JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Phytotoxic Activity and Metabolism of *Botrytis cinerea* and Structure–Activity Relationships of Isocaryolane Derivatives

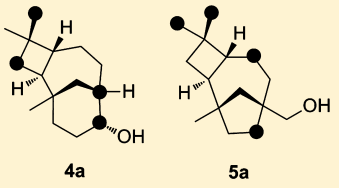
Jociani Ascari,[†] Maria Amélia Diamantino Boaventura,[†] Jacqueline Aparecida Takahashi,[†] Rosa Durán-Patrón,[‡] Rosario Hernández-Galán,[‡] Antonio J. Macías-Sánchez,*[,‡] and Isidro G. Collado*[,‡]

[†]Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil

[‡]Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain

Supporting Information

ABSTRACT: Research has been conducted on the biotransformation of (8*S*,9*R*)-isocaryolan-9-ol (**4a**) and (1*S*,2*S*,5*R*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]undecan-12-ol (**5a**) by the fungal phytopathogen *Botrytis cinerea*. The biotransformation of compound **4a** yielded compounds **6**–**9**, while the biotransformation of compound **5a** yielded compounds **10**–**13**. The activity of compounds **4a** and **5a** against *B. cinerea* has been evaluated. (8*R*,9*R*)-Isocaryolane-8,9-diol (**6**), a major metabolite of compound **4a**, shows activity compared to its parent compound **4a**, which is inactive. The effect of isocaryolanes **3**, **4a**, and **5a**, together with their biotransformation products **6**–**8**, **10**, and **14**–**17**, on the germination and radicle and shoot growth of *Lactuca sativa* (lettuce) has also been determined. Compounds **7**–**13** are described for the first time.

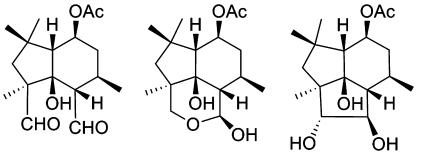


INTRODUCTION

Plant pathogens such as *Botrytis cinerea* are responsible for serious economic losses.[1] There is a considerable need to develop fungicides with novel modes of action to combat resistant strains of organisms,[2] lower persistence,[3] reduced toxicity[4] and phytotoxicity,[5] and specific rather than nonspecific antifungal activity.[6] These novel agents must be compatible with biological crop protection agents and avoid harmful effects on crops, workers, end consumers, and the environment.[7]

In phytopathogenic species, some metabolites play crucial roles in interactions with host plants.[8] Part of the interaction of *B. cinerea* with host plants involves the effect that botrydial, a nonspecific low molecular weight phytotoxin, has on the plant.[9] Botrydial induces a hypersensitive response, and its toxicity is modulated by host signaling pathways mediated by salicylic acid and jasmonic acid. Botrydial appears to have an effector role that allows *B. cinerea* to manipulate host defenses, promoting cell death and thus enabling it to feed on necrotic tissue.[10] Symptoms of the disease can be decreased by inhibiting the production of this phytotoxin.[11] Our research group has explored a method of fungal control using nonphytotoxic analogues of botrydial and related metabolites such as dihydrobotrydial[12] or analogues of biosynthetic intermediates[9,13] (such as **1**) en route to botrydial. Sesquiterpene derivatives with several carbon skeletons have been prepared for this purpose.[14−24]

Fungi are able to detoxify both synthetic and naturally occurring antifungal compounds. *B. cinerea* is able to transform plant defense compounds from active forms to inactive[25] or to detoxify antifungal compounds such as those produced by fungi of the *Tricoderma* species.[26] Furthermore, fungal metabolism can activate nontoxic compounds.[27] Therefore, the metabolism



of antifungal compounds should be examined to shed some light on the mechanism of action of active compounds. Work has been carried out on the metabolism by *B. cinerea* of a number of sesquiterpenes,[14−17,20−24] many of which are active against this fungus.

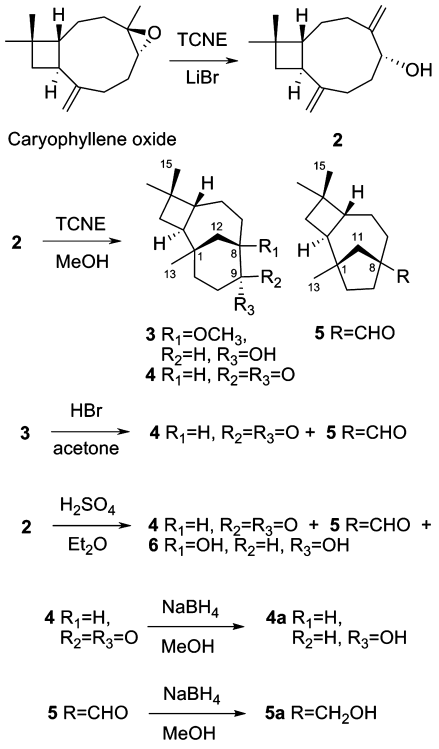
Isocaryolane (compounds **3**, **4**, **6**) and (1*S*,2*S*,5*R*,8*R*)-1,4,4,8-tetramethyltricyclo[6.2.1.0^{2,5}]undecane (compound **5**) derivatives have a similar structure to that of the biosynthetic precursors of the phytotoxin botrydial. They have been obtained by cyclization and rearrangement of (−)-*trans*-caryophyllene and caryophyllene oxide with several electrophilic reagents, by means of a caryophylladiene intermediate (**2**) (Scheme 1).[28] Separate treatment of **4** and **5** with $NaBH_4$ in MeOH leads to the more stable alcohols **4a**[29] and **5a**,[28] respectively.

We have recently described the activity and metabolism of isocaryolane derivatives **3** and **6** by the phytopathogenic fungus *B. cinerea*. The growth inhibition activity of reported isocaryolanes on *B. cinerea* seems to be correlated with the presence of an alkoxy group at C-8 and the hydroxylation level.[24] Therefore, the evaluation of the activity and metabolism of (8*S*,9*R*)-isocaryolan-9-ol (**4a**) and (1*S*,2*S*,5*R*,8*S*)-8-methyl-

Received: December 23, 2012
Published: June 3, 2013

dx.doi.org/10.1021/np3009013 | *J. Nat. Prod.* 2013, 76, 1016−1024

Scheme 1



ene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]undecan-12-ol (**5a**) by *B. cinerea*, together with previously reported data for compounds **3** and **6** and their metabolites, should clarify structure−activity relationships for this class of compounds and provide data on the way the fungus metabolizes the above-mentioned compounds. This, in turn, would help to develop these compounds as antifungal agent leads.

Fungal phytotoxins exhibit different levels and patterns of oxidation that apparently originate from the metabolism of less functionalized precursors.[30] In *B. cinerea*, oxido-reductive and degradative reactions on botrydial and related metabolites seem to be related to a self-protection mechanism since botrydial is toxic to the fungus itself.[31] Therefore, analogues of botrydial and its biosynthetic precursors may undergo oxidative metabolism, which may modify not only their antifungal properties but also their phytotoxicity. Therefore evaluation of the phytotoxicity of compounds **3**, **4a**, **5a**, and **6** and their metabolites is needed in order to unravel the structure−activity relationships of these compounds and their potential activity *in planta*.

In this paper we report the metabolism by the fungus *B. cinerea* of (8*S*,9*R*)-isocaryolan-9-ol (**4a**) and (1*S*,2*S*,5*R*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]undecan-12-ol (**5a**), the evaluation of their activity against *B. cinerea*, and the phytotoxic activity of **3**, **4a**, **5a**, and **6** and some of their fungal metabolites on the germination of seeds and the growth of radicals and shoots of *Lactuca sativa* (lettuce).[32]

RESULTS AND DISCUSSION

The chemical transformations carried out for the preparation of (8*S*,9*R*)-isocaryolan-9-ol (**4a**) and (1*S*,2*S*,5*R*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]undecan-12-ol (**5a**) are summarized in Scheme 1. Compounds **4a** and **5a** are obtained by treatment of (8*R*,9*R*)-isocaryolan-9-one (**4**) and (1*S*,2*S*,5*R*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]undecane-8-carbaldehyde (**5**), respectively, with $NaBH_4$ in MeOH. In turn, compounds **4** and **5** are obtained, together with (8*R*,9*R*)-8-methoxyisocaryolan-9-ol (**3**), from (1*R*,5*R*,9*S*)-caryophylla-4(12),8(13)-dien-5-ol (**2**) by treatment with tetracyanoethylene (TCNE) in methanol.[28] Compound **5** was obtained by treatment of caryophyllene oxide with LiBr and TCNE, in acetone.[33]

Compounds (8*S*,9*R*)-isocaryolan-9-ol (**4a**) and (1*S*,2*S*,5*R*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]-undecan-12-ol (**5a**) were each incubated separately with *B. cinerea* for 5 or 9 days. A certain amount of the characteristic metabolite of *B. cinerea*, dihydrobotrydial, was obtained in every culture. The metabolites isolated (Scheme 2) were tabulated

Scheme 2

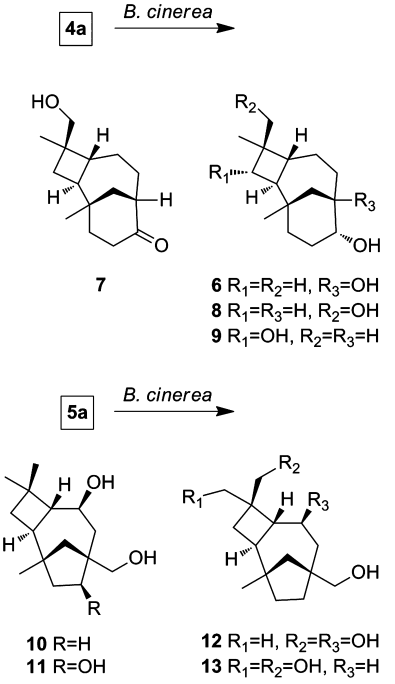


Table 1. Metabolites of Isocaryolane Derivatives Produced by *B. cinerea*

substrate	metabolites[a]
4a	DHB, **6**, **7**, **8**, **9**
5a	DHB, **10**, **11**, **12**, **13**

[a]DHB = dihydrobotrydial.

(see Table 1) and quantified for each culture (see Experimental Section). The metabolites were identified by their ^{1}H (see Table 2) and ^{13}C (see Table 3) NMR spectra, combined with 1D and 2D NMR techniques and by comparison with authentic samples, where appropriate.

The biotransformation of (8*S*,9*R*)-isocaryolan-9-ol (**4a**) led to the isolation of five products. Compounds **7** and **8** displayed similar signal patterns in their ^{13}C NMR spectra (Table 3). Compound **7** showed an HREIMS molecular ion peak at *m*/*z* 236.1779, consistent with the molecular formula $C_{15}H_{24}O_2$. Its IR spectrum showed absorptions at 3454 and 1695 cm^{-1}, indicating the presence of hydroxyl and carbonyl functions, corroborated by carbon resonances at δ_C 71.9 ppm and 215.6

dx.doi.org/10.1021/np3009013 | *J. Nat. Prod.* 2013, 76, 1016−1024

Table 2. ^{1}H NMR Spectroscopic Data for 7−13

	7	8	9	10	11	12	13
position	δ_H[b] (mult, J in Hz)	δ_H[b] (mult, J in Hz)	δ_H[a] (mult, J in Hz)	δ_H[a] (mult, J in Hz)	δ_H[a] (mult, J in Hz)	δ_H[a] (mult, J in Hz)	δ_H[b] (mult, J in Hz)
1							
2	α: 1.76−1.60 (m)	α: 2.21 ddd (12.0, 10.4, 8.0)	α: 1.85, dd (12.6, 8.7)	α: 1.79, dt (10.8, 8.1)	α: 1.63, ddd (11.4, 10.5, 7.8)	α: 1.86, dt (11.4, 11.4, 7.8)	α: 1.74 (m)
3	a: 1.76−1.60 (m)	a,b: 1.46−1.38	β: 3.41, d (8.7)	α: 1.53, dd (9.6, 8.1)	a: 1.57−1.50 (m)	a: 1.40−1.35 (m)	α: 1.70 (m)
	b: 1.43 (m)			β: 1.33 (m)	b: 1.34 (m)	b: 1.51−1.45 (m)	β: 1.33 (m)
4							
5	β: 1.85 ddd (11.2, 10.4, 3.4)	β: 1.89 dt (12.0, 6.8)	β: 1.20 (m)	β: 1.66−1.63 (m)	β: 1.69, t (10.5)	β: 1.78, t (11.4)	β: 1.78 (m)
6	1.48−1.37 (m)	1.57 (m)	a: 1.69−1.59 (m)	α: 3.78, ddd (10.2, 9.9, 6.0)	α: 3.76, ddd (10.5, 10.2, 5.7)	α: 3.69, ddd (11.4, 9.6, 6.0)	a: 1.83 (m)
			b: 1.41 (m)				b: 1.46 (m)
7	a: 2.27 dddd (12.4, 5.3, 5.2, 4.6)	a: 1.74−1.66 (m)	a: 1.83−1.75 (m)	α: 1.91, dd (12.6, 6.0)	α: 2.20, dd (12.6, 5.7)	α: 1.97, dd (13.2, 6.0)	a: 1.44 (m)
	b: 1.19 (m)	b: 1.46−1.38 (m)	b: 1.36 (m)	β: 1.18, dd (12.6, 9.9)	β: 0.86, dd (12.6, 10.2)	β: 1.26−1.23 (m)	b: 1.28 (m)
8	2.53−2.48 (m)	2.12 (m)	2.13 (m)				
9		β: 3.72, dt (11.2, 5.6)	β: 3.72, ddd (12.0, 6.0, 5.4)	a,b: 1.47−1.43	α: 4.16, dd (9.3, 6.9)	a: 1.51−1.45 (m) b: 1.26−1.23 (m)	a: 1.52−1.43 (m) b: 1.38 (m)
10	a: 2.48−2.37(m)	a: 1.80 (m)	a: 1.83−1.75 (m)	a,b: 1.37−1.31	α: 1.93 (m)	1.40−1.35 (m)	a,b: 1.42−1.35
	b: 1.76−1.60 (m)	b: 1.74−1.66 (m)	b: 1.67 (m)		β: 1.36, dd (11.4, 9.3)		
11	a: 2.53−2.37 (m)	a: 1.46−1.38 (m)	a: 1.27, td (13.2, 4.2)	a: 1.05, d (12.6)	a,b: 1.57−1.50	a: 1.08, d (12.6)	a: 1.62, d (12.4)
	b: 1.76−1.60 (m)	b: 1.21 dt (13.2, 4.4)	b: 1.49 (m)	b: 1.66−1.63 (m)		b:1.67, dd (12.6, 2.4)	b: 1.08, d (12.4)
12	a: 2.05−2.01 (m)	a: 1.74−1.66 (m)	a: 1.69−1.59 (m)	3.33 (s)	a: 3.55, d (11.4)	a,b: 3.38 (s)	3.32 (s)
	b: 1.76−1.61 (m)	b: 1.05, dd (14.4, 4.8)	b: 1.09 dd (13.8, 4.8)		b: 3.46, d (11.4)		
13	0.89 (s)	0.76 (s)	0.91 (s)	0.93 (s)	0.92 (s)	0.91 (s)	0.92 (s)
14α	0.97 (s)	1.04 (s)	0.91 (s)	1.14 (s)	1.12 (s)	1.21 (s)	a: 4.02, d (11.2) b: 3.90, d (11.2)
15β	a,b: 3.33 (s)	a,b: 3.34 (s)	1.01 (s)	1.10 (s)	1.09 (s)	a: 3.43, d (9.3)	a: 3.71, d (10.4)
						b: 3.32, d (9.3)	b: 3.66, d (10.4)

[a]Acquired in $CDCl_3$ (600 MHz). [b]Acquired in $CDCl_3$ (400 MHz).

Table 3. ^{13}C NMR Spectroscopic Data ($CDCl_3$, 100 MHz) for Compounds 4a, 5a, and 7−13

	4a[29]	5a[28]	7	8	9	10	11	12	13
position	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C,[a] mult.	δ_C,[a] mult.	δ_C,[a] mult.	δ_C,[a] mult.	δ_C, mult.
1	31.4, C	33.3, C	31.9, C	31.6, C	30.9, C	41.0, C	37.6, C	41.1, C	41.2, C
2	38.3, CH	46.1, CH	42.1, CH	39.3, CH	52.3, CH	41.6, CH	42.1, CH	42.1, CH	46.8, CH
3	36.0, CH_2	37.2, CH_2	30.3, CH_2	30.8, CH_2	75.8, CH	36.5, CH_2	36.5, CH_2	32.2, CH_2	28.8, CH_2
4	34.3, C	41.1 C	37.8, C	39.1, C	40.2, C	33.3, C	33.3, C	38.7, C	42.5, C
5	46.1, CH	49.1 CH	40.7, CH	40.8, CH	38.5, CH	53.9, CH	54.2, CH	52.6, CH	45.2, CH
6	24.2, CH_2	34.0, CH_2	26.0, CH_2	24.4, CH_2	23.9, CH_2	72.5, CH	72.0, CH	70.0, CH	27.0, CH_2
7	24.3, CH_2	28.5, CH_2	29.5, CH_2	24.2, CH_2	24.9, CH_2	44.8, CH_2	44.2, CH_2	43.9, CH_2	34.1, CH_2
8	39.5, CH	47.5, C	44.7, CH	38.2, C	38.3, C	45.9, C	48.0, C	46.7, C	47.5, C
9	73.1, CH	25.7, CH_2	215.6, C	73.1, CH	72.8, CH	28.9, CH_2	77.1, CH	29.8, CH_2	28.5, CH_2
10	27.9 CH_2	45.2, CH_2	36.5, CH_2	27.7, CH_2	28.2, CH_2	44.8, CH_2	55.6, CH_2	44.8, CH_2	45.1, CH_2
11	38.4 CH_2	47.8, CH_2	36.6, CH_2	38.2, CH_2	39.7, CH_2	47.3, CH_2	44.9, CH_2	47.5, CH_2	47.6, CH_2
12	40.2 CH_2	73.7, CH_2	41.9, CH_2	40.2, CH_2	40.5, CH_2	73.4, CH_2	68.8, CH_2	73.4, CH_2	73.6, CH_2
13	26.4, CH_3	30.5, CH_3	25.8, CH_3	26.3, CH_3	25.7, CH_3	22.3, CH_3	22.4, CH_3	22.2, CH_3	22.1, CH_3
14	20.6, CH_3	22.3, CH_3	17.1, CH_3	16.0, CH_3	14.7, CH_3	20.6, CH_3	20.4, CH_3	15.8, CH_3	65.9, CH_2
15	30.6, CH_3	20.4, CH_3	71.9, CH_2	72.4, CH_2	28.2, CH_3	31.9, CH_3	31.8, CH_3	73.4, CH_2	73.2, CH_2

[a]150 MHz.

ppm. Compound **8** showed an HREIMS molecular ion peak at m/z 238.1976, consistent with the molecular formula $C_{15}H_{26}O_2$. An IR spectrum showing a broad band absorption at 3230 cm^{-1} and ^{13}C NMR signals at δ_C 72.4 and 73.1 ppm suggested the presence of two hydroxyl groups in the molecule.

HMBC correlations of carbon resonances at δ_C 215.6 and 73.1 ppm for compounds **7** and **8**, respectively, are consistent with the assignment of these resonances to carbon C-9 in each compound (quaternary carbon for **7** and methyne for **8**). ^{13}C NMR resonances at δ_C 71.9 and 72.4 ppm for compounds **7**

dx.doi.org/10.1021/np3009013 | J. Nat. Prod. 2013, 76, 1016−1024

and **8**, respectively, are assigned to methylene groups that show HMBC correlations with H-3a, H-5β, and H_3-14 for **7** and H-3a,b and H_3-14 for **8**. These observations are consistent with the assignment of these carbon resonances to C-15 in each compound. Further confirmation of these assignments is supported by NOESY correlations among H_2-15 and H-5β and H_3-14 in both **7** and **8**. On this basis, the structures of these metabolites were determined as (4*R*,8*S*)-15-hydroxyisocaryolan-9-one (**7**) and (4*R*,8*S*,9*R*)-isocaryolane-9,15-diol (**8**), respectively.

Compound **9** presented an HREIMS molecular ion peak at *m*/*z* 238.1027, consistent with the formula $C_{15}H_{26}O_2$, and an IR absorption band at 3362 cm^{-1}, which reveals the presence of two hydroxyl groups. One of them is located at C-9 (δ_C 72.8 ppm), as in the starting material. The corresponding H-9β showed COSY correlations with hydrogens on C-10. The second oxygenated function was found to be located at C-3. On one hand, in the COSY experiment, the signal at δ_H 3.41 ppm, a doublet, was correlated with H-2α. On the other hand, NOESY experiment correlations between this signal and H-5β and methyl groups CH_3-13 and CH_3-15 were consistent with α stereochemistry for the OH group. Therefore, this metabolite was assigned as (3*S*,8*S*,9*R*)-isocaryolane-3,9-diol (**9**).

Finally, (8*R*,9*R*)-isocaryolane-8,9-diol (**6**), identified by comparison with authentic samples and its spectroscopic data,[28] was also isolated as a metabolite of (8*S*,9*R*)-isocaryolan-9-ol (**4a**).

Feeding of (1*S*,2*S*,5*R*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.$0^{2,5}$]undecan-12-ol (**5a**) yielded compound **10** as the major metabolite after 5 (4% yield) and 9 (10% yield) days of incubation. Its HREIMS exhibited a molecular ion peak at *m*/*z* 238.1933 consistent with the formula $C_{15}H_{26}O_2$. Resonances in the ^{13}C NMR spectrum of compound **10** at δ_C 73.4 and 72.5 ppm indicated two oxygenated functions in the molecule, the first related to the C-12 methylene alcohol of the starting material and the second to a hydroxylated methyne carbon, according to DEPT. This carbon was assigned to C-6 owing to a downfield displacement of C-5 and C-7 resonances from δ_C 46.1 and 24.3 ppm, respectively, in the starting material[28] to δ_C 53.9 and 44.8 ppm, respectively, in **10**. The ^1H NMR spectrum showed a signal at δ_H 3.78 ppm, which was assigned to H-6α. COSY correlations between the signals of H-6 and H-5β (δ_H 1.66−1.63), H-7α (δ_H 1.18), and H-7β (δ_H 1.91) were observed. The stereochemistry of the hydroxyl group at C-6 as β-OH has been confirmed by NOESY data on the basis of correlations observed between the H-6 signal (δ_H 3.78) and the methyl group H_3-14 (δ_H 1.14) and H-7α (δ_H 1.91) and between the latter and H_2-12 (δ_H 3.33). Signal assignments at δ_H 1.14 as H_3-14 and δ_H 1.10 as H_3-15 are supported by NOESY effects with H-2α and H_3-5β signals, respectively (see Figure 1). These data led to the assignment of the structure of this compound as (1*S*,2*S*,5*R*,6*S*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.$0^{2,5}$]undecane-6,12-diol (**10**).

Compound **11** possesses three hydroxyl groups, as shown by resonances at δ_C 72.0 (CH), 77.1 (CH), and 68.8 (CH_2) ppm, the latter referring to the hydroxymethylene group at C-12 already present in the starting material. The displacement of the C-9 chemical shift (δ_C 25.7 ppm in the starting material and δ_C 77.1 ppm in **11**), as well as the downfield resonances of C-8 (δ_C 48.0) and C-10 (δ_C 55.6), were indicative of a C-9 hydroxylation. COSY correlations were consistently found between H-9 and both H-10 (δ_H 1.36 and 1.93 ppm). The third

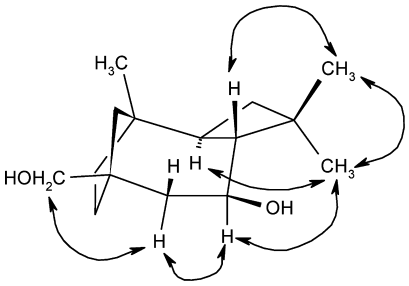


Figure 1. Selected NOESY enhancements for compound **10**.

hydroxyl group (δ_C 72.0, δ_H 3.76 ppm) was located at C-6, as in compound **10**, due to the downfield shifts of C-5 and C-7 and to the COSY correlations between the H-6 signal at δ_H 3.76 ppm and the resonances of H-7α (δ_H 2.20 ppm), H-7β (δ_H 0.86 ppm), and H-5β (δ_H 1.69 ppm). The stereochemistry of the C-6 and C-9 hydroxyl groups was determined as β based on a series of NOESY correlations observed between H-6α, C$\underline{H}_3$-14, and H-9α and between H-9α, H-6α, and H-2α. Lastly, the assignment of H_3-14 as a signal at δ_H 1.12 is supported by NOESY effects between this methyl and H-2α and H-6α (see Figure 2). This compound was therefore assigned as (1*R*,2*S*,5*R*,6*S*,8*R*,9*S*)-8-methylene-1,4,4-trimethyltricyclo-[6.2.1.$0^{2,5}$]undecane-6,9,12-triol (**11**).

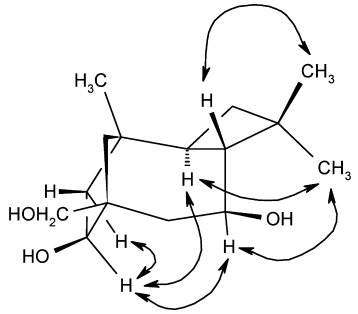


Figure 2. Selected NOESY enhancements for compound **11**.

Compound **12** presented an HREIMS molecular ion peak at *m*/*z* 254.1886, consistent with the formula $C_{15}H_{26}O_3$, which indicates a trihydroxylated derivative. Its ^{13}C NMR spectrum featured one methine hydroxylated carbon (δ_C 70.0 ppm) and two hydroxyl-methylene groups (both at δ_C 73.4 ppm). As with the previous assignments, two of the hydroxyl groups were located at C-12 (δ_C 73.4 ppm) (from the starting material) and at C-6 (δ_C 70.0 ppm; δ_H 3.69 ppm, H-6α). COSY correlations between H-6α and H-5β (δ_H 1.78 ppm) and between H-7α (δ_H 1.97 ppm) and H-7β (δ_H 1.26−1.23 ppm), as well as NOESY interactions between H-6α and CH_3-14α (δ_H 1.21 ppm), H-7α (δ_H 1.97 ppm), and H-2α (δ_H 1.86 ppm), were fully consistent with this assignment (Figure 3). These NOESY interactions, together with those observed between CH_3-14α and C$\underline{H}$H-15a (δ_H 3.43 ppm) and CH$\underline{H}$-15b (δ_H 3.32 ppm), on one hand, and between CH$\underline{H}$-15b (δ_H 3.32 ppm) and H-5β (δ_H 1.78 ppm) on the other, allowed the assignment of the remaining hydroxylation point at CH_2-15β. These data confirmed the structure of this metabolite as (1*S*,2*S*,4*R*,5*R*,6*S*,8*S*)-1,4-dimethyl-4,8-dimethylenetricyclo[6.2.1.$0^{2,5}$]undecane-6,12,15-triol (**12**).

Compound **13** showed an HREIMS molecular ion peak at 254.1880, also consistent with the formula $C_{15}H_{26}O_3$. Analysis

dx.doi.org/10.1021/np3009013 | *J. Nat. Prod.* 2013, 76, 1016−1024

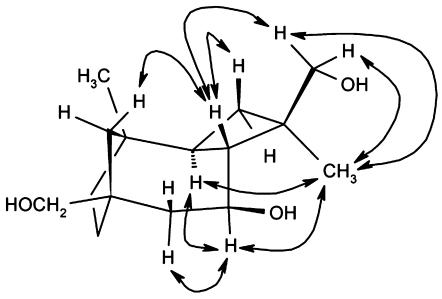


Figure 3. Selected NOESY enhancements for compound **12**.

of its ^{13}C NMR spectrum confirmed the presence of three hydroxymethylene groups at δ_C 65.9, 73.2, and 73.6 ppm, respectively. Analysis of 2D-NMR data suggested that both methyls on the *gem*-dimethyl group at **5a** had undergone hydroxylation. HMBC correlations among oxygen-substituted methylene signals at δ_C 65.9 ppm and signals at δ_H 1.70 (H-3α) and 1.33 (H-3β) and NOESY correlations of signals at δ_H 4.02 (CHH-14a) and 3.90 (CHH-14b) with those at δ_H 1.74 (H-2α) and 1.70 (H-3α), support the assignment of the hydroxymethylene group as CH_2-14α. Furthermore, HMBC correlations between the signals at δ_C 73.2 ppm (C-15) and those at δ_H 1.70 (H-3α) and 1.33 (H-3β) and NOESY correlations between signals at δ_H 3.71, 3.66 (H_2-15a,b), 1.78 (H-5β), and 1.33 (H-3β) led to the assignment of this hydroxymethylene group as CH_2-15β. The spectroscopic data support the proposed structure of this product as (1*S*,2*S*,5*R*,8*S*)-1-methyl-4,4,8-trimethylenetricyclo[6.2.1.02,5]-undecane-12,14,15-triol (**13**).

As described above, incubation of isocaryolane derivatives (8*S*,9*R*)-isocaryolan-9-ol (**4a**) and (1*S*,2*S*,5*R*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]undecan-12-ol (**5a**) with *B. cinerea* yielded compounds derived from oxidation of inactivated saturated positions, including the bridge-head position C-8 (compound **6**, 15% from **4a**). This compound is also obtained from the biotransformation of (8*R*,9*R*)-8-methoxyisocaryolan-9-ol (**3**) through ether cleavage on the methoxy substituent, as described previously (compound **6**, from **3**, 25% yield, 5 days feeding).[24]

Furthermore, further oxidation at position C-12 of metabolites of **5a** is not observed, and there is only one metabolite derived from **4a**, where C-9 shows further oxidation (compound **7**). This suggests that the hydroxyl group at these positions may be interacting with a binding site in the enzymatic complex responsible for hydroxylations. Occurrence of compound **7** may be explained by further oxidation of compound **8**, where binding on the enzymatic complex would take place at the hydroxyl group on C-15.

The antifungal properties of compounds **4a** and **5a** were determined against the growth of *B. cinerea* using the poisoned food technique[34] and compared with the previously reported activity for compounds **3**, **6**, and (4*R*,8*R*,9*R*)-8-methoxyisocaryolane-9,15-diol (**14**), a metabolite of **3**.[24] The commercial fungicide Euparen (dichlofluanid) was used as a standard for comparison in this test. Several levels of inhibition were observed (Figure 4). Compound **5a** showed inhibition levels similar to those reported for compounds **3** and **6**. Compound **4a** showed a lower activity level comparable to that reported for compound **14**.[24]

Therefore, the presence of an oxygen-bearing functional group at C-8 is essential for the antifungal activity of

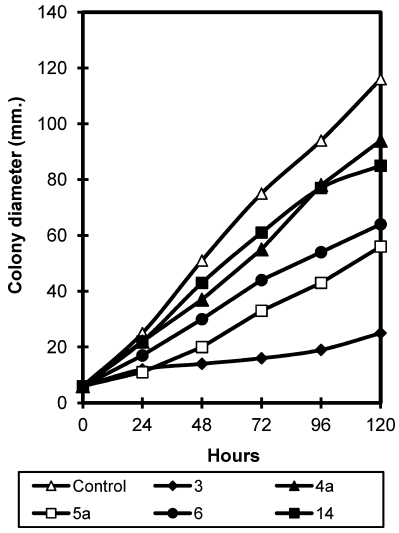


Figure 4. Comparison of fungal growth inhibition (*B. cinerea*) among compounds **3**, **4a**, **5a**, **6**, and **14** (100 ppm dose; **3** (0.40×10^{-3} M), **4a**, **5a** (0.45×10^{-3} M), **6** (0.42×10^{-3} M), and **14** (0.37×10^{-3} M)).

isocaryolane derivatives. Compound **5a**, more persistent than compound **4a**, **3**, or **6**,[35] bears a CH_2OH moiety at an equivalent position and maintains a similar activity to that of diol **6**.

The effect of previously reported (8*R*,9*R*)-8-methoxyisocaryolan-9-ol (**3**) and (8*R*,9*R*)-isocaryolane-8,9-diol (**6**), their metabolites **14**, (4*R*,8*R*,9*R*)-isocaryolane-8,9,15-triol (**15**), (3*S*,8*R*,9*R*)-isocaryolane-3,8,9-triol (**16**), and (6*R*,8*S*,9*R*)-isocaryolane-6,8,9-triol (**17**),[24] starting materials **4a** and **5a**, and biotransformation products **6**–**8** and **10** on germination (Figure 5) and radicle and shoot growth (Figure 6) of *L. sativa* (lettuce) was evaluated. *L. sativa* seeds have proven to be suitable for use in bioassays to evaluate phytotoxicity, specifically focusing on allelochemical detection, because they

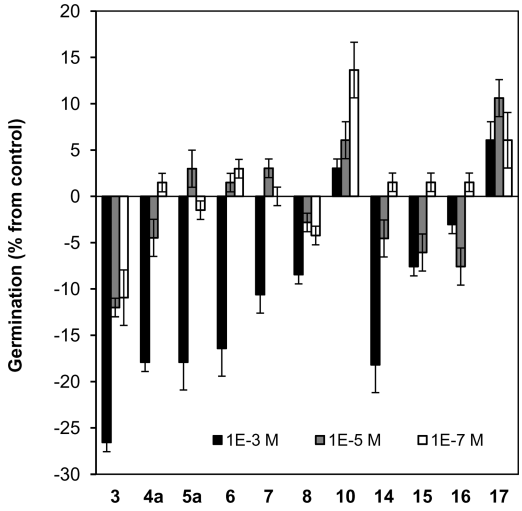


Figure 5. Effect of isocaryolane derivatives **3**, **4a**, **5a**, **6**–**8**, **10**, and **14**–**17** on germination of *L. sativa* (see biotransformation compounds in Table 1). Values are presented as percentage differences from the control: zero representing an observed value identical to the control, a positive value representing stimulation, and a negative value representing inhibition.

dx.doi.org/10.1021/np3009013 | *J. Nat. Prod.* 2013, 76, 1016–1024

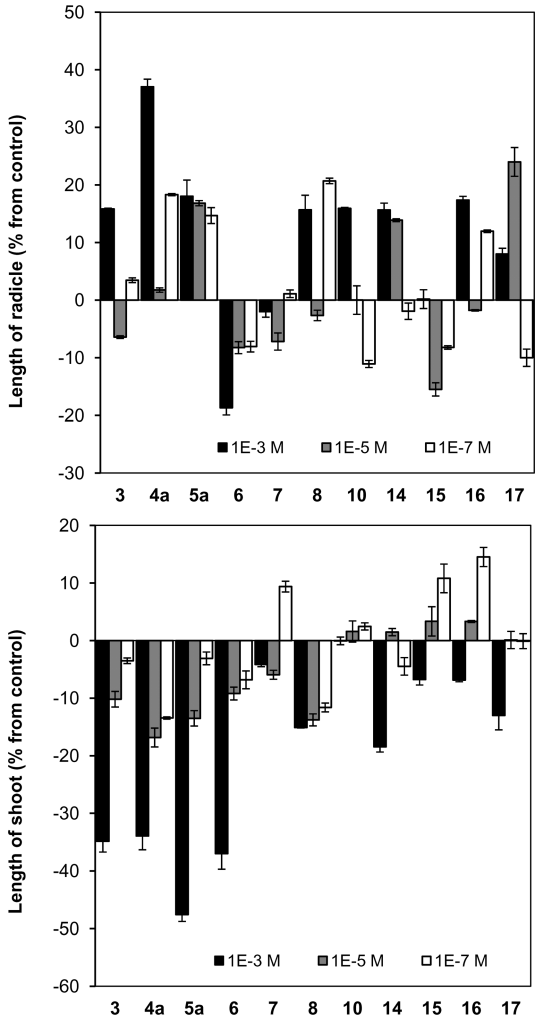
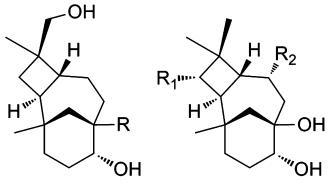


Figure 6. Effect of isocaryolane derivatives **3**, **4a**, **5a**, **6**–**8**, **10**, and **14**–**17** on *L. sativa* radicle and shoot length (see biotransformation compounds in Table 1). Values are presented as percentage differences from the control: zero representing an observed value identical to the control, a positive value representing stimulation, and a negative value representing inhibition.

are readily available and are characterized by swift, complete, and uniform germination.[32c]



14 R=OCH_3
15 R=OH
16 R_1=OH, R_2=H
17 R_1=H, R_2=OH

Predominance of germination inhibitory effect was observed at the higher concentration used (10^{-3} M). Compound **3** exhibited a consistent inhibitory effect on germination for all the concentrations evaluated, somewhat higher at 10^{-3} M (ca. 25%) (Figure 5). Compounds **14**, **15**, and **16** inhibited germination at 10^{-3} and 10^{-5} M, while compound **17** exhibited a stimulatory effect at all the concentrations evaluated.

Isocaryolane skeleton compounds **4a** and **6** and tetramethyltricyclo[6.2.1.$0^{2,5}$]undecane skeleton derivative **5a** have a similar effect on germination at the 10^{-3} M level, while (8*R*,9*R*)-8-methoxyisocaryolan-9-ol (**3**) showed higher inhibition at the 10^{-3} M level. On the other hand, biotransformation derivatives **7** and **8** showed decreased inhibitory levels (Figure 3).

Interestingly, compound **5a** and its biotransformation derivative **10** exhibited opposite effects: whereas the starting material **5a** inhibited germination (at 10^{-3} M), metabolite **10**, containing a further hydroxylation at C-6 in relation to **5a**, showed a slight stimulatory effect at all tested concentrations (Figure 5).

Postgermination effects were generally greater than those observed for germination. Regarding radicle and shoot growth, the tested compounds generally had the opposite effect, i.e., stimulation of radicle growth and inhibition of shoot growth, with the exception of compound **6**, which inhibited the growth of both shoots and radicles.

Compounds **3**, **4a**, **5a**, **6**, and **8** inhibited *L. sativa* shoot growth, this effect being concentration dependent. Biotransformation products **7**, **10**, **14**, **15**, **16**, and **17** showed similarly lower inhibitory effects at concentrations of 10^{-3} M. A clear pattern could not be drawn from observations at lower concentrations (Figure 6).

A greater inhibition effect on root growth was observed for **6** at 10^{-3} M, and **4a** had the highest stimulatory effect on root growth at 10^{-3} M. Behavior at lower concentrations was more erratic but generally had a mild stimulatory effect.

If we compare the fungal growth inhibition assay and the phytotoxicity data, compounds **3** and **4a**, along with their main metabolites **6**, **7**, and **14**, are moderately phytotoxic to *L. sativa* at a concentration (10^{-3} M) comparable to that tested against *B. cinerea* (0.37×10^{-3} to 0.42×10^{-3} M range; see Figure 4); in any case, their phytotoxicity decreases sharply at lower concentrations, i.e., at a natural concentration for biotransformation compounds. Therefore, the metabolism of isocaryolane skeleton compounds by *B. cinerea* probably does not increase phytotoxicity. Compound **5a** has greater phytotoxicity potential and was the strongest inhibitor of shoot development, reducing growth by nearly 50% at 10^{-3} M, and it is not as readily metabolized by the fungus as compounds **3**, **4a**, and **6**.[35] On the other hand, compound **5a** managed to promote only a modest increase in radicle growth at 10^{-3} M, and its main metabolite (compound **10**) is not phytotoxic.

In conclusion, the biotransformation of (8*S*,9*R*)-isocaryolan-9-ol (**4a**) and (1*S*,2*S*,5*R*,8*S*)-8-methylene-1,4,4-trimethyltricyclo[6.2.1.$0^{2,5}$]undecan-12-ol (**5a**) by *B. cinerea* produced a series of hydroxylated compounds with different levels of phytotoxicity against *L. sativa* and growth inhibition of *B. cinerea*. Compound **4a** was hydroxylated at C-3 (α position), C-6 (α position), and C-8 and C-15 (compounds **6**–**9**), while compound **5a** was hydroxylated at C-6 (β position), C-9 (β position), and C-14 and C-15 (compounds **10**–**13**). These hydroxylation patterns are consistent with those previously described in the biotransformation of (8*R*,9*R*)-8-methoxyisocaryolan-9-ol (**3**) and (8*R*,9*R*)-isocaryolane-8,9-diol (**6**).[24] Compound **6**, also a *B. cinerea* metabolite of (8*S*,9*R*)-isocaryolan-9-ol (**4a**), shows activity compared to its parent compound **4a**, which is inactive. Therefore, the presence of an oxygenated function at C-8 is essential for antifungal activity on isocaryolane derivatives.

Compounds **4a** and **5a** and their main metabolites exhibit a moderate phytotoxic effect on *L. sativa* but with little effect at concentrations found naturally for biotransformation com-

dx.doi.org/10.1021/np3009013 | *J. Nat. Prod.* 2013, 76, 1016–1024

pounds. Therefore, active isocaryolane derivatives are models of antifungal agents against *B. cinerea*, whose phytotoxicity against *L. sativa* is not enhanced by metabolism by the fungus.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured with a Reichert-Jung Kofler block and are uncorrected. Optical rotations were determined on a Perkin-Elmer 341 polarimeter. IR spectra were recorded on a Perkin-Elmer Spectrum BX FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on Varian INOVA 400 and INOVA 600 NMR spectrometers using tetramethylsilane as an internal reference. NMR assignments were made by a combination of 1D and 2D techniques and by comparison with assignments available in the literature for previously described compounds, where appropriate. Mass spectra were recorded on a Finnigan Voyager spectrometer at 70 eV. High-resolution mass spectra were recorded on a Micromass Autospec spectrometer at 70 eV. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with a UV−vis detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 F254, 0.2 mm thick. Silica gel (Merck) was used for column chromatography. Purification by HPLC was performed using a Si gel column (LiChrospher Si 60, 10 μm, 1 cm wide, 25 cm long).

Microorganism and Antifungal Assays. The culture of *Botrytis cinerea* strain UCA 992 employed in this work was isolated from Domecq vineyard grapes, Jerez de la Frontera, Cádiz, Spain. This culture of *B. cinerea* has been deposited at the Mycological Herbarium Collection (UCA), Facultad de Ciencias, Universidad de Cádiz. Antifungal bioassays were performed by measuring radial growth on agar medium in a Petri dish in the presence of test compounds. Test compounds were dissolved in EtOH to a final compound concentration in the culture medium of 50 to 200 mg L^{-1}. Solutions of test compounds were added to glucose-malt-peptone-agar medium (61 g of glucose-malt-peptone-agar per L, pH 6.5−7.0). The final EtOH concentration was identical in both the control and treated cultures. The medium was poured in 6 or 9 cm diameter sterile plastic Petri dishes, and a 5 mm diameter mycelia disk of *B. cinerea* cut from an actively growing culture was placed in the center of the agar plate. Radial growth was measured for six days. Every concentration was evaluated in triplicate.

General Culture Conditions. *B. cinerea* (UCA 992) was grown on a Czapek-Dox medium composed of (per L of distilled water), glucose (40 g), yeast extract (1 g), potassium dihydrogen phosphate (5 g), sodium nitrate (2 g), magnesium sulfate (0.5 g), ferrous sulfate (10 mg), and zinc sulfate (5 mg) in surface and shaking conditions. Surface culturing was carried out in Roux bottles (150 mL per flask), and shaken experiments were conducted using conical flasks (200 mL per flask). In both cases, growth was carried out at 25 °C for 4 days, and then the substrate, dissolved in ethanol, was added to each flask. Incubation lasted 5 or 9 days, after which the mycelium was filtered and washed with brine and EtOAc. The broth was saturated with sodium chloride, acidified (pH 2), and extracted with EtOAc. The extracts were separated into acidic and neutral fractions with aqueous sodium bicarbonate. The acid fraction was recovered in EtOAc. The extracts were dried over sodium sulfate, the solvent was evaporated, and the residues were chromatographed using silica gel and a gradient mixture of petroleum ether−EtOAc of increasing polarity. The acidic fractions were methylated with diazomethane prior to chromatography. Chromatography of the acidic fractions yielded inseparable mixtures.

Biotransformation of (8*S*,9*R*)-Isocaryolan-9-ol (4a) by *B. cinerea*. Compound **4a** (300 mg) was distributed in 20 flasks. Cultures were grown in shaken conical flasks for 5 and 9 days. Chromatography of the neutral fraction (5 days, 10 flasks) gave **4a** (40 mg), **6** (19 mg), **7** (3 mg), **8** (3 mg), **9** (2 mg), and dihydrobotrydial (4 mg). Chromatography of the neutral fraction (9 days, 10 flasks) gave **4a** (34 mg), **6** (10 mg), **7** (3 mg), **8** (6 mg), **9** (4 mg), and dihydrobotrydial (13 mg).

Biotransformation of (1*S*,2*S*,5*R*,8*S*)-8-Methylene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]undecan-12-ol (5a) by *B. cinerea*. Compound **5a** (330 mg) was distributed in 22 Roux bottles. Cultures were grown for 5 and 9 days. Chromatography of the neutral fraction (5 days, 11 flasks) gave **5a** (78 mg), **10** (7 mg), **11** (<1 mg), **12** (<1 mg), **13** (1 mg), and dihydrobotrydial (17 mg). Chromatography of the neutral fraction (9 days, 11 flasks) gave **5a** (24 mg), **10** (18 mg), **11** (1 mg), **12** (<1 mg), **13** (1 mg), and dihydrobotrydial (41 mg).

Bioassay for Germination and Growth of Lettuce Seeds. *Lactuca sativa* (cv. Grand Rapids) seeds were purchased from Isla Pak, RS, Brazil. All undersized and damaged seeds were discarded. The bioassay was conducted in 100 mm Petri dishes containing Whatman #1 filter paper (90 mm) as a support. *L. sativa* seeds (25 per dish), controls, and test compound solutions (10 mL) at concentrations of 10^{-3}, 10^{-5}, and 10^{-7} M were placed on the dishes. All solutions were prepared with deionized water, and the pH values, buffered with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), were adjusted to 6.0−6.5 with NaOH solution. Concentrations lower than 10^{-3} M were obtained by serial dilution. Dishes were wrapped with Parafilm to reduce evaporation and incubated in the dark at 25 °C in an environmental chamber. After 5 days, germinated seeds were counted (a seed was considered to be germinated when the radicle was at least 0.2 mm long) and the lengths of radicle and shoots were measured using a pachymeter. Dishes were kept at 4 °C during the measurement process to prevent subsequent growth. Osmotic pressure values were measured on a microsmometer and ranged between 30 and 38 mOsmolar.[32b] The experiment was carried out in triplicate.

Data Analysis. The effects of the test compounds on *L. sativa* germination and growth are given as percent differences from control, calculated from the differences (in cm) between mean values obtained upon addition of test compounds and mean values obtained for control (seeds grown without addition of tested compounds)/mean values for control × 100. Thus, zero represents the control, positive values represent stimulation of the studied parameter, and negative values represent inhibition. The data were evaluated using Student's *t* tests, and the differences between the experiment and control were significant at a value of $p \leq 0.0517$.

(4R,8S)-15-Hydroxyisocaryolan-9-one (7): oil; $[\alpha]^{25}_D$ −10 (*c* 0.22, $CDCl_3$); IR (film) ν_{max} 3454, 2933, 1695, 1458, 1038, 517 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 2; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 3; HMBC (selected correlations) C_2 → H_3-13, H12a,b ; C_3 →, H_3-14, H_2-15; C_9 → H-10a,b, H-11a,b; C_{10} → H-8, H-11a,b; C_{12} → H-3a,b, H-7a,b; C_{15} → H-3a, H-5β, H_3-14; EIMS *m*/*z* 236 [M]$^+$ (5), 218 (29), 179 (17), 165 (58), 147 (66), 122 (58), 109 (82), 95 (92), 81 (100), 55 (92); HREIMS *m*/*z* 236.1779 [M]$^+$ (calcd for $C_{15}H_{24}O_2$, 236.1776).

(4R,8S,9R)-Isocaryolane-9,15-diol (8): amorphous solid; $[\alpha]^{25}_D$ +22 (*c* 0.15, $CHCl_3$); IR (film) ν_{max} 3230, 2922, 1474, 1094 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 2; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 3; HMBC (selected correlations) C_1 → H_3-13, H-3a,b ; C_3 → H-5β, H_3-14, H_2-15; C_5 → H_3-14, H_2-15; C_7 → H-9β; C_9 → H-10a,b, H-11a,b; C_{14} → H-5β, H_2-15, H-3a,b; C_{15} → H_3-14, H-3a,b; EIMS *m*/*z* 238 [M]$^+$ (8), 220 (31), 175 (32), 149 (34), 118 (36), 109 (41), 82 (100), 81 (49), 41 (41); HREIMS *m*/*z* 238.1976 [M]$^+$ (calcd for $C_{15}H_{26}O_2$, 238.1933).

(3S,8S,9R)-Isocaryolane-3,9-diol (9): oil; $[\alpha]^{25}_D$ +6 (*c* 0.43, $CHCl_3$); IR (film) ν_{max} 3362, 2937, 1458,1054, 756 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz), see Table 2; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 3; HMBC (selected correlations) C_1 → H-3α, H-5β; C_2 → H_3-13, H-11a,b, H-6b, H-12a,b; C_3 → H-5β, H_3-14, H_3-15; C_6 → H-2α, H-8; C_7 → H-12a,b, H-9β; C_9 → H-7a,b, H-12ab; C_{10} → H-8; C_{14} → H-5β, H_3-15; EIMS *m*/*z* 238 [M]$^+$ (4), 220 (18), 193 (35), 174 (23), 148 (33), 135 (25), 107 (42), 93 (53), 82 (100), 72 (60), 69 (51), 41 (43); HREIMS *m*/*z* 238.1027 [M]$^+$ (calcd for $C_{15}H_{26}O_2$, 238.1933).

(1S,2S,5R,6S,8S)-8-Methylene-1,4,4-trimethyltricyclo[6.2.1.0^{2,5}]-undecane-6,12-diol (10): yellow oil; $[\alpha]^{25}_D$ +9 (*c* 0.46, $CDCl_3$); IR (film) ν_{max} 3361, 2948, 2864, 1458, 1037, 756 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz), see Table 2; ^{13}C NMR ($CDCl_3$, 150 MHz), see Table 3; HMBC (selected correlations) C_1 → H-9a,b; C_2 → H-11a,b; C_3 → H_3-14, H_3-15, C_5 → H-7α, H-3α, H-2α, H_3-14, H_3-15; C_7 →,

dx.doi.org/10.1021/np3009013 | *J. Nat. Prod.* 2013, 76, 1016−1024

H_2-12, H-5β, H-9a,b; C_9 → H_2-12, H-7α; C_{10} → H_3-13, H-11a,b; EIMS m/z 238 $[M]^+$ (6), 230 (18), 220 (24), 207 (78), 168 (38), 123 (42), 118 (52), 95 (54), 81 (62), 68 (100), 43 (44); HREIMS m/z 238.1933 $[M]^+$ (calcd for $C_{15}H_{26}O_2$, 238.1933).

*(1R,2S,5R,6S,8R,9S)-8-Methylene-1,4,4-trimethyltricycle-[6.2.1.0^{2,5}]undecane-6,9,12-triol (**11**):* oil; $[\alpha]^{25}_D$ +23 (c 0.12, $CDCl_3$); IR (film) ν_{max} 3421, 2948, 1462, 1042, 589 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz), see Table 2; ^{13}C NMR ($CDCl_3$, 150 MHz), see Table 3; HMBC (selected correlations) C_2 → H_3-13, H-11a,b; C_3 → H_3-14, H_3-15; C_5 → H-3a, H_3-14, H_3-15, H-7α; C_7 → H-5β, H-11a,b; C_{10} → H-2α, H_3-13; C_{12} → H-7α, H-7β; EIMS m/z 254 $[M]^+$ (0.8), 236 (6), 221 (23), 192 (35), 149 (43), 136 (60), 109 (99), 95 (86), 93 (92), 85 (100), 71 (43), 55 (70); HREIMS m/z 236.1772 $[M - 18]^+$ (calcd for $C_{15}H_{24}O_2$, 236.1776).

*(1S,2S,4R,5R,6S,8S)-1,4-Dimethyl-4,8-dimethylenetricycle-[6.2.1.0^{2,5}]undecane-6,12,15-triol (**12**):* oil; $[\alpha]^{25}_D$ +49 (c 0.14, $CDCl_3$); IR (film) ν_{max} 3377, 2944, 1457, 1034, 756 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz), see Table 2; ^{13}C NMR ($CDCl_3$, 150 MHz), see Table 3; HMBC (selected correlations) C_1 → H-5β, H-3a, H_3-13; C_5 → H-7α, H_3-14, H_2-15; C_7 → H_2-12; C_9 → H_2-12; C_{10} → H-11a, H_3-13; EIMS m/z 254 $[M]^+$ (5), 223 (28), 205 (26), 187 (15), 151 (35), 128 (44), 121 (43), 111 (76), 107 (93), 95 (84), 93 (100), 81 (68), 67 (29), 55 (33); HREIMS m/z 254.1886 $[M]^+$ (calcd for $C_{15}H_{26}O_3$, 254.1882).

*(1S,2S,5R,8S)-1-Methyl-4,4,8-trimethylenetricycle[6.2.1.02,5]-undecane-12,14,15-triol (**13**):* oil; $[\alpha]^{25}_D$ +32 (c 0.1, $CDCl_3$); IR (film) ν_{max} 3344, 2929, 1452, 1028, 752 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 2; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 3; HMBC (selected correlations) C_3 → H_2-14; H_2-15; C_5 → H_2-14, H_2-15; C_7 → H-11a, H_2-12; C_9 → H-7b, H-11a, H_2-12; C_{13} → H-11b; EIMS m/z 254 $[M]^+$ (1), 223 (11), 205 (22), 187 (25), 165 (46), 135 (100), 107 (85), 93 (82), 81 (59), 67 (24), 55 (22); HREIMS m/z 254.1880 $[M]^+$ (calcd for $C_{15}H_{26}O_3$, 254.1882).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **7**−**13**. This information is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(A.J.M.-S.) Tel: +34-956-016368. Fax: +34-956-016193. E-mail: antoniojose.macias@uca.es. (I.G.C.): Tel: +34-956-016368. Fax: +34-956-016193. E-mail: isidro.gonzalez@uca.es.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Use of NMR facilities at Servicio Centralizado de Ciencia y Tecnología (SCCYT) of the University of Cádiz is acknowledged. This research was supported by grants from MEC (AGL2012-39798-C02-01 and PHB2008-0067-PC). J.A. and J.A.T thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for research fellowships (CAPES-DGU 197/09).

■ REFERENCES

(1) (a) Oerke, E. C.; Dehne, H. W.; Schonbeck, F.; Weber, A. *Crop Protection and Crop Production*; Elsevier: Amsterdam, 1994. (b) Oerke, E. C. *J. Agric. Sci.* **2006**, *144*, 31−43.

(2) Fungicide Resistance Action Committee. http://www.frac.info/index.htm (accessed March 19, 2013).

(3) (a) Petit, A. N.; Fontaine, F.; Clement, C.; Vaillant-Gaveau, N. *J. Agric. Food Chem.* **2008**, *56*, 6761−6767. (b) European Food Safety Authority.. *EFSA Sci. Rep.* **2009**, *305*, 1−106.

(4) Mendes, C. A. C.; Mendes, G. E.; Cipullo, J. P.; Burdmann, E. A. *Clin. Toxicol.* **2005**, *43*, 117−118.

(5) Dias, M. C. *J. Bot.* **2012**, DOI: 10.1155/2012/135479.

(6) Leroux, P. *Pest. Sci.* **1996**, *47*, 191−197.

(7) Hueter, O. F. *Phytochem. Rev.* **2011**, *11*, 185−194.

(8) (a) Trichotecenes of *Fusarium* spp. (nonselective toxins): Desjardins, A. E.; Proctor, R. H.; Bai, G.; McCormick, S. P.; Shaner, G.; Buechley, G.; Hohn, T. M. *Mol. Plant−Microbe Interact.* **1996**, *9*, 775−781. (b) HC-toxin of the maize pathogen *Cochliobolus carbonum* (host selective): Walton, J. D. *Phytochemistry* **2006**, *67*, 1406−1413. (c) Polyketide of pathogen *Magnaporthe grisea* recognized by resistant rice (*Oryza sativa*) cultivars: Collemare, J.; Pianfetti, M.; Houlle, A. E.; Morin, D.; Camborde, L.; Gagey, M. J.; Barbisan, C.; Fudal, I.; Lebrun, M.-H.; Bohnert, H. U. *New Phytol.* **2008**, *179*, 196−208.

(9) Collado, I. G.; Macias-Sanchez, A. J.; Hanson, J. R. *Nat. Prod. Rep.* **2007**, *24*, 674−686.

(10) Rossi, F. R.; Gárriz, A.; Marina, M.; Romero, F. M.; Gonzalez, M. E.; Collado, I. G.; Pieckenstain, F. L. *Mol. Plant−Microbe Interact.* **2011**, *24*, 888−896.

(11) Siewers, V.; Viaud, M.; Jimenez-Teja, D.; Collado, I. G.; Gronover, C. S.; Pradier, J.-M.; Tudzynski, B.; Tudzynski, P. *Mol. Plant−Microbe Interact.* **2005**, *18*, 602−612.

(12) (a) Fehlhaber, H.-W.; Geipel, R.; Mercker, H.-J.; Tschesche, R.; Welmar, K.; Schönbeck, F. *Chem. Ber.* **1974**, *107*, 1720−1730. (b) Lindner, H. J.; Gross, B. v. *Chem. Ber.* **1974**, *107*, 3332−3336.

(13) (a) Hanson, J. R. *Pure Appl. Chem.* **1981**, *53*, 1155−1162. (b) Wang, S. C.; Tantillo, D. J. *Org. Lett.* **2008**, *10*, 4827−4830. (c) Pinedo, C.; Wang, C.-M.; Pradier, J.-M.; Dalmais, B.; Choquer, M.; Le Pecheur, P.; Morgant, G.; Collado, I. G.; Cane, D. E.; Viaud, M. *ACS Chem. Biol.* **2008**, *3*, 791−801.

(14) Caryophyllane: (a) Duran, R.; Corrales, E.; Hernandez-Galan, R.; Collado, I. G. *J. Nat. Prod.* **1999**, *62*, 41−44. (b) Duran, R.; Aleu, J.; Hernandez-Galan, R.; Collado, I. G. *J. Nat. Prod.* **2000**, *63*, 44−47.

(15) Clovane: (a) Collado, I. G.; Hanson, J. R.; Macias-Sanchez, A. J.; Mobbs, D. *J. Nat. Prod.* **1998**, *61*, 1348−1351. (b) Deligeorgopoulou, A.; Macias-Sanchez, A. J.; Mobbs, D. J; Hitchcock, P. B.; Hanson, J. R.; Collado, I. G. *J. Nat. Prod.* **2004**, *61*, 1348−1351. (c) Saiz-Urra, L.; Racero, J. C.; Macias-Sanchez, A. J.; Hernandez-Galan, R.; Hanson, J. R.; Perez-Gonzalez, M.; Collado, I. G. *J. Agric. Food Chem.* **2009**, *57*, 2420−2428.

(16) Ginsane: Aleu, J.; Hernandez-Galan, R.; Hanson, J. R.; Hitchcock, P. B.; Collado, I. G. *J. Chem. Soc., Perkin Trans. 1* **1999**, 727−730.

(17) Patchoulane: Aleu, J.; Hanson, J. R.; Hernandez-Galan, R.; Collado, I. G. *J. Nat. Prod.* **1999**, *62*, 437−440.

(18) Isoprobotryane: Collado, I. G.; Aleu, J.; Macias-Sanchez, A. J.; Hernandez-Galan, R. *J. Nat. Prod.* **1994**, *57*, 738−746.

(19) 1-Epiprobotryane: Collado, I. G.; Aleu, J.; Macias-Sanchez, A. J.; Hernandez-Galan, R. *J. Chem. Ecol.* **1994**, *20*, 2631−2644.

(20) Norprobotryane (norpresilphiperfolane): Collado, I. G.; Deligeorgopoulo, A.; Hanson, J. R.; Hitchcock, P. B.; Macias-Sanchez, A. J. *J. Chem. Res.* **2004**, 468−470.

(21) Daucane: Collado, I. G.; Hanson, J. R.; Macias-Sanchez, A. J.; Mobbs, D. J. *J. Chem. Res.* **2004**, 524−526.

(22) Eremophilane: Collado, I. G.; Deligeorgopoulou, A.; Hanson, J. R.; Hitchcock, P. B.; Macias-Sanchez, A. J. *J. Chem. Res.* **2004**, 527−529.

(23) Ginsane, patchoulane, isoprobotryane, cedrane, globulane: Aleu, J.; Hanson, J. R.; Hernandez-Galan, R.; Collado, I. G. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 329−334.

(24) Isocaryolane: Ascari, J.; Boaventura, M. A. D.; Takahashi, J. A.; Hernández-Galán, R.; Macías-Sánchez, A. J.; Collado, I. G. *J. Nat. Prod.* **2011**, *74*, 1707−1712.

(25) α-Tomatine: Quidde, T.; Osbourn, A.; Tudzynski, P. *Physiol. Mol. Plant Pathol.* **1998**, *52*, 151−165.

(26) 6-Pentyl-2*H*-pyran-2-one: Poole, P. R.; Whitaker, G. *J. Agric. Food Chem.* **1997**, *45*, 249−252.

(27) Activation of resveratrol: Schouten, A.; Wagemakers, L.; Stefanato, F. L.; van der Kaaij, R. M.; van Kan, J. A. L. *Mol. Microbiol.* **2002**, *43*, 883−894.
(28) Racero, J. C.; Macias-Sanchez, A. J.; Hernandez-Galan, R.; Hitchcock, P. B.; Hanson, J. R.; Collado, I. G. *J. Org. Chem.* **2000**, *65*, 7786−7791.
(29) Collado, I. G.; Hanson, J. R.; Hernandez-Galan, R.; Hitchcock, P. B.; Macias-Sanchez, A. J.; Racero, J. C. *Tetrahedron* **1998**, *54*, 1615−1626.
(30) Strange, R. N. *Nat. Prod. Rep.* **2007**, *24*, 127−144.
(31) Duran-Patron, R.; Cantoral, J. M.; Hernandez-Galan, R.; Hanson, J. R.; Collado, I. G. *J. Chem. Res.* **2004**, 441−443.
(32) (a) Macias, F. A.; Simonet, A. M.; Esteban, M. D. *Phytochemistry* **1994**, *36*, 1369−1379. (b) Macias, F. A.; Castellano, D. *J. Agric. Food Chem.* **2000**, *48*, 2512−2521. (c) Boaventura, M. A. D.; Pereira, R. G.; Freitas, L. B. O.; Reis, L. A.; Vieira, H. S. *J. Agric. Food Chem.* **2008**, *56*, 2985−2988. (d) Rocha, A. D.; dos Santos, G. C.; Fernandes, N. G.; Pfenning, L. H.; Takahashi, J. A.; Boaventura, M. A. D. *J. Nat. Prod.* **2010**, *73*, 1431−1433.
(33) Collado, I. G.; Hanson, J. R.; Macías-Sánchez, A. J. *Tetrahedron* **1996**, *52*, 7961−7972.
(34) Patil, I. S.; Kulkarni, S.; Hedge, R. K. *Pesticides* **1986**, 30−31.
(35) Conversion after 5 days of feeding for **5a** is only 50%, compared to more than 75% for **4a**, **3**, and **6** (see ref 24).