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Lasiojasmonates A–C, three jasmonic acid esters produced by *Lasiodiplodia* sp., a grapevine pathogen

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ABSTRACT

In this study, a strain (BL 101) of a species of *Lasiodiplodia*, not yet formally described, which was isolated from declining grapevine plants showing wedge-shaped cankers, was investigated for its ability to produce *in vitro* bioactive secondary metabolites. From culture filtrates of this strain three jasmonic acid esters, named lasiojasmonates A–C and 16-*O*-acetylbotryosphaerilactones A and C were isolated together with (1*R*,2*R*)-jasmonic acid, its methyl ester, botryosphaerilactone A, (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone and (3*R*,4*S*)-botryodiplodin. The structures of lasiojasmonates A–C were established by spectroscopic methods as (1*R**,2*R**,3′*S**,4′*R**,5′*R**)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone, (1*R**,2*R**,3′*S**,4′*R**,5′*R**,10′*R**,12′*R**,13′*R**,14′*S**) and (1*R**,2*R**,3′*S**,4′*R**,5′*R**,10′*S**,12′*R**,13′*R**,14′*S**)-4-(4-hydroxymethyl-3,5-dimethyltetrahydro-furan-2-yloxymethyl)-3,5-dimethyldihydro-2-furanones jasmonates (**1**, **4** and **5**). The structures of 16-*O*-acetylbotryosphaerilactones A and C were determined by comparison of their spectral data with those of the corresponding acetyl derivatives obtained by acetylation of botryosphaerilactone A. The metabolites isolated, except **4** and **5**, were tested at 1 mg/mL on leaves of grapevine cv. Cannonau and cork oak using the leaf puncture assay. They were also tested on detached grapevine leaves at 0.5 mg/mL and tomato cuttings at 0.1 mg/mL. In all phytotoxic assays only jasmonic acid was found to be active. All metabolites were inactive in the zootoxic assay at 50 μg/mL.

1. Introduction

The Botryosphaeriaceae represent a well-known family of plant pathogenic fungi associated with fruit rot, leaf spots, dieback, cankers and root rot of Angiosperms and Gymnosperms worldwide (Phillips et al., 2013). Over the past decades, several species of the Botryosphaeriaceae have been recognized as important pathogens of grapevine in the many growing areas (Larignon et al., 2001; Phillips, 2002; van Niekerk et al., 2006; Pitt et al., 2010; Úrbez-Torres, 2011). In particular, to date, 21 different *taxa* of *Botryosphaeriaceae* have been reported as weak or aggressive pathogens on grapevine. Among these, four species belonging to the *Lasiodiplodia* genus namely *Lasiodiplodia crassispora* T.I. Burgess & Barber, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Lasiodiplodia missouriana* Úrbez-Torres, Peduto & Gubler and *Lasiodiplodia viticola* Úrbez-Torres, Peduto & Gubler have been recognized to be highly virulent on grapevine (Úrbez-Torres, 2011). The main disease symptoms caused by *Lasiodiplodia* spp. on grapevine consist of sunken cankers associated with wedge-shaped lesions of the vascular tissues.

Results of a recent study conducted in several Sardinian vineyards and aimed at clarifying the aetiology of grapevine canker and dieback, have led to the identification and characterization of 9 different species of *Botryosphaeriaceae* from symptomatic grapevine tissues (Linaldeddu et al., unpublished data). Among these, a *Lasiodiplodia* species morphologically (shape and size of conidia) and phylogenetically (ITS and EF1-α sequence data) distinct from all known species was isolated. Its formal description will be addressed in a future publication currently in preparation.

At present, 18 species are recognized in *Lasiodiplodia* (Phillips et al., 2013) and recent studies, based on ITS and EF-1α sequence data, have led to the clarification of systematics of this genus and to the identification of cryptic species within the *L. theobromae* species complex (Alves et al., 2008; Abdollahzadeh et al., 2010; Begoude et al., 2010; Úrbez-Torres et al., 2012).

The nature of symptoms (vascular necrosis) caused by this new species of *Lasiodiplodia* on grapevine suggests that phytotoxic

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metabolites may be involved in the host-pathogen interaction. On the other hand, it is well-known that *L. theobromae*, which is considered the type species of the *Lasiodiplodia* genus, biosynthesizes a variety of lipophilic and hydrophilic metabolites that exhibit interesting biological activities (Aldridge et al., 1971; Husain et al., 1993; Nakamori et al., 1994; Matsuura et al., 1998; Yang et al., 2000; He et al., 2004; Miranda et al., 2008; Kitaoka et al., 2009; Abdou et al., 2010; Pandi et al., 2010; da Cunha et al., 2012).

Therefore, the main aims of this work were: (1) to isolate and identify the main secondary metabolites produced *in vitro* by a selected strain (BL 101) of this new *Lasiodiplodia* species; (2) to evaluate their biological activities such as phytotoxicity and zootoxicity.

2. Result and discussion

The fungal culture filtrates were exhaustively extracted with EtOAc at pH 2, and the corresponding organic extract was purified by combined column and TLC chromatography as detailed reported in the experimental section, yielding 10 metabolites (**1**–**10**, Fig. 1). The preliminary ^{1}H NMR investigation showed that the metabolites belong to different classes of natural compounds. The structures of the known compounds were confirmed by physical and spectroscopic methods (OR, IR, UV, ^{1}H and ^{13}C NMR, ESI and/or APCI MS) and by comparison of the obtained data with those reported in the literature for (1*R*,2*R*)-jasmonic acid and its methyl ester (**6** and **7**) (Husain et al., 1993; Yukimune et al., 2000), botryosphaerilactone A (**8**) (Rukachaisirikul et al., 2009), (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone (**9**) (Ravi et al., 1979), and (3*R*,4*S*)-botryodiplodin (**10**) (Ramezani et al., 2007). The identification of jasmonic acid and its methyl ester was confirmed by comparison by their behavior TLC (silica gel eluent A *Rf* 0.43, and on reversed phase eluent C *Rf* 0.50; silica gel eluents A *Rf* 0.8 and B *Rf* 0.13, respectively) and ^{1}H NMR with those of commercial samples of (±)-jasmonic acid and its methyl ester. Some other new spectroscopic data as ESI and/or APCIMS spectra and in particular the ^{13}C NMR data for the trisubstituted dihydrofuranone **9** were detailed reported in the corresponding paragraphs of the experimental section. In addition, the identification of **8** was also supported by data from its COSY, HSQC and HMBC spectra. Finally, as (3*R*,4*S*)-botryodiplodin (**10**) was obtained as an inseparable anomeric mixture (α/β, 65:35), its structure was confirmed by acetylation carried out in the usual conditions. In fact, this reaction yielded only the 2,3-*trans*-botryodiplodin acetate, whose physic and spectroscopic data were very similar to those previously reported (Arsenault and Althaus, 1969).

Furthermore, the preliminary ^{1}H and ^{13}C NMR investigation of **1** showed its close correlation with both jasmonic acid and 3,4,5-trisubstituted dihydrofuranone **9** and, being a new compound as described below, it was named lasiojasmonate A. These suggestions were also confirmed by the band typical of ketone ester and olefinic groups (Nakanishi and Solomon, 1977) and the end absorption maximum (Scott, 1964) observed in the IR and UV spectra, respectively. It showed a molecular weight of 336 associated with the molecular formula of $C_{19}H_{28}O_5$ with six hydrogen deficiens as deduced from the HRESIMS spectrum. The ^{1}H NMR spectrum, also compared to that of jasmonic acid (Husain et al., 1993) and of standard commercial sample (Table 1), showed the signal patterns of the jasmonyl residue. For the 2-pentenyl moiety the multiplets of the protons (H-10 and H-9) of the *cis* double bond, the two allylic methylene groups (H_2-8 and H_2-11) and the triplet (J = 7.4 Hz) of the terminal methyl group (Me-12) were observed at δ 5.45, 5.26, 2.37, 2.04 and 0.95, respectively. For the 2,3-disubstituted cyclopentanone residue the multiplets of H-1 and H-2, the double doublet (J = 20.0 and 8.2 Hz) and the multiplet, and the multiplets of H_2-4 and H_2-5 were observed at δ 2.10, 1.90, 2.74 and 2.37, and 2.33 and 1.50

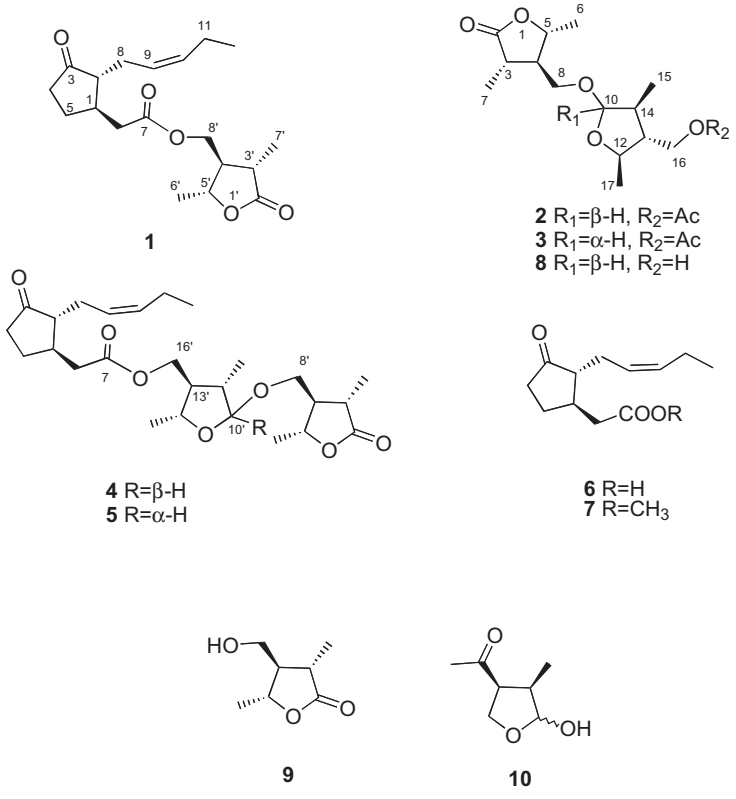

Fig. 1. Structures of lasiojasmonate, 16-*O*-acetylbotryosphaerilactone A and C, (−)-(1*R*,2*R*)-jasmonic acid and its methyl ester, botryosphaerilactone A, (3*R*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone and (−)-(3*R*,4*S*)-botryodiplodin (**1, 4, 5, 2, 3, 6**–**10**) produced by *Lasiodiplodia* sp.

Table 1
NMR data of lasiojasmonate A (**1**).[a,b]

Position	**1**		
	δC[c]	δH (*J* in Hz)	HMBC
1	37.6 *d*	2.10 (1H) *m*	H-2, H_2-5, H_2-6
2	53.9 *d*	1.90 (1H) *m*	H_2-4, H_2-8, H-9
3	218.3 *s*	–	H-1, H_2-5, H-8
4	38.7 *t*	2.74 (1H) dd, (20.0, 8.2) 2.37(1H) *m*	H_2-5
5	27.2 *t*	2.33 (1H) *m* 1.50 (1H) *m*	H-1, H_2-4
6	37.9 *t*	2.32(2H) m	
7	171.7 *s*	–	H_2-5, H_2-6, H_2-8′
8	25.5 *t*	2.37 (2H) *m*	H-9, H-10
9	124.9 *d*	5.26 (1H) *m*	H_2-8, H-11
10	134.2 *d*	5.45 (1H) *m*	H_2-8, H_2-11, Me-12
11	20.6 *t*	2.04 (2H) *m*	Me-12
12	14.1 *q*	0.95 (3H) *t* (7.4)	H_2-11
2′	177.6 *s*	–	H-3′, Me-7′, H_2-8′B
3′	38.6 *d*	2.49 (1H) *dq* (11.0, 7.1)	Me-7′
4′	50.5 *d*	2.04 (1H) *m*	H-3′, Me-6′, Me-7′
5′	77.0 *d*	4.26 (2H) *m*	Me-6′, H_2-8′
6′	19.9 *q*	1.47 (3H) *d* (6.1)	H-3′
7′	14.1 *q*	1.28 (3H) *d* (7.1)	H-3′
8′	62.5 *t*	4.26 (2H) *m* 4.21 (1H) *dd* (11.0, 5.7)	H-3′, H-5′

[a] The chemical shifts are in δ values (ppm) from TMS.
[b] 2D ^{1}H, ^{1}H (COSY) ^{13}C, ^{1}H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons.
[c] Multiplicities were assigned by DEPT spectrum.

respectively (Pretsch et al., 2000). As regard the signal system of the 4-hydroxy-3,5-dimethyl-3,4-dihydrofurane residue, which were also compared to those reported in literature (Ravi et al., 1979) (Table 1) the protons of hydroxymethylene H_2C-8′ showed, in particular, the typical AB system appearing as a mutiplet and double doublet (*J* = 11.0 and 5.7 Hz) at δ 4.26 and 4.21 significantly downfield shifted (Δδ 0.43 and 0.46), as a consequence of the esterification with the jasmonic acid. The other signals of the same moiety appeared as a double quartet (*J* = 11.0 and 7.1 Hz) for H-3′, two multiplets for H-4′ and H-5′ (the latter being overlapped with the signal of H-8′A) and two doublets (*J* = 6.1 and 7.1 Hz) for the two secondary methyl groups (Me-6′ and Me-7′) at δ 2.49, 2.04, 4.26, and 1.47 and 1.28, respectively (Pretsch et al., 2000). All these attributions were confirmed by the couplings observed in the COSY and HSQC spectra (Berger and Braun, 2004), with the latter also contributing to assigning the chemical shifts of protonated carbons in the ^{13}C NMR spectrum as reported in Table 1. The signals of the remaining quaternary carbons observed at δ 218.3, 177.6 and 171.7 typical of a ketone and two ester carbonyl groups (Breitmaier and Voelter, 1987) were assigned to C-3, C-2′ and C-7 with the last two attributed on the basis of the couplings observed in the HMBC spectrum (Table 1) (Berger and Braun, 2004) between C-2′ with H-3′, Me-7′ and H-8′B and C-7 with H_2-5, H_2-6 and H_2-8′. The coupling observed in the HMBC spectrum between C-7 with H_2C-8′ was very significant in supporting the jasmonate esterification of the 4-hydroxytmethyl-3,5-dimethyl-3,4-dihydrofuranone.

On the basis of these results lasiojasmonate A was formulated as 4-hydroxymethyl-3,5-dimethyldihydro-2-furanone jasmonate (**1**).

The structure of **1** was confirmed by all of the other couplings observed in the HMBC spectrum and by data from the HRESI and APCI MS spectra which showed, respectively, the sodium cluster $[M+Na]^+$ and the pseudomolecular ion $[M+H]^+$ at *m*/*z* 359.1913 and 337.

The relative stereochemistry of **1** as depicted in Fig. 1 was deduced by the comparison of the couplings constant measured in its ^{1}H NMR spectrum with those reported for (1*R*,2*R*)-jasmonic acid (Husain et al., 1993) and (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone. This stereochemistry was also confirmed by the correlations observed in the NOESY spectrum (Berger and Braun, 2004). In fact, this spectrum showed the correlations between the protons of the *cis* double bond H-10 and H-9 and that this latter with the protons of allylic methylene H_2-8, and that of H-3′ with H-5′. Thus, lasiojasmonate A can be formulated as (1*R**,2*R**,3′*S**,4′*R**,5′*R**)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone jasmonate.

2 and **3**, being two epimeric acetals, both showed a molecular weight of 314, as deduced from their ESIMS. They were obtained as an inseparable mixture (α/β, 45:55). All of the attempts carried out for their separation, using different chromatographic methods on direct and reverse phase and different solvent mixtures, failed. Their ^{1}H NMR spectrum (Table 2) showed the presence of two doublets (*J* = 2.3 and 4.6 Hz) typical of anomeric protons (H-10) for the β and α isomers at δ 4.67 and 4.79, respectively. The comparison of this spectrum with that of botryosphaerilactone **8** (Rukachaisirikul et al., 2009) indicates that **2** and **3** were the acetyl derivatives of botryosphaeriolactones A and C, or of their respective enantiomers. In fact, their ^{1}H NMR spectra (Table 2) differed from that of **8** and botryosphaerilactone C (Rukachaisirikul et al., 2009) essentially for the downfield shift of H_2-16 (Δδ 0.46 and 0.35; and 0.41 and 0.40, for β and α isomers respectively) resonating as two double doublets (*J* = 11.0, 5.6 and 11.0, 7.9 Hz) for β anomer (**2**) at δ 4.15 and 4.04, while for the α anomer (**3**) at δ 4.21 and 4.06 (*J* = 11.3, 4.5 and 11.3, 7.6 Hz). Furthermore, two singlets of the acetyl groups resonated at δ 2.06 and 2.07 for **2** and **3** respectively. The chemical shifts of all protons of two anomers were assigned (Table 2) by comparison of their ^{1}H NMR spectrum with that of **8**. The epimeric nature of **2** and **3** was also confirmed by the usual acetylation of botryosphaerilactone A with pyridine and acetic anhydride. As expected this reaction yielded both the β and α anomers the latter (**3**) being the main product of the reaction.

The structures assigned to **2** and **3** were also confirmed by data of both ESI and APCIMS spectra. The ESIMS spectrum showed dimeric sodiated form $[2M+Na]^+$and the sodium cluster $[M+Na]^+$ at *m*/*z* 651 and 314, respectively. The APCIMS spectrum showed ions originating from the pseudomolecular ion $[M+H]^+$ recorded at *m*/*z* 315 by fragmentation mechanisms typical of asymmetrical ethers (Pretsch et al., 2000) as reported in Fig. 2. In fact, the pseudomolecular ion by cleavage of the acetalic bond C(10)-O (mechanism a) originated the γ-lactonyl and the trisubstituted tetrahydrofurane ions recorded at *m*/*z* 145 and 172, respectively. This

Table 2
^{1}H NMR data of 16-*O*-acetylbotryosphaerilactones A and C (**2** and **3**).[a]

Position	**2** δH (*J* in Hz)	**3** δH (*J* in Hz)
2		
3	2.53 (1H) *dq* (9.2, 6.7)	2.57 (1H) *dq* (11.3, 7.1)
4	1.90 (1H) *m*	1.91 (1H) m
5	4.29 (1H) *dq* (8.4, 6.0)	4.32 (1H) *dq* (9.2, 6.2)
6	1.43 (3H) *d* (6.2)	1.45 (3H) *d* (6.2)
7	1.26 (3H) *d* (7.1)	1.26 (3H) *d* (7.1)
8	3.83 (1H) *dd* (10.3, 4.3) 3.41 (1H) *dd* (10.3, 4.8)	3.84 (1H) *dd* (10.2, 5.4) 3.41 (1H) *dd* (10.2, 4.8)
10	4.67 (1H) *d* (2.3)	4.79 (1H) *d* (4.6)
12	3.91 (1H) *dq* (12.3, 6.2)	3.97 (1H) *dq* (12.3, 6.2)
13	1.51 (1H) *m*	1.51 (1H) *m*
14	2.03 (1H) *m*	2.05 (1H) *m*
15	1.13 (3H) *d* (7.0)	1.04 (3H) *d* (7.0)
16	4.15 (1H) *dd* (11.0, 5.6) 4.04 (1H) *dd* (11.0, 7.9)	4.21 (1H) *dd* (11.3, 4.5) 4.06 (1H) *dd* (11.3, 7.6)
17	1.31 (3H) *d* (6.2)	1.30 (3H) *d* (6.2)
$COCH_3$	2.06 (3H) *s*	2.07 (3H) *s*

[a] The chemical shifts are in δ values (ppm) from TMS.

latter, by loss of AcOH, generated a fragment ion at *m*/*z* 113 (Fig. 2). Furthermore, the pseudomolecular ion by the alternative cleavage of the acetalic bond C(8)-O (fragmentation mechanism b) generated the γ-lactonyl fragment ion observed at *m*/*z* 127 (Fig. 2).

Compounds **4** and **5** showed a molecular weight of 464, as deduced from their HRESIMS spectrum recorded in positive mode associated with the molecular formula of $C_{26}H_{40}O_7$. They were obtained as an inseparable epimeric mixture (α/β, 30:70). As above reported for **2** and **3**, all attempts made using different chromatographic methods failed. By preliminary ^{1}H NMR investigation, they appeared to be closely related to both botryosphaerilactones A and C and jasmonic acid, suggesting for **4** and **5** a structure of jasmonate esters and therefore were named lasiojasmonates B and C. Their ^{1}H NMR spectrum (Table 3) showed the presence of two doublets (J = 2.0 and 4.5 Hz) typical of anomeric protons (H-10′) at δ 4.68 and 4.80 for the β and α isomers, respectively. Significant differences from the ^{1}H NMR spectra of botryosphaerilactones A and C were observed for the protons of the ABX systems HC-(13′)-H_2C-(16′). As expected the H_2-16′ protons were downfield shifted (Δδ 0.48, 0.41, and 0.43, 0.41 for **4** and **5**) with respect to the

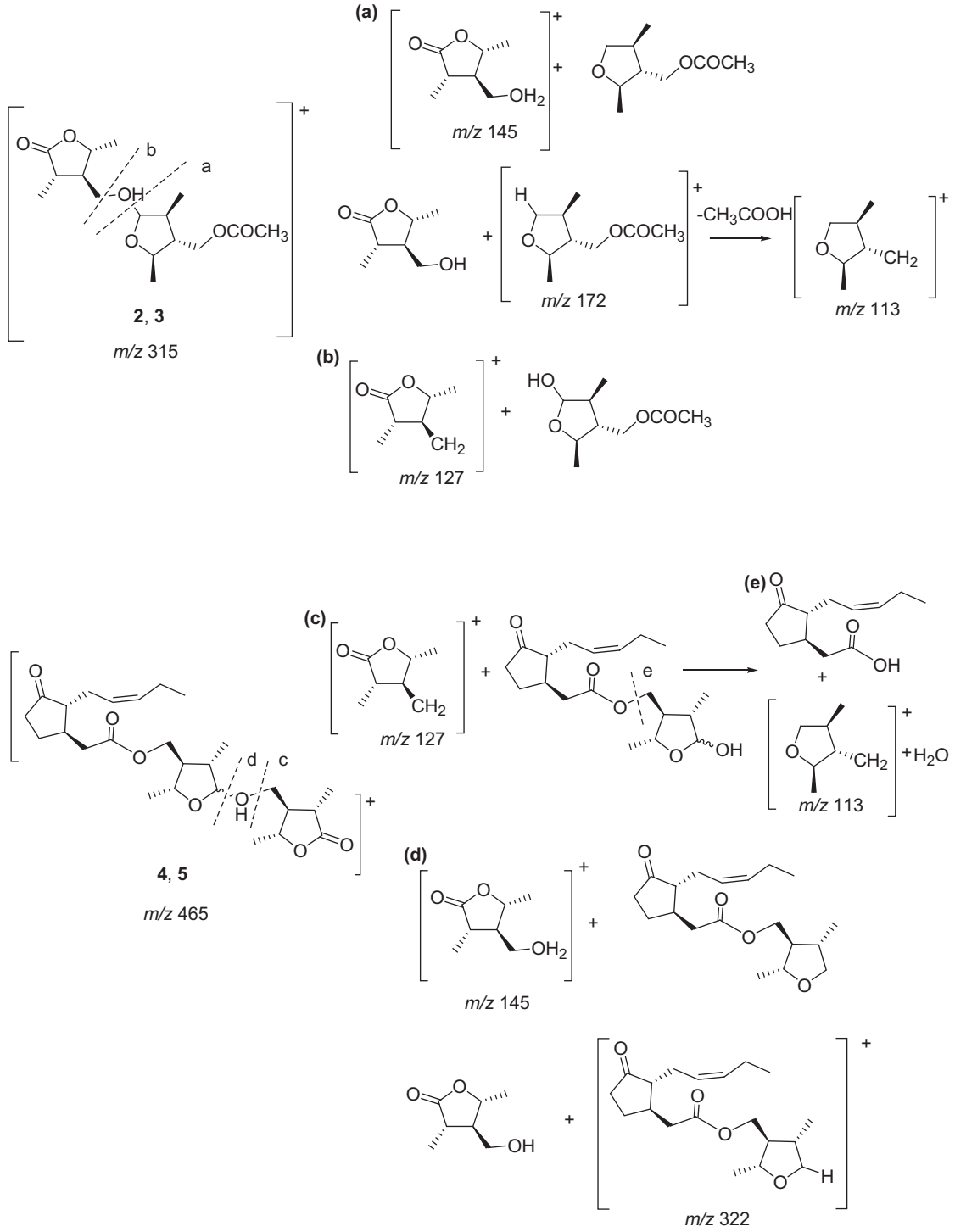

Fig. 2. Mechanisms of fragmentation observed for **2**–**5** in the APCI MS (+).

Table 3
^{1}H NMR data of lasiojasmonates B and C (**4** and **5**).[a]

Position	**4** δH (*J* in Hz)	**5** δH (*J* in Hz)
1	2.13 *dq* (17.5, 9.8)	2.13 *dq* (15.5, 9.8)
2	1.91 *m*	1.91 *m*
4	2.70 *dd* (17.5, 6.1)	2.70 *dd* (17.5, 6.1)
	2.25 *m*	2.25 *m*
5	2.37 *m*	2.37 *m*
	1.63 *ddd* (14.3, 6.9, 4.6)	1.63 *ddd* (14.3, 6.9, 4.6)
6	2.37 *m*	2.37 *m*
8	2.37 *m*	2.37 *m*
9	5.25 *m*	5.25 *m*
10	5.46 *m*	5.46 *m*
11	2.04 *dq* (7.5, 1.0)	2.04 *dq* (7.5, 1.0)
12	0.96 *t* (7.5)	0.96 *t* (7.5)
3′	2.54 m	2.54 *m*
4′	2.00 m	2.00 *m*
5′	4.30 (m)	4.30 *m*
6′	1.44 *d*, (6.2)	1.45 *d* (6.2)
7′	1.26 *d* (7.1)	1.26 *d* (7.1)
8′	3.86 *dd* (10.0, 4.3)	3.87 *dd* (10.0, 4.3)
	3.41 *dd* (10.0, 5.6)	3.41 *dd* (10.0, 5.3)
10′	4.68 *d* (2.0)	4.80 *d* (4.5)
12′	3.90 *dq* (12.3, 6.1, 2.8)	3.96 *dq* (12.6, 6.2, 1.6)
13′	1.50 *m*	1.50 *m*
14′	2.03 *m*	2.03 *m*
15′	1.13 *d* (7.2)	1.05 *d* (7.0)
16′	4.17 *dd* (11.0, 6.9)	4.23 *dd* (11.3, 4.5)
	4.10 *dd* (11.0, 7.2)	4.07 *dd* (11.3, 5.8)
17′	1.32 *d* (6.1)	1.30 *d* (6.2)

[a] The chemical shifts are in δ values (ppm) from TMS.

values reported for the same protons in botryosphaerilactones A and C (Rukachaisirikul et al., 2009). These protons appeared as doublet of doublets (*J* = 11.0, 7.2, and 11.0, 6.9) and (*J* = 11.0, 5.8, and 11.3, 4.5) at δ 4.10 and 4.17, and δ 4.07 and 4.23 for **4** and **5**. Thus, all chemical shifts of all protons were assigned to **4** and **5** in comparison with those reported for **2** and **3** and jasmonic acid as reported in Table 3. Therefore, **4** and **5** can be formulated as (1*R**,2*R**,3′*S**,4′*R**, 5′*R**,10′*R**,12′*R**,13′*R**,14′*S**)- and (1*R**,2*R**,3′*S**,4′*R**,5′*R**,10′*S**,12′*R**, 13′*R**,14′*S**)-4-(4-hydroxymethyl-3,5-dimethyltetrahydro-furan-2-yloxymethyl)-3,5-dimethyldihydro-2 furanone jasmonates.

These structures were confirmed through fragmentation ions observed in APCIMS spectrum recorded in positive mode. Also in this case the spectrum exhibited ions originating by pseudomolecular ion by two different fragmentation mechanisms generated by the cleavage of the acetalic bond as reported in Fig. 2. The cleavage of the bond C(8′)-O (mechanism c) generated the fragment ion *m*/*z* 127 and the 4-hydroxy-3,5-dimethyl-4-hydroxy jasmonate esters. This latter generated jasmonic acid and fragment ion *m*/*z* 113 and H_2O (mechanism e). Alternatively, the pseudomolecular ion by cleavage of the acetalic bond C(10′)-O (mechanism d) generated two fragment ions recorded at *m*/*z* 145 and 322.

The presence in lasiojasmonates A–C of (1*R*,2*R*)-jasmonic acid was confirmed by their mild alkaline hydrolysis carried out according to Farmer et al. (1992). In fact jasmonic acid obtained from **1**, **4** and **5**, reported in detail in the experimental section, and **6** showed the same R*f* by TLC analysis (silica gel eluent A *Rf* 0.43, and on reversed phase eluent C *Rf* 0.50) also by co-injection and OR and ^{1}H NMR recorded in the same conditions.

In addition, the keto-enolization process of C-2 of jasmonic acid, as reported in literature by Miersch et al. (1987), was ruled out for the compounds **1**,**4**–**7** isolated from *Lasiodiplodia* sp. as a result of their optical (OR) and/or spectroscopic (essential ^{1}H NMR) properties. So that compounds **1**, **4**–**7** are not artefact.

Pathogenicity of the new species of *Lasiodiplodia* was verified by wound inoculation of excised canes under controlled laboratory conditions.

Fifty days after inoculation, the lignified canes inoculated with the pathogen displayed dark-brown to black discoloration on bark and vascular tissues, measuring 7.4 ± 2.7 cm (mean ± S.D.). In cross section all canes showed a wedge-shaped necrotic sector. The pathogen was successfully re-isolated from the margin of symptomatic tissues, thus fulfilling Koch's postulates. Artificially obtained symptoms were congruent with field observations. Control canes inoculated with sterile PDA plugs remained symptomless.

The phytoxicity data obtained in the leaf-puncture assay on cork oak and grapevine leaves showed that (1*R*,2*R*)-jasmonic acid (**6**) was the only active metabolite (Table 4). It had remarkable toxicity at 1.0 mg/mL causing necrotic lesions to leaves of both species tested (necrosis area: 7.04 and 11.96 mm^2 on grapevine and cork oak, respectively). All of the other metabolites tested did not show any effect in this bioassay.

All compounds, except **4** and **5**, were also tested on grapevine detached leaves at 0.25 and 0.5 mg/mL. In this bioassay only jasmonic acid caused vein necrosis, slowly spreading to large areas of the leaf lamina at 0.5 mg/mL. When assayed on tomato cuttings at 0.1 mg/mL jasmonic acid caused withering within 7 days from treatment. None of the compounds showed activity in the assay performed for zootoxicity (*Artemia salina*) at 50 μg/mL.

In conclusion, from the culture filtrates of *Lasiodiplodia* sp. were isolated three new jasmonic acid esters, namely lasiojasmonates A–C (**1**, **4** and **5**), and for the first time 16-*O*-acetyl derivatives of botryosphaerilactones A and C (**2** and **3**), together with (1*R*,2*R*)-jasmonic and its methyl ester (**6** and **7**), botryosphaerilactone A (**8**), and (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyl-dihydro-2-furanone (**9**) and (3*R*,4*S*)-botryodiplodin (**10**).

Jasmonic acid is the basic structure of a naturally occurring family of compounds named jasmonates which regulate many aspects of growth, development and defence responses in plants (Miersch et al., 1999a). In particular, jasmonic acid is a signaling molecule in plant defence, activating responses to wounding, herbivores and necrotrophic pathogens.

6 and relate compounds were also produced by fungi (Miersch et al., 1999b and literature cited). In particular, *Lasiodiplodia* spp. are among the few fungal species which synthesize enantiomeric pure form of the different enantiomers as (1*R*,2*S*) diasteromer, called also (+)-7-*iso*-jasmonic acid, isolated from *L. theobromae* (Miersch et al., 1987). Jasmonic acid is biosynthesised by the octadecanoid pathway in plants (Schaller and Stinzi, 2009) and fungi (Tsukada et al., 2010). Recently jasmonate biosynthesis by a linolenate 9*R*-dioxygenase and an allene oxide synthase has been described in *L. theobromae* (Jernerèn et al., 2012).

Botryosphaerilactone A and (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyl-dihydro-2-furanone (**8** and **9**) were recently reported as secondary metabolites of the endophytic fungus *L. theobromae*

Table 4
Phytotoxicity of compounds **1**–**10** evaluated at 1 mg/mL by leaf punctured assay on two plant species. Area lesions (mm^2) ± error standard.

Compound	Plant species	
	Cork oak	Grapevine
1	n.a.	n.a.
2	n.a.	n.a.
3	n.a.	n.a.
4	n.t.	n.t.
5	n.t.	n.t.
6	11.96 ± 1.82	7.04 ± 0.91
7	n.a.	n.a.
8	n.a.	n.a.
9	n.a.	n.a.
10	n.a.	n.a.

n.a. = not active.
n.t. = not tested.

isolated from leaves of *Garcinia mangostana* (Rukachaisirikul et al., 2009). When they were assayed against *Staphylococcus aureus*, they did not show antibacterial activity (Rukachaisirikul et al., 2009).

(3*R*,4*S*)-Botryodiplodin was first isolated from cultures of *L. theobromae* Pat. the causal agent of fruit rot in tropical fruits trees (Gupta et al., 1966). Subsequently, it was found in other cultures of phytopathogenic fungal species such as *Penicillium roquefortii* strains (Nielsen et al., 2006), *Penicillium stipitatum* (Fuska et al., 1988) and *Macrophomina phaseolina* (Ramezani et al., 2007).

(3*R*,4*S*)-Botryodiplodin exhibits anticancer, antibacterial, antifungal, phytotoxic, mitogenic, and antifertility activities, and may play a role in plant diseases. Unusual structural properties allow oligomerization of (−)-botryodiplodin to pigments and cross-linking agents, which may be responsible for some of its biological activities (Shier et al., 2007).

This study represents the first report on the expression of secondary metabolites by a new species of *Lasiodiplodia* which is closely related phylogenetically to *L. pseudotheobromae*. The results here reported have permitted the increase of the knowledge on secondary metabolites produced by members of *Botryosphaeriaceae*, a family of ascomycetes capable of producing a plethora of bioactive compounds (Evidente et al., 2010, 2012; Andolfi et al., 2011, 2012). However, they do not permit the elucidation of the true biological function of the only phytotoxin isolated, the jasmonic acid, in the pathogenesis process. Moreover, the data available in the literature concerning the role of this metabolite in host–pathogen interaction is uncertain. In a previous study it was suggested that in the *Brassica nigra* – *L. theobromae* pathosystem, the development of systemic acquired resistance does not occur due to the deficiency of salycilic acid whose biosynthesis is inhibited by jasmonic acid produced by fungus (Thakkar et al., 2004). In addition, it is possible to hypothesize that the lasiojasmonates and methyl esters (**1**, **4**, **5** and **7**) represent a reserve that could generate jasmonic acid by hydrolysis at physiological pH as frequently observed in other natural metabolites and this conversion is known as lethal metabolism (Hassal, 1990).

Finally, further investigation must be addressed at acquiring a body of data in order to speculate on the potential use of these metabolites in the resolution of the taxonomic status of *Botryosphaeriaceae* species, in particular the cryptic species closely related phylogenetically to *L. theobromae* complex.

3. Experimental

3.1. General

Optical rotations were measured in $CHCl_3$, unless otherwise noted, on a Jasco polarimeter; IR spectra were recorded as glassy films on a Perkin–Elmer Spectrum One FT-IR spectrometer and UV spectra were taken in MeOH solution on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer; ^1H and ^{13}C NMR spectra were recorded at 600 and at 125 MHz, respectively, in $CDCl_3$, unless otherwise noted, on Bruker spectrometers. The same solvent was used as internal standard. DEPT, COSY-45, HSQC, HMBC and NOESY experiments (Berger and Braun, 2004) were performed using standard Bruker microprograms. HRESI and ESI, and APICMS spectra were recorded on Waters Micromass Q-TOF and Agilent Technologies 6120 Quadrupole LC/MS instrument. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively) or reverse phase (Whatman, KC18 F_{254}, 0.20 mm) plates. The spots were visualized by exposure to UV radiation (253), or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography (CC) was performed on silica gel column (Merck, Kieselgel 60, 0.063–0.200 mm). Solvent systems: (A) $CHCl_3$-*i*-PrOH (95:5); (B) *n*-hexane-Me_2CO (95:5), (C) EtOH-H_2O (6:4); (D) $CHCl_3$-*i*-PrOH (93:7); (E) *n*-hexane-Me_2CO (6:4). (±)-Jasmonic acid and its methyl ester were purchased from Sigma–Aldrich.

3.2. Fungal strains

The strain BL 101 of *Lasiodiplodia* sp. used in this study was originally isolated from wood tissues of a wedge-shaped canker collected in a vineyard in northern Sardinia (Italy). The strain was identified on the basis of morphological characters and analysis of internal transcribed spacer (ITS) of rDNA and part of the translation elongation factor 1-α gene (EF1-alpha). Fungal DNA extraction, PCR amplification reactions, and DNA sequencing were carried out as reported by Linaldeddu et al. (2013). Representative sequences of both loci were deposited in GenBank: (ITS: accession number KJ170150), (EF1-α: accession number KJ170151).

Pure cultures were maintained on potato-dextrose-agar (PDA, Fluka, Sigma–Aldrich Chemic GmbH, Buchs, Switzerland) and stored at 4 °C in the collection of the ''Dipartimento di Agraria – Sezione di Patologia vegetale ed Entomologia'', University of Sassari, Italy, as BL 101.

3.3. Pathogenicity test

Pathogenicity of the new species of *Lasiodiplodia* from grapevine was verified by inoculating a selected strain (BL101) on nine excised grapevine canes (30 cm in length) of cv. Cannonau. A mycelial plug (3 to 4 mm^2) taken from the margin of an actively growing colony on PDA was placed in a shallow wound (~3 mm) made by a scalpel on the middle of each cane. The inoculation point was covered with cotton wool soaked in sterile water and wrapped with Parafilm®. A sterile PDA plug was placed on the wound in control canes. The inoculated canes were enclosed in a transparent plastic bag at 25 °C. Fifty days after inoculation, the length of the dark-brown to black necrotic lesions were measured. Re-isolation was attempted by transferring to PDA 10 pieces of inner bark and xylem tissue taken from the margin of each lesion.

3.4. Extraction and purification of fungal metabolites (*1*–*10*)

The fungus was grown in 2 L Erlenmeyer flasks containing 400 mL of Czapek medium amended with corn meal (pH 5.7). Each flask was seeded with 5 mL of a mycelial suspension and then incubated at 25 °C for 4 weeks in darkness. The culture filtrates (3 L) were acidified to pH 2 with 2 N HCl and extracted exhaustively with EtOAc. The organic extracts were combined, dried with Na_2SO_4, and evaporated under reduced pressure to give a brown-red oil residue (2.3 g), having a high phytotoxic activity on grapevine leaves by the puncture leaf bioassay at 4 mg/mL. The residue was submitted to a bioassay-guided fractionation through CC on silica gel, eluted with the solvent system A. Height homogenous fraction groups were collected and screened for their phytotoxic activity. The residue (18.7 mg) of the first fraction was purified by TLC on silica gel eluted with solvent system B yielding a homogeneous oil identified as (1*R*,2*R*)-methyl ester of jasmonic acid (**7**, 3.4 mg, 1.1 mg/L, *Rf* 0.8 and 0.13, eluent A and B respectively). The residue (27.7 mg) of the second fraction was purified by TLC on reversed phase eluted with the solvent system C to give three main bands. The first band corresponded to an uncoloured oil (1.6 mg, 0.6 mg/L, *Rf* 0.30, eluent C), which was characterized as lasiojasmonates B and C (**4** and **5**), obtained as an inseparable mixture. The second band corresponded to another uncoloured oil (5.2 mg, 1.7 mg/L, *Rf* 0.40, eluent C) which was characterized as lasiojasmonate A (**1**). The third band corresponded to an amorphous solid (5.2 mg, 1.7 mg/L, R_f 0.50, eluent C), which was characterized as 16-*O*-botryosphaerilactone A and C acetylates (**2** and **3**), obtained as an

inseparable mixture. The residue (587.4 mg) of the third fraction of the original column appeared to be the main metabolite, obtained as a pure yellow oil (*Rf* 0.43, TLC of silica gel eluent A and *Rf* 0.50, TLC on reversed phase eluent C), which was identified as (1*R*,2*R*)-jasmonic acid (**6**). The residue (294.4 mg) of the fourth fraction was dissolved in EtOAc and then washed with a saturated solution of $NaHCO_3$ to remove jasmonic acid (110.3 mg, 236.6 mg/L). The organic phase was dried with Na_2SO_4, and evaporated under reduced pressure affording a brown oil residue (175.8 mg). This residue was further purified by CC on silica gel, eluted with solvent system D, yielding seven homogeneous fraction groups. The residue (48.3 mg) of the second fraction was purified by TLC on reversed phase eluted with solvent system C, yielding an amorphous white solid (6.6 mg, 2.2 mg/L, *Rf* 0.68, eluent C), which was characterized as (3*R*,4*S*)-botryodiplodin (10), and an uncoloured oil (27.6 mg, 9.2 mg/L, *Rf* 0.64, eluent C), which was characterized as botryosphaerilactone A (**8**). The residue (18.3 mg) of the fifth fraction of the same column was purified by TLC on reversed phase eluted with eluent system C yielding a homogeneous amorphous solid (4.4 mg, 1.7 mg/L, *Rf* 0.76, eluent C), which was characterized as the (3*S*,4*R*,5*R*)-4-hydroxymethyl-3,5-dimethyldihydro-2-furanone (**9**).

3.5. Lasiojasmonate A (*1*)

Compound **1**: $[\alpha]_D^{25}$ −15 (*c* = 0.4); UV λ_{max} nm (log ε) 204 (3.98); IR ν_{max} cm^{-1} 1747, 1695, 1634, 1240, 1207; ^1H and ^{13}C NMR spectra: see Table 1; HRESIMS (+) *m*/*z*: 359.1913 $[M+Na]^+$ (calcd. for $C_{19}H_{28}NaO_5$ 359.1834); APCIMS (+) *m*/*z*: 337 $[M+H]^+$.

3.6. 16-*O*-Acetylbotryosphaerilactone A and C (*2* and *3*)

Compounds **2** and **3**: UV λ_{max} nm (log ε) 203 (4.01); IR ν_{max} cm^{-1} 1764, 1731, 1681, 1438, 1379; ^1H NMR, see Table 2; ESIMS (+) *m*/*z*: 651 [2 M+Na]$^+$, 314 $[M+Na]^+$; APCIMS (+) *m*/*z*: 315 $[M+H]^+$, 172 $[C_9H_{16}O_3]^+$, 145 $[C_7H_{13}O_3]^+$, 127 $[C_7H_{11}O_2]^+$, 113 $[C_7H_{13}O]^+$.

3.7. Lasiojasmonates B and C (*4* and *5*)

Compounds **4** and **5**: UV λ_{max} nm (log ε) 205 (4.00); IR ν_{max} cm^{-1} 1765, 1701, 1624, 1238, 1211; ^1H NMR ($CDCl_3$), see Table 3; ESIMS (+) *m*/*z*: 487.1682 $[M+Na]^+$ (calcd. for $C_{26}H_{40}NaO_7$ 487.2672); APCIMS (+) *m*/*z*: 482 $[M+H_2O]^+$, 465 $[M+H]^+$, 322 $[C_{19}H_{30}O_4]^+$, 145 $[C_7H_{13}O_3]^+$, 127 $[C_7H_{11}O_2]^+$, 113 $[C_7H_{13}O]^+$.

3.8. (1*R*,2*R*)-Jasmonic acid (*6*)

Compound **6**: $[\alpha]_D^{25}$ −75 (*c* = 0.3, MeOH); UV λ_{max} nm (log ε) 206 (3.72); IR ν_{max} cm^{-1} 3571, 1749, 1707, 1636, 1259; [lit, Aldridge et al., 1971: $[\alpha]_D^{25}$ −73 (*c* = 0.1, MeOH); lit, Nielsen and Smedsgaard, 2003: UV absorption (nm) in% of UV-max (MeOH) end; lit, Husain et al., 1993: ν_{max} 1740, 1700 cm^{-1}]; ^1H and ^{13}C NMR were similar to data previously reported (Husain et al., 1993); ESIMS (+) *m*/*z*: 233 $[M+Na]^+$; APCIMS (+) *m*/*z* 211 $[M+H]^+$.

3.9. (1*R*,2*R*)-Methyl jasmonate (*7*)

Compound **7**: $[\alpha]_D^{25}$ −58 (*c* = 0.4); UV λ_{max} nm (log ε) 205 (3.67), IR ν_{max} cm^{-1} 1754, 1678, 1426, 1165 [lit. (Nishida et al., 1985) $[\alpha]_D^{25}$ −58 (*c* = 0.2, MeOH); lit (Nielsen and Smedsgaard, 2003) UV absorption (nm) in% of UV-max (MeOH) end; lit. (Takeda et al., 2006) IR (neat) ν_{max}: 1738, 1438, 1198, 1160 cm^{-1}]; ^1H NMR spectrum was similar to data reported (Takeda et al., 2006); ESIMS (+) *m*/*z*: 247 $[M+Na]^+$; APCIMS (+) *m*/*z* 225 $[M+H]^+$.

3.10. Botryosphaerilactone A (*8*)

Compound **8**: $[\alpha]_D^{25}$ −4.0 (*c* = 0.3, MeOH); UV λ_{max} nm (log ε) 209 (3.81); IR ν_{max} cm^{-1} 3458, 1764, 1678, 1613, 1453, 1187 [lit: $[\alpha]_D^{27}$ −1.2 (c 0.77, MeOH); UV (MeOH) λ_{max} (log ε) 286 (1.30) nm; IR (neat) ν_{max} 3438, 1770 cm^{-1} (Rukachaisirikul et al., 2009)]; ^1H and ^{13}C NMR were very similar to those reported previously (Rukachaisirikul et al., 2009); ESIMS (+) spectrum *m*/*z*: 567 $[2M+Na]^+$, 295 $[M+Na]^+$; ESI (−) *m*/*z* 271 $[M-H]^-$; APCIMS (+) *m*/*z*: 145 $[C_7H_{13}O_3]^+$, 129 $[C_7H_{13}O_2]^+$, 127 $[C_7H_{11}O_2]^+$, 111 $[C_7H_{11}O]^+$.

3.11. (3*S*,4*R*,5*R*)-4-Hydroxymethyl-3,5-dimethyldihydro-2-furanone (*9*)

Compound **9**: $[\alpha]_D^{25}$ −18 (*c* = 0 0.3); UV λ_{max} final absorption; IR ν_{max} cm^{-1} 3427, 1747, 1635; 1456, 1385, 1183; lit, Ravi et al. (1979): IR ν_{max} cm^{-1} 3400, 1770 (Ravi et al., 1979); ^1H NMR spectrum was similar to data previously reported (Ravi et al., 1979), ^{13}C NMR δ: (179.0, *s*, C-2), (76.1, *d*, C-5), (60.7, *t*, C-8), (52.7, *d*, C-4), (37.6, *d*, C-3), (20.1, *q*, C-6), (14.2, *q*, C-7); ESIMS (+) *m*/*z*: 167 $[M+Na]^+$; ESIMS (−) *m*/*z*: 143 $[M-H]^-$.

3.12. (3*R*,4*S*)-Botryodiplodin (*10*)

Compound **10**: UV λ_{max} nm (log ε) 205 (3.92); IR ν_{max} cm^{-1} 3457, 1707, 1467, 1343; ^1H NMR was similar to data previously reported (Ramezani et al., 2007); ESIMS (+) *m*/*z*: 167 $[M+Na]^+$, APCIMS (+) *m*/*z*: 145 $[M+H]^+$, 127 $[M-OH]^+$.

3.13. Acetylation of botryosphaerilactone A

Botryosphaerilactone A (**8**, 5.0 mg) dissolved in pyridine (30 μL), was converted into the corresponding 16-*O*-acetyl derivatives (α and β anomers) by acetylation with Ac_2O (30 μL) carried out at room temperature for 10 min. The reaction was stopped by addition of MeOH and the azeotrope formed by addition of benzene was evaporated with a N_2 steam. The oily residue (6.0 mg) was purified by TLC on silica gel, solvent system E, yielding a mixture of two anomeric acetyl derivatives **3** and **2** (4.1 mg, *Rf* 0.39, α/β, 90:10).

3.14. Acetylation of (3*R*,4*S*)-botryodiplodin

(3*R*,4*S*)-Botryodiplodin (2.0 mg) was acetylated with pyridine (35 μL) and Ac_2O (35 μL) in the same conditions above reported to converted **8** in the corresponding 2-*O*-acetyl derivative. Also the reaction work-up is the same and the residue (2.4 mg) was purified by TLC on silica gel, eluent system A, to give 2,3-*trans*-botryodiplodin acetate (0.8 mg, *Rf* 0.88, eluent A), whose physic and spectroscopic data were very similar to those previously reported (Arsenault and Althaus, 1969).

3.15. Alkaline hydrolysis of lasiojasmonates A–C

Lasiojasmonate A (**1**, 3,0 mg) was dissolved in MeOH (200 μl) and H_2O (10 μl) and hydrolyzed with K_2CO_3 (6.0 mg). The mixture was stirred at 60 °C for 2 h following the procedure previously reported (Farmer et al., 1992). Then the mixture was diluted with distilled water (2 mL) and acidified to pH 4.0 with 2 N HCl and extracted with EtOAc (2 mL × 3). The organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure. The residue (2.8 mg) was purified by TLC, eluted with solvent system A, and the jasmonic acid (0.8 mg) obtained as an oil was compared (TLC, OR and ^1H NMR) with the authentic sample of (1*R*,1*R*)-jasmonic acid. Lasiojasmonates B and C (**4** and **5**) were hydrolyzed in the same conditions used to treat **1**, yielding (1*R*,2*R*)-jasmonic acid ascertained as above reported.

3.16. Biological activities

Grapevine bioassay: All compounds, except **4** and **5**, were assayed on detached leaves of grapevine cv Cannonau (red vine). The leaves with their petioles were immersed in 2 mL toxic solution until complete absorption. After this they were transferred to distilled water. Toxicity symptoms were recorded 48 h later. Each compound was tested at 0.25 and 0.5 mg/mL.

Leaf Puncture Assay: Young cork oak and grapevine leaves were utilized for this assay. Each compound was assayed at 1.0 mg/mL. Compounds were first dissolved in MeOH, and then a stock solution with sterile distilled water was made. A droplet (20 μl) of test solution was applied on the adaxial sides of leaves that had previously been needle punctured. Droplets (20 μl) of MeOH in distilled water (4%) were applied on leaves as control. Each treatment was repeated three times. The leaves were then kept in a moist chamber to prevent the droplets from drying. Leaves were observed daily and scored for symptoms after 7 days. The effects of the toxins on the leaves were observed for up to 7 days. Lesions were estimated using APS Assess 2.0 software (Lamari, 2002) following the tutorials in the user's manual. The lesion size was expressed in mm^2.

Tomato cutting assay: Tomato cuttings were taken from 21-day-old seedling and each compound was assayed at 0.1 mg/mL. Cuttings were placed in the test solutions (2 mL) for 72 h and then transferred to distilled water. Symptoms were visually evaluated for up to 7 days.

Artemia salina bioassay: All compounds, except **4** and **5**, were assayed on brine shrimp larvae (*Artemia salina* L.). The assay was performed in cell culture plates with 24 cells (Corning) as already described (Favilla et al., 2006). The metabolites were dissolved in methanol and tested at 50 μg/mL. Tests were performed in quadruplicate. The percentage of larval mortality was determined after incubation for 24 and 36 h at 27 °C in the dark.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.03.016. These data include MOL files and InChiKeys of the most important compounds described in this article.

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