unambiguous assignment of the gross structure as shown in **2**. It was initially anticipated that the stereochemistry would be analogous to that of **1**. Indeed, a NOESY correlation of H-6 with H-10 required a cis-1,3-diaxial-type orientation of these protons,

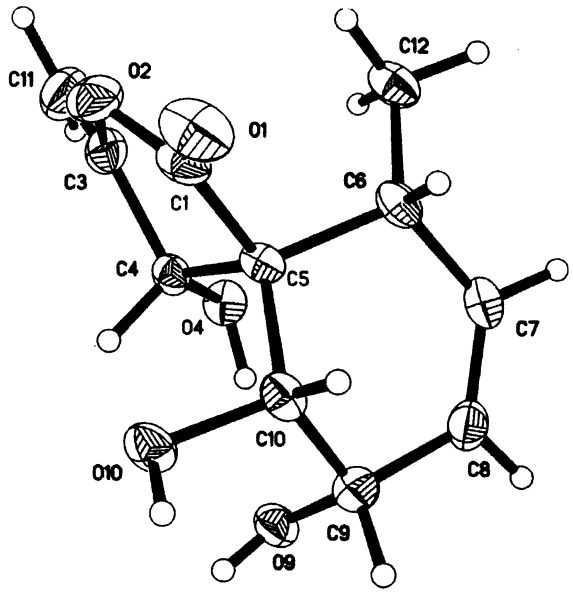


Figure 1. Final X-ray model of massarigenin A (**1**). The ellipsoid probability level is 35%.

as was the case in **1**, and was consistent with observation of a large *J*-value (13 Hz) between H-7ax and H-6. However, NOESY correlations of H-4 with H_3-12 and with H-7_{ax} in **2** revealed that these protons are all spatially close. Given the relative configurations at C-6 and C-10, the latter correlations would not both be expected if the stereochemistry at C-4 in **2** were the same as in **1**. In fact, they would only be likely for a system with the relative stereochemistry at C-4 and C-5 shown in **2**.

The molecular formula of massarigenin C (**3**) was found to be $C_{11}H_{12}O_5$ on the basis of HRFABMS and NMR data. The NMR spectra for **3** (Tables 1 and 2) were again similar to those of **1** except for the presence of a ketone carbonyl signal and the absence of signals for one of the oxygenated methine units (δ 4.29/66.7) observed in the spectra for **1**. The chemical shifts of the ketone and 1,2-disubstituted olefin carbon signals in the ^{13}C NMR spectrum of **3** (δ 196.1, 151.4, 127.8) suggested the presence of an α,β-unsaturated ketone moiety. The locations of two hydroxy groups in **3** were verified by ^{1}H NMR analysis of diacetate **5** formed upon treatment of **3** with acetic anhydride, as the signals for both H-4 and H-10 were shifted downfield by approximately 1.5 ppm in the diacetate. A series of selective INEPT experiments (Table 3) led to confirmation of the gross structure as shown in **3**.

The possible spatial relationships among substituents of the more conformationally restricted cyclohexenone ring in **3** are significantly different from those of the corresponding rings in **1** and **2**. NOESY correlations of H-4 with H_3-12 and of H-6 with H-10 were again noted. Although the latter correlation was much weaker than in **1**, these data suggested that **3** possesses the same relative stereochemistry as in **2**. This assignment was supported by NOESY data for diacetate **5**, which showed the same two key correlations, including a more robust H6/H10 interaction.

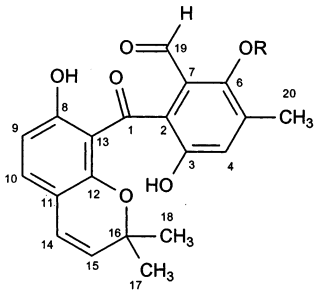
A compound very similar to **3** has been previously reported and assigned a diastereomeric structure differing only in the relative configuration at C-4.[10,11] The physical and spectral properties described were similar to those of **3**, though there were some limited differences in the NMR, mp, $[\alpha]_D$, and UV data. NOE data reported for this metabolite included an H-6/H-10 interaction, but, as would be expected from the proposed structural difference, an H-4/H_3-12 correlation was not noted for the literature compound.

HRFABMS and NMR data revealed that massarigenin D (**4**) is an isomer of **3**. The NMR data for **4** (Tables 1 and 2) were very similar to those of **3** except for chemical shift differences associated mostly with the protons of the cyclohexenone unit. These data suggested a diastereomeric relationship between **3** and **4**. In the case of **4**, the H_3-12-to-H-4 NOESY correlation was still present, but the H-6/H-10 and H-4/H-10 correlations were completely absent. Instead, additional correlations were observed between H-4 and H-6 and between H-10 and H_3-12. This result led to the conclusion that the stereochemistry at C-10 is inverted in **4** relative to **1**−**3**.

The unusual 2-oxaspiro[4,5]decane skeleton found in **1**−**4** has been reported for fungal secondary metabolites from *Mycosphaerella rosigena*,[3] *Arthropsis truncata*,[4,5] *Coniothyrium sporulosum*,[6] a mutant strain of *Rhodotorula glutinis*,[7,8] and a *Microsphaeropsis* sp. known primarily for production of the macrosphelides.[10] A number of fungal metabolites containing the similar, nitrogen-containing 2-azaspiro[4,5]decane skeleton have been isolated from *Drechslera tritici-repentis*[9] and from both wild-type and mutant strains of *Staphylotrichum coccosporum* (spirostaphylotrichins).[12−15] The numbering system employed for **1**−**4** is based on that originally reported for **8**−**9** and for the spirostaphylotrichins.

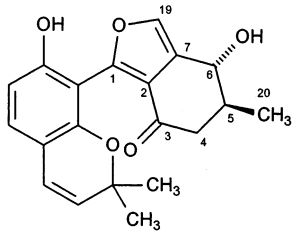
Massarigenins A−D (**1**−**4**) also bear close resemblance to massarilactone A (**9**),[2] a compound we previously reported from *M. tunicata* that contains an additional ether bridge linking O-4 to C-7. The relative stereochemistry of **9** is analogous to that of **1**. The absolute stereochemistry depicted in **9** was determined by X-ray crystallographic analysis of its *p*-bromobenzoate ester. Although the absolute configurations of **1**−**4** were not directly determined, the stereostructures shown for **1**−**4** seem most likely by analogy. However, in related compounds bearing a ketone functionality at C-4 (presumed precursors of **1**−**4**),[4] it has been proposed that epimerization can occur at positions 5 and/or 10 via a retroaldol process involving opening and re-closing of the six-membered ring at the C5−C10 bond.[4,9] Thus, the absolute stereochemistry of the compounds isolated in this instance (particularly **2**−**4**) remains uncertain.

The biosynthetic origin of this class of compounds has not been clearly demonstrated. On the basis of biogenetic pathways proposed for rosigenin,[3] and for metabolites containing the oxaspiro[4,5]decane skeleton or the azaspiro[4,5]decane skeleton,[5,9,14] compounds **1**−**4** may be derived from a pentaketide precursor, or a tetraketide precursor and a malic acid unit.



6 R = CH_3

10 R = $CH_2CH{=}C(CH_3)_2$



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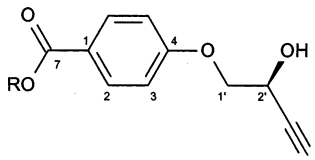

11 R = H

12 R = CH_3

The molecular formula of massarinin A (**6**) was assigned as $C_{21}H_{20}O_6$ (12 unsaturations) on the basis of HREIMS and ^{13}C NMR data. The ^1H and ^{13}C NMR data (Table 4) contained signals for pentasubstituted and 1,2,3,4-tetrasubstituted benzene rings, a 1,2-disubstituted olefin, an aryl methyl group, two coincident or identical methyl groups linked to an oxygenated quaternary carbon, a methoxy group, a ketone carbon, an aldehyde moiety, and two phenolic OH groups with shifts (δ 9.71 and 12.77) suggesting differing levels of intramolecular hydrogen bonding. Analysis of HMQC and HMBC data for **6** (Table 4) provided evidence for a 2,2-dimethylchromene unit substituted with the hydrogen-bonded phenolic OH group at δ 12.77. A search of the literature led to the hypothesis that this compound is closely related to arugosin E (**10**), a polyketide metabolite from *Aspergillus silvaticus* originally identified by X-ray crystallography.[16] Comparison of the chemical shift data with those of **10** supported this conclusion, but because other possible substitution patterns for the pentasubstituted aromatic ring could be envisioned, analysis of the 2D NMR data was necessary to verify the structure and to unambiguously locate the hydroxy and methoxy groups.

The methoxy group was located on the basis of an HMBC correlation of H_3-21 with C-6. HMBC correlations of H_3-20

Table 4. NMR Data for Massarinins A (**6**) and B (**7**)

	6[a]			**7**[b]		
C#	δ_H (mult., *J*)[c]	δ_C	HMBC (C#)[d]	δ_H (mult., *J*)[c]	δ_C	sel. INEPT (C#)[c]
1		200.6			152.0	
2		128.9			119.6	
3 (OH)	9.71 (s)	149.0	2, 3		198.8	
4	7.04 (s)	124.3	1, 2, 3, 6, 20	2.70 (dd; 17, 3.6) 2.40 (dd; 17, 10)	45.1	2, 3, 5, 6, 20
5		132.6		2.29 (m)	39.1	3, 6, 7, 20
6		154.1		4.59 (br d; 7.5)	69.1	2, 4, 5, 7, 19, 20
7		126.8			128.8	
8 (OH)	12.77 (s)	162.6	8, 9, 13	7.96 (s)	156.8	8, 9, 13
9	6.44 (d; 8.1)	108.8	8, 11, 13	6.62 (d; 8.1)	112.5	
10	7.18 (d; 8.1)	133.5	8, 12, 14	6.98 (d; 8.4)	129.4	
11		112.5			115.7	
12		154.3			153.0	
13		111.2			109.5	
14	6.24 (d; 9.9)	121.4	10, 11, 12, 16	6.26 (d; 9.6)	121.9	
15	5.42 (d; 9.9)	126.8	11, 16	5.48 (d; 9.9)	128.3	
16		76.8			77.0	
17	0.86 (s)	26.7	15, 16	1.37 (s)	27.7	
18	0.86 (s)	26.7	15, 16	1.37 (s)	27.8	
19	10.15 (s)	190.0	2, 7	7.5 (d; 1.2)	138.5	1, 2, 7
20	2.28(s)	15.1	4, 5, 6	1.15 (d; 6.3)	17.2	
21	3.80 (s)	63.4	6		–	

[a] DMSO-d_6. [b] $CDCl_3$. [c] 300 MHz. [d] 600 MHz (^{1}H dimension).

with C-4, C-5, and C-6 placed the aryl methyl group ortho to both the isolated aromatic proton and the methoxy group (i.e., at C-5). A correlation of the aldehyde proton H-19 with C-7 appeared as a pair of satellite peaks reflecting a $^1H-^{13}C$ coupling constant of 23 Hz, which is consistent with the expected value for the two-bond $^1H-^{13}C$ coupling in an aldehyde group.[17] This observation allowed direct connection of the aldehyde carbonyl C-19 to C-7. The remaining phenolic proton (δ 9.71) showed correlations to C-2 and C-3, placing this OH group at C-3, and ortho to C-2. Correlations of H-19 and H-4 with C-2 further supported arrangement of the substituents as shown in **6**. The ketone carbon (C-1) was connected to C-2 and to C-13 to complete the structure assignment for **6** based on a weak four-bond HMBC correlation between H-4 and C-1, together with the requirement that OH-8 be involved in a strong intramolecular hydrogen bond.

NMR data and HREIMS analysis of massarinin B (**7**) indicated that it has the molecular formula $C_{20}H_{20}O_5$. The ^{1}H and ^{13}C NMR spectra of **7** (Table 4) again contained signals for a hydroxy-substituted 2,2-dimethylchromene unit. In this instance, the 8-OH proton appeared at δ 7.96, suggesting the absence of an ortho ketone group as is present in **6**, although a ketone resonance (δ 198.8) was observed. In addition, only four aromatic/olefinic ^{13}C NMR resonances were unaccounted for by the 2,2-dimethylchromene unit, indicating the absence of a second benzene ring in **7**. Interestingly, the ^{1}H NMR shifts of the methyl groups of the 2,2-dimethylchromene unit are significantly downfield-shifted compared to those in **6**. This observation is consistent with the absence of a benzene ring anisotropic shielding effect in **7** that is present in **6**. $^1H-^1H$ decoupling and selective INEPT (Table 4) data revealed the presence of a spin-system corresponding to the C4−C6/C20 portion of **7**. The ketone carbon (C-3) was linked to C-4 on the basis of selective INEPT correlations with H-5 and H_2-4. One of the remaining double bonds (C-7/C-19) was linked to oxymethine C-6 on the basis of correlations of H-5 with C-7 and of H-6 with C-19. Another sp^2 carbon (δ 119.6; C-2) was linked to the ketone carbon on the basis of correlations observed with H_2-4. The only remaining carbon signal (C-1; δ 152.0) must be paired in a double bond with C-2 and was connected to C-19 via oxygen on the basis of a correlation with H-19, together with its chemical shift. C-7 and C-2 were also connected by virtue of selective INEPT correlations of each with both H-5 and H-19. These connections completed a second subunit consisting of a furan ring fused to a cyclohexanone unit. On the basis of chemical shift considerations, the remaining OH group (indicated by the formula and DEPT data) must be located at C-6, and C-1 and C-13 must be connected to complete the gross structure of massarinin B (**7**).

The ^{1}H NMR *J*-values for H_{ax}-4 and H_{eq}-4 with H-5 (10.2 and 3.6 Hz, respectively) suggested that H_{ax}-4 and H-5 adopt a near trans-diaxial relationship, placing the H_3-20 methyl group in an equatorial (or pseudoequatorial) position with respect to the cyclohexanone ring. A strong NOESY correlation between H-6 and H_{ax}-4 indicated that these groups are spatially close, suggesting that these groups have a cis-1,3-diaxial relationship. On the basis of these results, the relative stereochemistry of massarinin B was proposed as shown in **7**.

Massarinins A and B (**6**−**7**) share a skeleton that is most likely derived from the polyketide pathway. The structure of massarinin A (**6**) is analogous to that of arugosin E (**10**),[16] and the numbering system shown for **6** and **7** was chosen to be consistent with the numbering system for arugosins A−C.[18] It has been suggested that the arugosins are derived from cleavage of an anthraquinone/anthrone precursor,[19,20] and biosynthetic studies of similar compounds support this hypothesis.[21,22] Although several representatives of this class have been reported,[16,18−20] arugosin E (**10**) is the only previously described member that possesses a 2,2-dimethylchromene unit as found in **6**−**7**.[16] The isobenzofuran skeleton in massarinin B (**7**) could be envisioned as forming via adjustment of oxidation state and cyclization reactions of the aldehyde (C-19) and ketone (C-1) groups present in **6**. Isobenzofuran (and tetrahydroisobenzofuranone) units are precedented among natural products,[23] but are not common.

The known compound **11** [4-(2-hydroxybutynoxy)benzoic acid] was also isolated from *M. tunicata* in this investigation. This compound has been previously reported only as a metabolite of an unidentified soil fungus.[24] The structure was independently confirmed by NMR, MS, and IR analysis, although it afforded distinctive results in some of the

NMR experiments (e.g., initially misleading DEPT data) by virtue of the large (52 Hz) heteronuclear ^2J-value observed between H-4′ and C-3′. To determine the absolute configuration at C-2′of **11**, the *R*- and *R*/*S*-phenylbutyrate ester derivatives of its corresponding methyl ester were prepared. Treatment of **11** with trimethylsilyldiazomethane in CH_3OH yielded methyl ester **12**. Separate reactions of this product with *R*- and *R*/*S*-phenylbutyric acid in the presence of 1,3-dicyclohexylcarbodiimide and 4-*N*,*N*-dimethylaminopyridine produced the desired derivatives, as confirmed by HRFABMS data and ^{1}H NMR results, including a downfield shift of H-2′ from δ 4.77 to 5.75. It was possible to assign the relevant proton signals of the *S*-2-phenylbutyrate ester by comparison of the ^{1}H NMR data for the product mixture with the data for the independently prepared *R*-ester. A significant downfield shift of the H-4′ resonance and upfield shifts of the H_2-1′, H-2, and H-3 signals for the *S*-derivative relative to those of the *R*-derivative were observed. On the basis of Helmchen's rules,[25] the *S*-configuration was assigned to C-9.

In standard disk assays, compounds **1**, **3**, **4**, **6**, **7**, and **11** were active against *Bacillus subtilis* (ATCC 6051), causing zones of inhibition of 11, 9, 14, 17, 23, and 15 mm, respectively, at 200 μg/disk. Compound **2** was not active in this assay. Compounds **6** and **7** also showed activity against *Staphylococcus aureus* (ATCC 29213), affording zones of inhibition of 7 and 12 mm, respectively, at the same concentration. None of the compounds showed significant activity against *Candida albicans* (ATCC 14053), *Aspergillus flavus* (NRRL 6541), or *Fusarium verticillioides* (NRRL 25457) at the same level.

Experimental Section

General Experimental Procedures. NMR spectra were recorded using $CDCl_3$, DMSO-d_6, or acetone-d_6 solutions, and chemical shifts were referenced relative to the corresponding residual solvents signals (δ_H 7.24/δ_C 77.0, δ_H 2.49/δ_C 39.5, and δ_H 2.04/δ_C 29.8, respectively). Carbon multiplicities were established by DEPT experiments. Selective INEPT NMR experiments were carried out at 75 MHz on a Bruker AC-300 spectrometer. HMQC and HMBC experiments were optimized for $^nJ_{CH}$ = 135 and 8 Hz, respectively, and conducted using a Bruker AMX-600 spectrometer. FABMS, HRFABMS, and HREIMS data were obtained on a VG ZAB-HF mass spectrometer, and EIMS data were obtained on a VG TRIO 1 quadrupole instrument at 70 eV. Reagents for chemical reactions were purchased from Aldrich Chemical Co. Other general procedures and instrumentation employed have been described previously.[26]

Isolation, Cultivation, and Extraction of *M. tunicata*. The strain of *Massarina tunicata* used in this study (culture number A25-1 = ATCC 201760) was isolated from a decorticated submerged twig collected from the Lemonweir River, Adams County, WI, on July 31, 1992, by C.A.S. This fungus is classified in the Lophiostomataceae, Pleosporales, Loculoascomycetes. The cultivation procedure was analogous to that reported earlier,[1] except that it was carried out on a larger scale. Twenty flasks, each containing 400 mL of potato dextrose broth (Difco) which had been sterilized at 120 °C for 15 min and then cooled to room temperature, were individually inoculated with 1 cm^2 agar plugs taken from stock cultures of *M. tunicata*. Flask cultures were inoculated at 25−28 °C and aerated by agitation on an orbital shaker at 150 rpm for period of 30 days. Extraction of the filtered broth with EtOAc (5 × 1 L) provided an organic phase which was dried with $MgSO_4$ and then concentrated using a rotary evaporator to yield 4.0 g of crude extract.

Isolation of Metabolites 1−4, 6, 7, and 11. The EtOAc extract (4.0 g) was subjected to silica gel VLC using a stepwise gradient elution of CH_3OH in CH_2Cl_2, collecting 200−400 mL fractions. The material was eluted with 1 L of CH_2Cl_2, followed by 1.5 L of 1% CH_3OH in CH_2Cl_2, 500 mL each of 2%−7% CH_3OH in CH_2Cl_2, 300 mL each of 10% CH_3OH in CH_2Cl_2, 20% CH_3OH in CH_2Cl_2, and 30% CH_3OH in CH_2Cl_2, and 500 mL of 50% CH_3OH in CH_2Cl_2. Fractions of similar composition were pooled on the basis of TLC analysis (9:1 CH_2Cl_2−CH_3OH). The fraction that eluted at 2% CH_3OH (320 mg) contained **1** and **7** and was subjected to Sephadex LH-20 column chromatography with a step-gradient elution sequence of hexane−CH_2Cl_2 (1:4), CH_2Cl_2−acetone (4:1), and CH_2Cl_2−acetone (2:3). Massarigenin A (**1**) eluted in the CH_2Cl_2−acetone (2:3) subfraction, and white crystals of **1** were obtained upon slow evaporation of the solvent. The fraction containing **7** was eluted at CH_2Cl_2−hexane (4:1), and this fraction was purified by semipreparative reversed-phase HPLC using a gradient from 50 to 100% CH_3CN in H_2O over 30 min (Alltech 100 HS BDS C_{18} column; 1.0 × 25 cm; 8 μm particle size; 2 mL/min; UV detection at 215 nm) to yield **7** (9.6 mg).

The fraction that eluted with 1% CH_3OH in CH_2Cl_2 from silica gel VLC (540 mg) contained **2**, **3**, **4**, and **6** and was further separated by Sephadex LH-20 column chromatography with the same solvent step-gradient elution sequence used in isolating **1**. A subfraction containing massarigenins B and C (**2** and **3**; 71 mg) eluted with CH_2Cl_2−acetone (4:1) and was further separated by reversed-phase HPLC as above using a gradient from 40 to 53% CH_3CN in H_2O over 10 min to yield **2** (11 mg) and **3** (8.8 mg). An adjoining subfraction containing **6** was also eluted at CH_2Cl_2−acetone (4:1), and this fraction was subjected to reversed-phase HPLC as above using a gradient program of 40−47% CH_3CN in H_2O over 10 min, 47−55% CH_3CN in H_2O over 5 min, and 55−62% CH_3CN in H_2O over 10 min to yield **6** (3.8 mg). A fraction containing massarigenin D (**4**; 34 mg) was eluted with hexane−CH_2Cl_2 (1:4) and was similarly subjected to reversed-phase HPLC using a gradient from 35 to 65% CH_3CN in H_2O over 35 min to yield **4** (11 mg).

The material that eluted from the silica gel VLC column with 4% CH_3OH in CH_2Cl_2 (335 mg) was subjected to Sephadex LH-20 column chromatography as described above. Slow evaporation of a subfraction eluted with 4:1 CH_2Cl_2−acetone afforded 40 mg of 4-(2-hydroxybutynoxy)benzoic acid (**11**) as a white powder.

Massarigenin A (1): white crystals; mp 169−171 °C; $[\alpha]_D$ −5.6° (*c* 0.26 g/dL; 24 °C; CH_3OH); UV (CH_3OH) 210 (ϵ 2000); IR 3590, 2928, 1806 (ester group), 1682 (vinyl ether group), 1605, 1275, 1174, 1103, 1027 cm^{-1}; EIMS (70 eV) *m*/*z* 208 [(M − H_2O)$^+$, rel int 9], 193 (36), 175 (15), 152 (25), 140 (32), 125 (29), 105 (29), 91 (32), 84 (100), 77 (34), 55 (66), 43 (56); ^{1}H NMR data, Table 1; ^{13}C NMR data, Table 2; HMBC data, Table 3; NOESY data (acetone-d_6, H-# ↔ H-#) H-6 ↔ H-10; H_2-11 ↔ H_3-12; HRFABMS (NaI/3-NBA matrix) obsd *m*/*z* 249.0735, calcd for $C_{11}H_{14}O_5$+Na, 249.0739.

Massarigenin B (2): white solid; mp 78−79 °C; $[\alpha]_D$ −9.3° (*c* 0.35 g/dL; 24 °C; CH_3OH); HPLC t_R 8.8 min; UV (CH_3OH) 212 (ϵ 2700); IR 3509, 2970, 1802, 1727, 1678, 1190, 1153, 1111, 1039 cm^{-1}; EIMS (70 eV) *m*/*z* 226 (M$^+$, rel int 69), 208 (7), 190 (5), 156 (49), 137 (48), 127 (92), 111 (61), 97 (51), 81 (75), 69 (38), 55 (99), 43 (100); ^{1}H NMR data, Table 1; ^{13}C NMR data, Table 2; selective INEPT data, Table 3; NOESY data ($CDCl_3$, H-# ↔ H-#) H-4 ↔ H-7ax; H-4 ↔ H_3-12; H-6 ↔ H-10; H-6 ↔ H-8ax; HRFABMS (LiI/3-NBA matrix) obsd *m*/*z* 233.0987, calcd for $C_{11}H_{14}O_5$+Li, 233.1001.

Massarigenin C (3): white crystals; mp 81−83 °C; $[\alpha]_D$ −123° (*c* 0.34 g/dL; 24 °C; CH_3OH); HPLC t_R 8.0 min; UV (CH_3OH) 238 (ϵ 2900); IR 3538, 2977, 1798, 1698, 1677, 1381, 1179, 1147, 1090, 1042 cm^{-1}; EIMS (70 eV) *m*/*z* 224 (M$^+$, rel int 2), 141 (7), 123 (5), 107 (4), 95 (4), 82 (100), 67 (4), 54 (14), 43 (15); ^{1}H NMR data, Table 1; ^{13}C NMR data, Table 2; selective INEPT data, Table 3; NOESY data (acetone-d_6, H-# ↔ H-#) H-4 ↔ H_3-12; H-6 ↔ H-10; HRFABMS (NaI/3-NBA matrix) obsd *m*/*z* 247.0575, calcd for $C_{11}H_{12}O_5$+Na, 247.0582.

Acetylation of Massarigenin C (3). A solution of **3** (1.1 mg), 4-*N*,*N*-dimethylaminopyridine (0.5 mg), and acetic anhydride (0.5 mL) in acetone (2 mL) was stirred for 20 h at room temperature. The solvent was then evaporated under N_2 flow.

The residue was redissolved in 1.5 mL of EtOAc and extracted with H_2O (2 × 2 mL). The organic phase was dried ($MgSO_4$) and evaporated to yield diacetate **5** (1.3 mg, 86% yield): ^1H NMR data ($CDCl_3$, 300 MHz) δ 1.27 (d; J_{H-H} = 7.2 Hz; H_3-12), 1.99 (s, $COCH_3$-4*), 2.15 (s, $COCH_3$-10*), 3.45 (m, H-6), 4.53 (dd; 3.3, 2.0; H-11a), 4.93 (dd; 3.3, 2.4; H-11b), 5.72 (s, H-10), 6.00 (dd; 10.2, 3.2; H-8), 6.20 (t; 2.1; H-4), 6.60 (dd; 10.2, 2.1; H-7); NOESY data (acetone-d_6, H-# ↔ H-#) H-4 ↔ H_3-12; H-6 ↔ H-10; H-6 ↔ H-7; H-7 ↔ H_3-12; *these assignments are interchangeable.

Massarigenin D (4): white solid; mp 117−121 °C; $[\alpha]_D$ −96° (*c* 0.54 g/dL; 23 °C; CH_3OH); HPLC t_R 9.0 min (under the conditions above); UV (CH_3OH) 244 (ϵ 3100); IR 3482, 2976, 1803, 1695, 1681, 1373, 1168, 1136, 1101, 1051 cm^{-1}; EIMS (70 eV) *m/z* 224 (M^+, rel int 0.9), 141 (13), 123 (7), 107 (3), 95 (3), 82 (100), 67 (4), 54 (16), 43 (20); ^1H NMR data, Table 1; ^{13}C NMR data, Table 2; NOESY data (acetone-d_6, H-# ↔ H-#) H-4 ↔ H_3-12; H-4 ↔ H-6; H-10 ↔ H_3-12; HRFABMS (NaI/3-NBA matrix) obsd *m/z* 247.0570, calcd for $C_{11}H_{12}O_5$+Na, 247.0582.

X-ray Crystallographic Analysis of Massarigenin A (1).[27] A crystal of **1** (0.45 × 0.25 × 0.09 mm) was orthorhombic (space group $P2_12_12_1$) with cell dimensions a = 8.251(1), b = 16.147(2), c = 8.229(1) Å. Data were collected on an Enraf-Nonius CAD4 diffractometer (Mo Kα radiation; graphite monochromator) at 203 K (N_2 cold gas stream) using $\theta-2\theta$ scans. The structure was solved using a MULTAN direct methods program and refined using full-matrix least-squares with the XL computer program from the SHELXTL v5.0 package. Computer programs from the MoLEN package were used for data reduction. The 2872 measurements yielded 1879 independent reflections (201 parameters) after equivalent data were averaged and Lorentz and polarization corrections were applied. The final refinement gave R_1 = 0.0318, wR_2 = 0.0680.

Massarinin A (6): pale yellow oil; HPLC t_R 27.4 min (BDS column conditions described above); UV (CH_3OH) 222 (ϵ 14000), 274 (ϵ 12000), 355 (ϵ 3400); IR 3567, 3063, 2978, 2931, 1685, 1652, 1616, 1474, 1244, 1206, 1119 cm^{-1}; EIMS (70 eV) *m/z* 368 (M^+, rel int 25), 353 (49), 339 (43), 319 (18), 193 (15), 187 (57), 161 (100), 77 (19); ^1H, ^{13}C, and HMBC data (DMSO-d_6), Table 4; ^1H NMR data ($CDCl_3$, 300 MHz) δ 7.00 (s, H-4), 7.01 (d; J_{H-H} = 6.9, H-10), 6.49 (d; J_{H-H} = 6.9, H-9), 6.11 (d; J_{H-H} = 8.3, H-14), 5.26 (d; J_{H-H} = 8.3, H-15), 0.93 (s, H_3-17,-18), 10.22 (s, H-19), 2.33 (s, H_3-20), 3.82 (s, H_3-21), 12.41 (s, 8-OH); ^{13}C NMR data ($CDCl_3$, 75.5 MHz) δ 200.3 (C-1), 128.5 (C-2), 147.8 (C-3), 125.2 (C-4), 134.0 (C-5, 10), 155.5 (C-6), 127.8 (C-7), 163.5 (C-8), 109.6 (C-9), 112.7 (C-11), 154.5 (C-12), 111.7 (C-13), 121.8 (C-14), 126.4 (C-15), 77.3 (C-16), 27.3 (C-17, 18), 189.3 (C-19), 15.5 (C-20), 63.6 (C-21); FABMS (3-NBA matrix) $(M + H)^+$ *m/z* 369, $(M + Na)^+$ *m/z* 391; HREIMS obsd *m/z* 368.1256, calcd for $C_{21}H_{20}O_6$, 368.1260.

Massarinin B (7): yellow solid; mp 123−125 °C; $[\alpha]_D$ −204° (*c* 0.6 g/dL; 25 °C; CH_3OH); HPLC t_R 19.3 min (under the conditions above); UV (CH_3OH) 229 (ϵ 8500), 291 (ϵ 5200); IR 3601, 3575, 3948, 2978, 2930, 1656, 1605, 1537, 1480, 1125 cm^{-1}; EIMS (70 eV) *m/z* 340 (M^+, rel int 18), 325 (100), 307 (26), 279 (6), 251 (6), 187 (14), 161 (10), 127 (12), 115 (16), 105 (14); ^1H, ^{13}C, and selective INEPT NMR data, Table 4; HREIMS obsd *m/z* 340.1314, calcd for $C_{20}H_{20}O_5$, 340.1311.

4-(2-Hydroxybutynoxy)benzoic acid (11): white powder; mp 126−127 °C; $[\alpha]_D$ +80° (*c* 0.4 g/dL; 24 °C; CH_3OH); UV (CH_3OH) 213 (ϵ 2900), 253 (ϵ 6800); IR 3306, 3024, 3012, 2125, 1710, 1607, 1425, 1363, 1255, 1170 cm^{-1}; EIMS (70 eV) *m/z* 206 (M^+, rel int 23), 151 (38), 138 (34), 121 (100), 105 (20), 93 (17), 65 (75); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.99 (distorted d, *J* = 7.5 Hz, H-2/6), 7.06 (distorted d, 7.5, H-3/5), 4.74 (ddd, 6.7, 4.7, 2.2, H-2′), 4.20 (dd, 9.7, 4.7, H-1′), 4.16 (dd, 9.7, 6.7, H-1′), 2.96 (d, 2.2, H-4′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.3 (C-7), 163.5 (C-4), 132.6 (2C, C-2/6), 124.1 (C-1), 115.3 (2C, C-3/5), 83.5 (C-3′), 74.8 (C-4′), 72.8 (C-1′), 61.3 (C-2′); HMBC (600 MHz) H-2/6 → C-6/2, 4, 7; H-3/5 → C-1, 4, 5/3; H_2-1′ → C-2′, 3′, 4; H-2′ → C-1′, 3′, 4′; H-4′ → C-1′, 2′, 3′; FABMS (3-NBA matrix) $(M + H)^+$ *m/z* 207; HREIMS obsd *m/z* 206.0583, calcd for $C_{11}H_{10}O_4$, 206.0579.

Methyl 4-(2-hydroxybutynoxy)benzoate (12). To a solution of 2.8 mg (14 μmol) of **11** in 1 mL of CH_3OH was added a 2 M solution of trimethylsilyldiazomethane ($TMSCHN_2$) in hexane (140 μL) until the solution stayed yellow. After stirring for 3 h, the solution was concentrated under N_2 flow to give methyl ester **12** (2.8 mg, 93%): ^1H NMR ($CDCl_3$, 300 MHz) δ 7.99 (distorted d, J_{H-H} = 8.5 Hz, H-2/6), 6.94 (distorted d, 8.5, H-3/5), 4.77 (m, H-2′), 4.19 (dd, 9.6, 3.8, H-1′), 4.12 (dd, 9.6, 6.9, H-1′), 3.87 (s, OCH_3), 2.53 (d, 2.4, H-4′); HREIMS obsd *m/z* 220.0734, calcd for $C_{12}H_{12}O_4$, 220.0736.

***R*-2-Phenylbutyrate Ester of 12.** To a solution of 1,3-dicyclohexylcarbodiimide (5.4 mg, 26 μmol) in THF (3 mL) were added *R*-2-phenylbutyric acid (4 μL, 26 μmol), compound **12** (1.8 mg, 8.1 μmol), and a catalytic amount of 4-*N*,*N*-dimethylaminopyridine (0.5 mg). After the mixture was stirred for 48 h, TLC analysis confirmed the disappearance of starting material and the solvent was evaporated under N_2 flow. The residue was then redissolved in 1.5 mL of Et_2O, and the resulting solution was extracted sequentially with 2% CH_3COOH (2 mL), 3% $NaHCO_3$ (2 mL), and H_2O (2 × 2 mL). The organic phase was dried ($MgSO_4$) and evaporated to give a crude reaction product. This material was subjected to semipreparative reversed-phase HPLC (Beckman Ultrasphere C_8 column; 1.0 × 25 cm; 5 μm particle size; 2 mL/min; UV detection at 215 nm) using a gradient elution from 80 to 100% CH_3OH in H_2O over 30 min to yield 2.4 mg of the *R*-2-phenylbutyrate ester of **12**: HPLC t_R 14.0 min (under the conditions above); ^1H NMR data ($CDCl_3$, 300 MHz) δ (mult, J_{H-H} in Hz, H-#) 7.97 (distorted d, 8.5, H-2/6), 7.21−7.29 (ov. m, Ar-H_5), 6.87 (distorted d, 8.5, H-3/5), 5.75 (ddd, 6.6, 5.0, 2.3, H-2′), 4.23 (ov. m, H_2-1′), 3.87 (s, OCH_3), 3.49 (dd, 8.3, 7.5, H-2″), 2.44 (d, 2.3, H-4′), 2.08 (m, H_a-3″), 1.79 (m, H_b-3″), 0.88 (dd, 7.5, 7.5, H_3-4″); HRFABMS (thioglycerol matrix) obsd *m/z* 367.1527, calcd for $C_{22}H_{22}O_5$+H, 367.1545.

***R/S*-2-Phenylbutyrate Esters of 12.** (*R*/*S*)-2-Phenylbutyric acid (2.5 mg, 15 μmol), compound **12** (1.2 mg, 5.5 μmol), and catalytic amount of 4-*N*,*N*-dimethylaminopyridine (0.5 mg) were added to a solution of 1,3-dicyclohexylcarbodiimide (3.1 mg, 15 μmol) in THF (3 mL). The reaction mixture was stirred for 7 days and was then worked up as described above to yield a mixture of *R*- and *S*-derivatives. Chemical shift assignments for the *S*-phenylbutyrate ester were made by comparison of the ^1H NMR data for the mixture with those of the independently prepared *R*-derivative (see above): ^1H NMR data (*S*-phenylbutyrate ester; $CDCl_3$, 300 MHz) δ (mult, J_{H-H} = Hz, H-#) 7.91 (distorted d, 8.5, H-2/6), 6.73 (distorted d, 8.5, H-3/5), 5.75 (m, H-2′), 4.14 (m, H_2-1′), 3.86 (s, OCH_3), 2.51 (d, 2.4, H-4′).

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Supporting Information Available: ^1H and ^{13}C NMR spectra for massarigenins A−D (**1**−**4**) and massarinins A and B (**6**, **7**), key selective INEPT data for massarinin B (**7**), and tables of X-ray data for massarigenin A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(27) Crystallographic data for compound **1** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 193811). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223−336033 or e-mail: deposit@ccdc.cam.ac.uk).

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