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Peer-reviewed version available at *Molecules* **2018**, *23*, 1529; doi:10.3390/molecules23071529

Article

(+)-*epi*-Epoformin, a phytotoxic fungal cyclohexenepoxide: Structure activity relationships

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Abstract: (+)-*epi*-Epoformin (**1**), is a fungal cyclohexene epoxide isolated together with diplopimarane and sphaeropsidins A and C, a *nor-ent*-pimarane and two pimaranes, from the culture filtrates of *Diplodia quercivora*, a fungal pathogen for cork oak in Sardinia, Italy. Compound **1** possesses a plethora of biological activities including: antifungal, zootoxic and phytotoxic activity. The last activity and the peculiar structural feature of **1** suggested to carry out a structure activity relationship study, preparing eight key hemisynthetic derivatives and their phytotoxicity was assayed. The complete spectroscopic characterization and the activity in the etiolated wheat coleoptile bioassay of all the compounds is reported. Most of the compounds inhibited growth and some of them had comparable or higher activity than the natural product and the reference herbicide Logran. As regards the structure-activity relationship, the carbonyl proved to be essential for their activity of **1**, as well as the conjugated double bond, while the epoxide could be altered with no significant loss.

Keywords: *Diplodia quercivora*; oak; *epi*-epoformin; cyclohexeneoxide; etiolated wheat coleoptile bioassay; phytotoxicity; allelopathy; SAR

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1. Introduction

(+)-*epi*-Epoformin (**1**, Figure 1) is a cyclohexene eoxide, isolated together with some *nor-ent*-pimaranes and pimarane diterpenes, namely diplopimarane and sphaeropsidins A and C, from culture filtrates of the fungus *Diplodia quercivora*, isolated from declining *Quercus canariensis* trees in Tunisia as a canker-causing agent [1]. The fungi belonging to *Diplodia* genus are well known as pathogens of forest plants and as producers of several bioactive metabolites belonging to different classes of natural compounds [2].

Compound **1** was also previously produced by an unidentified fungus isolated from a diseased leaf of crepe myrtle (*Lagerstroemia indica* L. Pers.) [3] and showed inhibition of lettuce seed germination. Its total enantioselective and asymmetric synthesis [4–7] was also developed as well as the assignment of its absolute configuration was determined by time-dependent density together with that of other close naturally occurring cyclohexene oxides [8].

When isolated from *D. quercivora,* **1** was tested for its phytotoxic activity using different bioassays. In leaf-puncture tests on holm oak (*Quercus ilex* L.), cork oak (*Quercus suber* L.), and tomato (*Lycopersicum esculentum* Mill.) leaves, **1** caused necrotic lesions on holm and cork oak (area lesion sizes of 6.7 and 9.5 mm^2, respectively), while induced irregular necrotic lesions (13.5 mm^2 area) on tomato. In a tomato cutting bioassay, **1** caused, to different extents, stewing on stem at 0.2 and 0.1 mg/mL. It also showed antifungal activity against some plant pathogens and *Phytophthora cinnamomi* and *Phytophthora plurivora* were the most sensitive target organisms followed by *Athelia rolfsii*, whereas *Diplodia corticola* was the most resistant as its mycelial growth was only slightly inhibited (39.7% inhibition) [1]. Finally, when assayed on brine shrimps (*Artemia salina* L.) **1** caused more than 50% larval mortality at 200 µg/ mL after 36 h to metabolites exposure [1].

More recently, **1** was also used in a preliminary screening to evaluate the potential of plant and fungal metabolites, belonging to different classes of natural compounds, for the biological control of several rusts species belonging to the genera *Puccinia* and *Uromices*, which are pathogens of very important crops including legumes. Compound **1** showed significant inhibition of all the species tested also exhibiting an effective penetration [9]. The potential fungicide activity of **1** also in plants against *U. pisi* and *P. triticina* was successively confirmed [10].

In this manuscript, **1** was isolated and tested with eight hemisynthetic derivatives (**2–9**) in order to obtain clues about the structure-activity (SAR) requirements of these compounds, where we acetylated and oxidized the hydroxy group, open the epoxide, reduce the carbonyl group or saturated the double bond.

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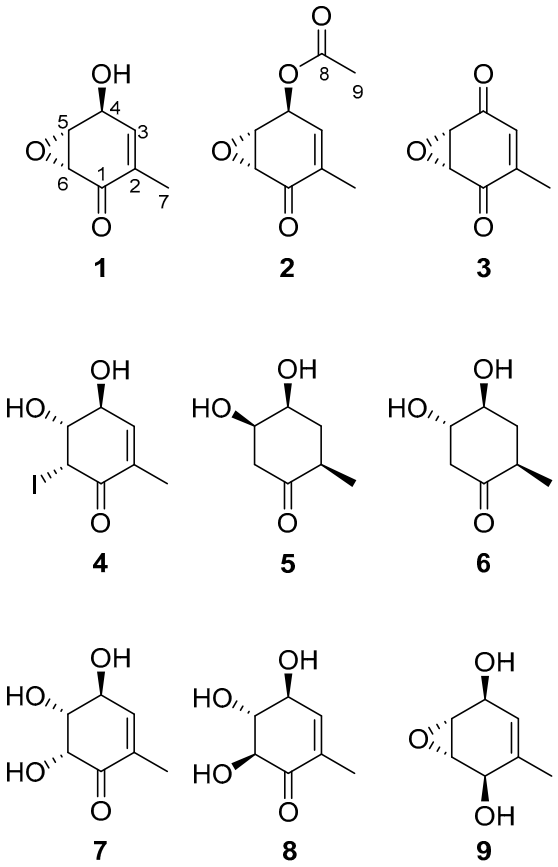

Figure 1. Structure of (+)-*epi*-epoformin (**1**) and derivatives (**2–9**).

2. Results and Discussion

The structure-activity relationship study of (+)-*epi*-epoformin (**1**) was carried out preparing eight of its hemisynthetic derivatives. Although **1** was previously described in the literature as a fungal phytotoxic compound [11], there is no information available regarding its activity in etiolated wheat coleoptiles. Thus, the phytotoxic activity of the parent compound (**1**) and its eight hemisynthetic derivatives was assayed using this test.

The culture filtrates of *D. quercivora* were extracted and purified as reported in detail in the section 3.2. **1** was obtained as a white solid and identified comparing its physical ($[\alpha]^{20}_D$) and spectroscopic (^{1}H and ^{13}C NMR and ESI-MS) properties with those previously reported [1,3].

The acetylation of **1** to obtain **2**, in a moderate yield (67%), was successfully carried out using acetic anhydride [12]. Its NMR spectra (section 3.4) showed that an acetyl group was incorporated to the compound by the new singlet at δ 2.13 (H-9) in the ^{1}H NMR and the corresponding carbons of the acetyl group in the ^{13}C NMR [13], with a carbonyl at δ 193.5 (C-8) and the carbon of the methyl at δ 20.7. A significant downfield shift (from δ 4.67 to δ 5.72) was also observed for H-4 when the ^{1}H NMR spectrum of **1** was compared to that of **2** [14]. In addition, the OH broad band of **1** disappeared in the FTIR spectrum. 2D NMR experiments [15] allowed confirming the structure of **2**. HSQC spectrum was employed to assign each carbon to their corresponding hydrogens and the HMBC showed the correlations to elucidate the position of the acetyl group: the signal at δ 2.13 (H-9) correlated with that at δ 64.5 (C-4), while the signal at δ 5.72 (H-4) correlated with that at δ 169.8 (C-8).

On the other hand, the oxidation of the hydroxyl group with Dess-Martin periodinane [16] was repeated several times changing the reaction times. The highest yield was 44% for **3**, which was obtained after 16 hours, recovering a 51% of the starting material (**1**). A longer reaction time did not significantly increase the yield and less starting material would be recovered. The success on synthesizing **3** was confirmed firstly by the absence of any hydroxyl group and the presence of two

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narrow bands at 1737 and 1715 cm^{-1} corresponding to the two conjugated carbonyls in the FTIR spectrum [17]. The NMR experiments showed two signals of conjugated carbonyl carbons in the ^{13}C NMR at δ 192.3 (C-1) and 191.2 (C-4) and the signal of the H-4 of **1** disappeared in the ^{1}H NMR. In the HMBC the correlation of the signal at δ 192.3 (C-1) with the methyl signal at δ 2.02 (H-7) was observed allowing to assign that signal to the C-1. Due to the proximity of the signals at δ 54.1(C-5) and δ 53.7 (C-6) the HSQC experiment was indispensable to assign them to their corresponding signals in the ^{1}H NMR at δ 3.79 (H-5) and δ 3.84 (H-6). These signals were assigned to their positions in the compounds thanks to the correlations in the COSY experiment, where the signal at δ 3.79 (H-5) correlated with the signal at δ 6.43 (H-3).

Following the procedure described by Nicolaou et al. [18] we attempted the opening of the epoxide to obtain a dihydroxylated compound in C-4 and C-5. Unexpectedly, the integrals of the ^{1}H NMR of the product showed that there was only one hydrogen at C-6 at δ 4.77 (H-6), too high for hydrogens in α position to the carbonyl at C-1. After studying all the NMR spectra we determined that the structure was that of **4**, obtained with a high yield (83%), but the compound did not ionize properly to observe the mass corresponding to the product. The presence of iodine in the compound was confirmed by the presence of an I^- ion in the MS (ES^-), with m/z 126.9045, acting as a leaving group in an intramolecular nucleophilic substitution, which leads to the corresponding neutral epoxide. The stereochemistry of the hydroxyl at C-5 was confirmed by the coupling constants value of H-5 at δ 2.80 with δ 4.77 (H-6) and δ 4.21 (H-4) being J_{56} = 4.1 Hz (equatorial-equatorial) and J_{45} = 7.7 Hz (axial-axial).

In order to study the influence of the double bond at C-2 in the bioactivity of **1**, this compound was treated with carbon supported palladium. After several tries, only traces of the expected compound with the epoxide at C-5 and the reduced double bond at C-2 were found. Instead, the major compounds were **5** and **6**, derived from the reductive opening of the epoxide that occurred at the same time that the reduction of the double bond. By using carbon supported platinum **5** and **6** were again the major products. Interestingly, by using Pd/C the yields were slightly higher for obtaining compounds **5** and **6** (29 and 33%, respectively) than by using Pt/C (19% and 21%, respectively). Only by direct infusion and solving the compounds in MeOH (0.1% formic acid) was possible to ionize these compounds, obtaining the $[M+Na]^+$ ions (*m*/*z* 167.3 for $C_7H_{12}O_3Na$). The lack of conjugation with a double bond in **5** and **6** moved the carbonyl bands to higher wavenumbers in the FTIR spectrum, from the starting 1674 cm^{-1} in **1** to 1704 and 1711 cm^{-1} in **5** and **6**, respectively. The ^{1}H NMR spectra of both compounds showed that the typical signal of H_2-3 moved to higher field, being located at δ 1.99 in **5** and δ 2.22 and 1.35 in **6**. Although the stereochemistry of the hydroxyl at H-5 could be assigned by using the values of coupling constants of H-5 with H-4 in both compounds (J_{45} = 4.3 Hz in **5** and J_{45} = 8.8 Hz in **6** indicating equatorial-axial and axial-axial couplings, respectively), NOE- and ROE-difference experiments were needed to assign the relative position of the methyl at C-2. By irradiating H-2, H-4 and H-5 in **5** with a ROE-1D experiment, it was confirmed that H-2 (δ 2.78) was close in space to H-4 (δ 4.02) and H-5 (δ 4.18). In addition, H-4 was close in the space to H-6a (δ 2.43). On the other hand, H-4, H-5 and H-3b were irradiated in **6** with a NOE-1D experiment. In this case, it was observed that the signal for H-3b (δ 1.35) was close in space with H-5 (δ 3.65). The latter was close to H-6b (δ 2.75) and H-4 (δ 3.88) was close in space to H-2 (δ 2.46) and H-3a (2.22), thus confirming the opposite stereochemistry of H-5 in **6**. There was one signal for each hydrogen at C-6 in the ^{1}H NMR of both compounds (δ 2.91 and δ 2.43 for **5**, and δ 2.75 and δ 2.46 for compound **6**). In the case of **6**, this was also observed for the hydrogens at C-3 (δ 2.22 and δ 1.35). The COSY and the HSQC experiments were used to assign each pair of signals to their corresponding carbons, while the HMBC allowed differentiating the close carbons at δ 74.0 (C-5, correlated with both H-6) and δ 73.8 (C-4 correlated with both H-3) in **6**.

The opening of the epoxide was performed using basic and acidic media (0.1 M NaOH, 0.1 M HCl and 0.05 M H_2SO_4) and none of them worked properly. Surprisingly, while using acyl chlorides with DMAP to acetylate the hydroxyl at C-4 using the Yamaguchi sterification [20], the epoxide open spontaneously, yielding both compounds **7** and **8** with high yields (39 and 45%, respectively) and no amount of the desired esters. At first, we checked the literature to compare the spectra of our

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trihydroxylated compounds with the already reported gabosines A and N [21], but the signals were different. Therefore, the spectra were analysed to determine their structures. Firstly, it was observed the opening of the epoxide in the shift of the signals to lower fields, from δ 3.78 (H-5) and δ 3.53 (H-6) to δ 4.05 and 4.57 (**7**) or δ 3.84 and 4.38 (**8**). H-5 and H-6 were assigned by observing the COSY, where H-5 and H-6 were correlated among themselves, and the HMBC where H-5 correlated with δ 69.3 (C-4), and H-6 correlated with δ 190.6 (C-1) and δ 74.6 (C-5) in the spectrum of **7**. The same correlations were found in **8**, H-5 and H-6 were correlated in the COSY, and in the HMBC H-5 correlated with δ 71.4 (C-4), and H-6 correlated with δ 189.7 (C-1) and δ 78.3 (C-5). Regarding the stereochemistry, the analysis of the NOE-difference experiments and the coupling constants [19] indicated that H-5 and H-6 of **7** were close in the space, H-5 had a high coupling constant with H-4 (J = 7.1 Hz) and a lower constant with H-6 (J = 3.5 Hz), indicating the 4*S*,5*R*,6*R* stereochemistry. Regarding **8**, the coupling constants of H-5 were with H-4 (J = 8.2 Hz) and H-6 (J = 11.3 Hz), both high, indicating axial-axial coupling in both cases and the 4*S*,5*R*,6*S* stereochemistry.

Finally, **1** was treated with $NaBH_4$ and $CeCl_3$ in MeOH to reduce the carbonyl without affecting the double bond or the epoxide, obtaining **9** in a moderate 39% yield. The FTIR showed clearly the presence of an OH broad band at 3234 cm^{-1} and the missing narrow band of the carbonyl. Regarding the ^{1}H NMR, all the signals shifted to a higher field. The lack of the carbonyl was clearly observed in the ^{13}C NMR with only one quaternary signal at δ 135.2 (C-2). The close signals of the epoxide hydrogens and carbons at positions 5 and 6 were assigned with the aid of the COSY, HSQC and HMBC experiments. The signal at δ 3.30 (H-5) correlated with δ 3.36 (H-6) in the COSY, with δ 51.9 in the HSQC, and was a dd with J = 3.6 (H-5 with H-4) and 1.5 Hz (H-5 with H-6). On the other hand, the signal at δ 3.36 (H-6) correlated with the signals at δ 3.30 (H-5) and δ 4.25 (H-1) in the COSY, with signals at δ 53.0 (C-6) in the HSQC, and with δ 135.2 (C-2) in the HMBC and was a dd with J = 3.5 (H-6 with H-1) and 1.5 Hz (H-6 with H-5). The signal for H-1 was assigned at δ 4.25 after the assignment of the signal at δ 4.46 to H-4 by observing the correlation of δ 4.46 (H-4) with δ 5.51 (H-3) in the COSY. The stereochemistry was assigned using the NOESY experiment, where δ 4.46 (H-4) and δ 4.25 (H-1) where correlated, meaning that they were close in the space. These data confirm the structure and stereochemistry shown in Figure 1 for **9**.

2.1. SAR study

The results from the etiolated wheat coleoptile bioassay of compounds **1–9** are showed in Figure 2. According to their IC_{50} values (Table 1), the most active compounds were **3** and then **4**. Compound **3** was one order of magnitude more active than **4** and comparable to the synthetic herbicide Logran®, however, the activity profile was inconsistent with the increasing concentration. The 50% inhibition for **3** was found at the lowest concentration tested (10 μM) and this value did not change significantly at higher concentrations. Although the test solutions of **3** were clear and no precipitate was observed, this is typically observed with problems of solubility at the highest concentration. If this or the nature of the compound caused the strange profile, it is unknown.

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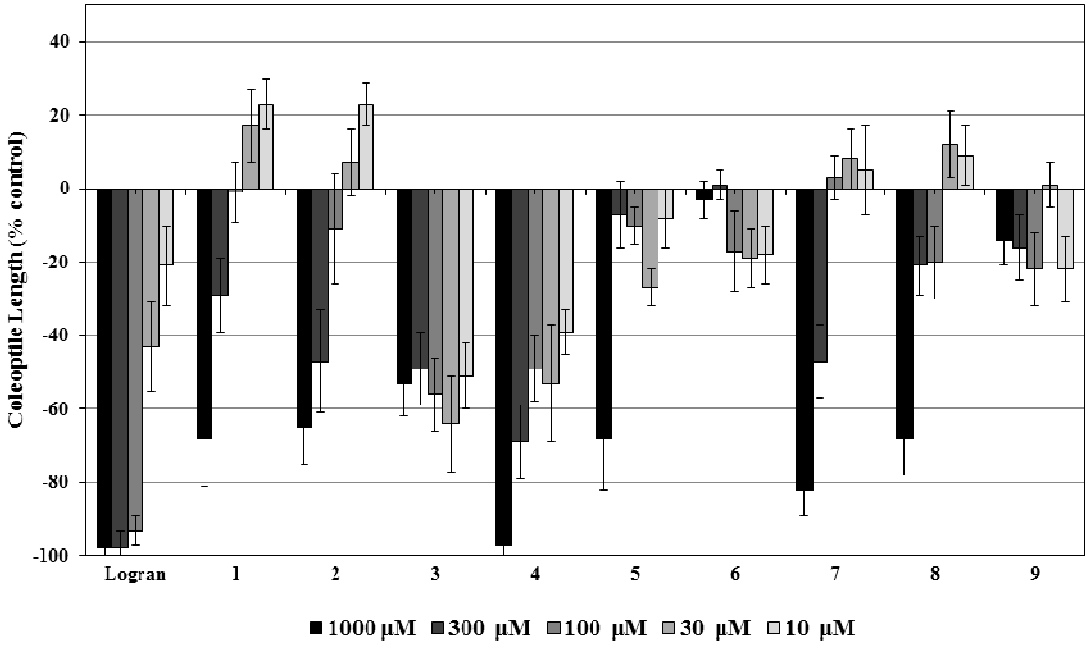

Figure 2.. Activity of compounds **1–9** in the etiolated wheat coleoptile bioassay. Results are given in % from the control coleoptile length. Positive values indicate a higher growth than the control and negative values a lower growth than the control.

Compound	Alog*P*	IC_{50}(µM)	R^2
1	-0.12	457	0.9737
2	0.26	326	0.9630
3	0.13	10.0[a]	-
4	1.00	117	0.9913
5	-0.43	762	1.000
6	-0.43	-[b]	-
7	-0.88	307	0.9887
8	-0.88	580	0.9916
9	-0.36	-[b]	-
Logran	-	33.1	0.9898

Table 1.$IC_{50}$and Alog*P* values for compounds **1–9**. IC_{50} was calculated only for those that reached the 50% of inhibition in the bioassay.[a]Tested concentration at which the inhibition was -50%. [b]Tested compound which not reached 50% inhibition.

Compound **4**, containing a iodine in the structure was the most active and three times more active than the next compounds **2** and **7**. The halogen in this structure seems to have a key role in this results, since the other dihydroxylated compound with the same stereochemistry (**6**) and the other with different stereochemistry (**5**) were much less active or not active at all. However, the lack of a double bond removing the α-β-unsaturated system could play also a role. Since the reaction for removing the iodine did not work, we could not explore further this hypothesis.

Alog*P* values varied between -0.88 and 1. Only compounds **2** and **4** followed the Lipinski's rule of five [22,23], having the appropriate molecular weight and Alog*P*. As predicted by Lipinski, these two compounds were part of the most active compounds of the collection. However, **7**, violating both requirements, also exhibited a remarkable activity. The stereochemistry seems to play an important role in this compound, since its epimer **8** had almost half the activity.

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The lowest active compounds were **5**, **6** and **9**, showcasing that the double bond and the carbonyl were both indispensable for the activity studied. The epoxide, on the other hand, proved to be disponsable, since the opening of the epoxide did not meant the drop of activity (compounds **4**, **7** and **8**) and the compound preserving the epoxide and losing the carbonyl had zero activity (**9**).

In summary, the addition of a iodine in the molecule (**4**) and an extra hydroxyl with the apropriate stereochemistry (**7**) had a positive effect in the bioactivity, while the acetylation (**2**) of the hydroxyl naturally present had little effect and the reduction of the carbonyl (**9**) or the double bond (**5** and **6**) had a negative effect.

A possible explanation of the higher activity of derivatives **3** and **4** was that **3** has a hemiquinone structure and derivative **4** by elimination of HI via E2 could be converted in the corresponding hemiquinone derivative and thus in the corresponding quinone. This could also occurr in derivative **7** but the leaving group from C-6 is OH^- in respect to the good one I^- in **4**. The hemiquinone generated from **7**, being also an enolic compound could be stabilized from the hydrogen bond between OH-6 and O=C-1. The other derivatives **5**,**6**, **8** and **9**, and the parent compound **1** are at low oxidation level and as these latter and derivative **2** would be more difficult to convert in the corresponding hemiquinone.

This hypothesis on the mode of action of *epi*-epoformin is in full agreement with the SAR study carried out using some derivatives of sphaeropsidones, two epimeric phytotoxic cyclohexene oxides isolated from *Diplodia cupressi* (a cypress pathogen in the Mediterranean basin) [24] and the testing their phytotoxic and antifungal activities on non-host plants and on five fungal pathogenic species belonging to the genus *Phytophthora* [25]. Successively, when sphaeropsidones and the same and new other derivatives were tested by haustorium-inducing activity in *O. cumana*, *O. crenata* and *S. hermonthica*, the results obtained showed that the activity is due to the possibility to convert the natural sphaeropsidones and natural and hemisynthetic derivatives in the corresponding 3-methoxyquinone [26].

This hypothesis on the mode of action of sphaeropsidone, that as above reported could work also for *epi*-epoformin, is in full agreement with the results obtained using natural and synthetic quinones as sorghum xenognosin and dimethoxybenzoquinones, in studies carried out on haustoria and the chemistry in host recognition parasitic angiosperms. Quinone/hydroquinone structures serve as cofactors in many metabolic pathways, playing critical chemical roles in oxidation/reduction processes [27,28].

3. Materials and Methods

3.1. General experimental procedures

The purities of the compounds were determined by ^{1}H NMR spectroscopy and every compound was purified in the HPLC prior bioassays. ^{1}H NMR and ^{13}C NMR spectra were recorded on Agilent™ 400 and 500 MHz spectrometers using $CDCl_3$ (MagniSolv;™ Merck) as solvent. The residual peaks of the solvent at 7.26 ppm (^{1}H NMR) and 77.0 ppm (^{13}C NMR) were used as the internal reference. Carbon multiplicities were determined by DEPT spectra [16]. DEPT, COSY, HSQC, HMBC, and NOESY experiments were performed using Varian vnmrj microprograms. Exact masses were measured on a UPLC-QTOF ESI (Waters Synapt G2, Manchester, UK) high-resolution mass spectrometer (HRTOFESIMS). Mass spectra were recorded in the negative- or positive-ion mode in the range m/z 100–2000, with a mass resolution of 20,000 and an acceleration voltage of 0.7 kV. FTIR spectra were obtained on Perkin-Elmer Spectrum TWO IR spectrophotometer. Major absorptions in the infrarred are given as wavenumbers $\bar{\upsilon}$ in cm^{-1}.Optical rotations were measured in $CHCl_3$ on a JASCO P-2000 polarimeter. HPLC in the isocratic mode was performed on a Merck Hitachi D-7000 equipped with a LiChroCART 250-10 Si 60 (10 µm) column. Column chromatography (CC) was performed using silica gel (Merck, Geduran® Si 60, 0.063-0.200 mm) and

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C_{18}-reversed phase silica gel (Sigma-Aldrich, C_{18} phase 90 A pore size). Analytical and preparative TLC were performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively) and on reversed phase (Merck, Kieselgel 60 RP-18, F_{254}, 0.20 mm) plates. The spots were visualized by exposure to UV radiation (253 nm), or iodine vapour, 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. Either Sigma-Aldrich Co. (St. Louis, Missouri), Merck (Darmstadt, Germany) or Alfa Aesar (Ward Hill, Massachusetts) supplied the reagents and the solvents. Seeds for the etiolated wheat coleoptile bioassay were kindly supplied by Fitó (Barcelona, Spain).

3.2. Fungal strain

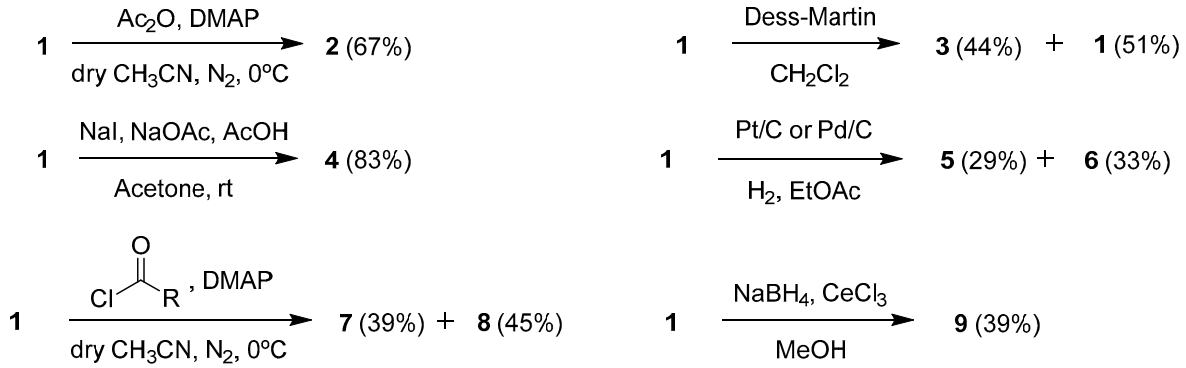
The fungal strain of *D. quercivora* used in this study was originally isolated from a symptomatic branch of *Q.canariensis* Willd. collected in a natural area in Tunisia as previously described [1].

*3.3. Extraction and purification of (+)-epi-epoformin (**1**) from* D. quercivora

The fungus was and grown *in vitro* as previously described [1]. The culturefiltrates (6.7 L) were acidified to pH 4 with 2 M 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 oil residue (1.14 g). This latter was fractionated through CC on silica gel, eluted with $CHCl_3$-*i*-PrOH (95:5). Eight homogeneous fraction groups were collected. The residue of the fourth fraction (474.6mg) was purified by CC on reverse phase eluted with Me_2CO–H_2O (7:3), yielding **1** (276.1 mg) as a white solid.

The spectroscopic data of **1** are as follows: HRMS, m/z (M^+) calcd for $C_7H_9O_3$ 141.0552, found 141.0601 $[M+H]^+$; IR $\tilde{\upsilon}_{max}$ 3357 (O-H), 1674 (C=O) cm^{-1}, $[\alpha]^{20}_D$= +139.3° (c= 0.08). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 6.46 (brs, 1H, H-3), 4.67 (brs, 1H, H-4), 3.78 (m, 1H, H-5), 3.53 (m, 1H, H-6), 1.86 (s, 3H, H-7).

*3.4. Synthesis of (+)-epi-epoformin (**1**) derivatives (**2–9**)*



Scheme 1. Synthesis of compounds (**2–9**).

(1*R*,2*S*,6*R*)-4-methyl-5-oxo-7-oxabicyclo[4.1.0]hept-3-en-2-yl acetate (2). 10.8 mg of **1** (0.077mmol) were solved in 2 mL of dry CH_3CN in a 10 mL pear-shaped flask, in N_2 atmosphere at 0°C. Then 4-dimethylaminopyridine (10.5 mg, 0.085 mmol) and acetic anhydride (29 µL, 0.308 mmol) were added and stirred slowly reaching room temperature (scheme 1). After 10 minutes, the reaction was quenched with 2 mL brine and extracted ×3 with EtOAc. The organic layers were combined, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure in a rotatory evaporator obtaining 14.7 mg of crude extract. After purification in column chromatography using hexane:acetone, 9:1, 9.4 mg of **2** (67% yield) were obtained as a light-yellow oil. Spectroscopic data are as follows: HRMS, m/z (M^+) calcd for $C_9H_{11}O_4$ 183.0657, found 183.0504 $[M+H]^+$; IR $\tilde{\upsilon}_{max}$ 2926 (C-H), 1741 (C=O), 1687 (C=O) cm^{-1}, $[\alpha]^{20}_D$ = +102.8° (c = 0.13).^1H NMR (500 MHz, $CDCl_3$, δ, ppm): 6.37 (m, 1H, H-3), 5.72 (dd, *J* = 5, 1.2 Hz, 1H, H-4), 3.72 (m, 1H, H-5), 3.53 (dd, *J* = 3.6, 1.1 Hz, 1H, H-6), 2.13 (s, 3H, H-9), 1.86 (dd, *J* = 1.4, 1.2 Hz, 3H, H-7). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 193.5 (s, C-1),

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169.8 (s, C-8), 136.6 (s, C-2), 134.3 (d, C-3), 64.5 (d, C-4), 55.0 (d, C-5), 52.9 (d, C-6), 20.7 (q, C-9), 16.0 (q, C-7).

(1*R*,6*S*)-3-methyl-7-oxabicyclo[4.1.0]hept-3-ene-2,5-dione(3). 9.8 mg of **1** (0.070 mmol) were solved in 1 mL of CH_2Cl_2 in a 10 mL pear-shaped flask. Then Dess-Martin periodinane (45.9 mg, 0.105 mmol) was added in small portions while stirring at rt. After 16 hours, the reaction was quenched with 1 mL of aqueous saturated $Na_2S_2O_3$ and extracted ×3 with CH_2Cl_2. The organic layers were combined and washed with 1 mL aqueous saturated $NaHCO_3$, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure in a rotatory evaporator. The crude was purified in column chromatography in a gradient of hexane:EtOAc from 1:0 to 2:3, and later in the HPLC using hexane:acetone 9:1 with a retention time of 12 min. Compound **3** was obtained as a colorless oil in a 44% yield (4.3 mg) and 5 mg of **1** (51%) were recovered. Spectroscopic data are as follows: HRMS, m/z (M^+) calcd for $C_7H_7O_3$ 139.0395 $[M+H]^+$, found 139.0401; IR $\tilde{\upsilon}_{max}$ 2925 (C–H), 1737 (C=O), 1715 (C=O) cm^{-1}, $[\alpha]^{20}_D$ = +5.8° (c = 0.02). ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm): 6.43 (brs, 1H, H-3), 3.84 (dd, *J* = 3.7, 0.7 Hz, 1H, H-6), 3.79 (dd, *J* = 2.5, 0.7 Hz, 1H, H-5), 2.02 (d, *J*= 1.4 Hz, 3H, H-7). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 192.3 (s, C-1), 191.2 (s, C-4), 146.8 (s, C-2), 133.3 (d, C-3), 54.1 (d, C-5), 53.7 (d, C-6), 16.4 (q, C-7).

(4*S*,5*R*,6*R*)-4,5-dihydroxy-6-iodo-2-methylcyclohex-2-en-1-one (4). 9.8 mg of **1** (0.070 mmol) were solved in 1 mL of acetone in a 10 mL pear-shaped flask. Then NaI (37 mg, 0.252 mmol), NaOAc (2.3 mg, 0.252 mmol) and AcOH (14 μL, 0.252 mmol) were added and stirred at rt. After 7 hours, the reaction was quenched with 1 mL of aqueous saturated $Na_2S_2O_3$ and evaporated the acetone under reduced pressure. The resultant aqueous solution was extracted ×3 with EtOAc. The organic layers were combined and washed with 1 mL H_2O, 1 mL aqueous saturated $NaHCO_3$ and 1 mL brine, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure in a rotatory evaporator. The crude was purified in column chromatography in a gradient of hexane:EtOAc from 1:0 to 2:3, and furnished 13.8 mg of a fraction with a mixture of two compounds. This fraction was later purified in the HPLC with hexane:acetone 7:3 obtaining two peaks at 15 (**4**) and 17 min. A total of 8.2 mg of **4** (83% yield) as a colorless oil were isolated. Spectroscopic data are as follows: HRMS, m/z (M^-) calcd for I 126.9050, found 126.9045 $[M]^-$; IR $\tilde{\upsilon}_{max}$ 3264 (O-H), 2925 (C–H), 1683 (C=O) cm^{-1}, $[\alpha]^{20}_D$ = +67.3° (c = 0.05). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 6.59 (m, 1H, H-3), 4.77 (d, *J* = 4.1 Hz, 1H, H-6), 4.21 (dt, *J* = 7.7, 2.1 Hz, 1H, H-4), 2.80 (dd, *J* = 7.7, 4.1 Hz, 1H, H-5), 1.87 (dd, *J* = 2.1, 1.5 Hz, 3H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 192.3 (s, C-1), 143.9 (d, C-3), 133.8 (s, C-2), 73.6 (d, C-4), 73.5 (d, C-5), 35.2 (d, C-6), 15.9 (q, C-7).

(2*R*,4*S*,5*R*)-4,5-dihydroxy-2-methylcyclohexan-1-one (5) and (2*R*,4*S*,5*S*)-4,5-dihydroxy-2-methylcyclohexan-1-one (6). 9.8 mg of **1** (0.070 mmol) were solved in 2 mL of EtOAc in a 10 mL pear-shaped flask and N_2 atmosphere. Then carbon supported Pt (23 mg, 0.004 mmol) was added followed by H_2 atmosphere and a H_2 balloon was connected while stirring at rt. After 10 minutes, no more starting material was observed in TLC and the crude of reaction was filtered through celite. Then, it was purified by column chromatography with a gradient of hexane:acetone from 1:0 to 2:3, furnishing a major fraction of 4.4 mg containing the mixture of reduced products. This fraction was purified in the HPLC with hexane:acetone 7:3 observing two peaks at 25 (**5**) and 32 min. (**6**), with a total mass of 1.9 mg (19% yield) and 2.2 mg (21%), respectively and both as colorless oils. Alternatively, using Pd/C(3 mg, 0.003 mmol) instead of Pt/C, and 9.2 mg of **1** (0.066 mmol), 2.7 mg of compound **6** (29% yield) and 3.2 mg of **6** (33% yield) were obtained. Spectroscopic data of **5** are as follows: MS, m/z (M^+) calcd for $C_7H_{12}O_3Na$ 167.0684, found at low resolution 167.3 $[M+Na]^+$; IR $\tilde{\upsilon}_{max}$ 3385 (O-H), 2926 (C–H), 1704 (C=O) cm^{-1}, $[\alpha]^{20}_D$ = +13.1° (c = 0.03). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 4.18 (ddd, *J*= 4.9, 4.3, 3.8 Hz, 1H, H-5), 4.02 (dd, *J* = 8.4, 4.3 Hz, 1H, H-4), 2.91 (dd, *J* = 14.4, 3.8 Hz, 1H, H-6b), 2.78 (m, 1H, H-2), 2.43 (dd, *J* = 14.4, 4.9 Hz, 1H, H-6a), 1.99 (dd, *J* = 8.4, 4.0 Hz, 2H, H-3), 1.08 (d, *J* = 6.8 Hz, 3H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 211.5 (s, C-1), 74.0 (d, C-5), 69.0 (d, C-4), 44.6 (t, C-6), 40.1 (d, C-2), 36.9 (t, C-3), 14.8 (q, C-7). Spectroscopic data of **6** are as follows: MS, m/z (M^+) calcd for $C_7H_{12}O_3Na$ 167.0684, found at low resolution 167.3 $[M+Na]^+$; IR $\tilde{\upsilon}_{max}$ 3392 (O-H), 2930 (C–H), 1711 (C=O) cm^{-1}, $[\alpha]^{20}_D$ = +11.5° (c = 0.03). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 3.88 (ddd, *J* = 13.3, 8.8, 4.5 Hz, 1H, H-4), 3.65 (ddd, *J* = 12.2, 8.8,

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5.3 Hz, 1H, H-5), 2.75 (ddd, *J* = 13.7, 5.3 Hz, 1H, H-6b), 2.46 (m, 1H, H-2), 2.46 (m, 1H, H-6a), 2.22 (ddd, *J* = 13.2, 5.7, 4.5 Hz, 1H, H-3a), 1.35 (td, *J* = 13.2, 11.4, 1H, H-3b), 1.06 (d, *J* = 6.6 Hz, 3H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 207.9 (s, C-1), 74.0 (d, C-5), 73.8 (d, C-4), 47.0 (t, C-6), 42.4 (d, C-2), 37.1 (t, C-3), 13.8 (q, C-7).

(4*S*,5*R*,6*R*)-4,5,6-trihydroxy-2-methylcyclohex-2-en-1-one (7) and (4*S*,5*R*,6*S*)-4,5,6-trihydroxy-2-methylcyclohex-2-en-1-one (8). 7.1 mg of **1** (0.051mmol) were solved in 2 mL of dry CH_3CN in a 10 mL pear-shaped flask. Then 4-dimethylaminopyridine (6.9 mg, 0.056 mmol) and hexanoyl chloride (29 µL, 0.204 mmol) were added while stirring at 0 °C and gradually reached rt. After 5 hours and 30 min. no more starting material was observed in TLC and the reaction was quenched with 2 mL $NaHCO_3$ and extracted ×3 with EtOAc. The organic layers were combined, dried over anhydrous Na_2SO_4 and the solvent evaporated in a rotatory evaporator. The crude was purified by column chromatography with a gradient of hexane:acetone from 1:0 to 2:3, furnishing two fractions. These fractions were purified in the HPLC with hexane:acetone 7:3 observing two peaks at 16 (**7**) and 17 min. (**8**), with a total mass of 3.2 mg (39% yield) and 3.6 mg (45%), respectively, both as colorless oils. The same compounds and similar yields were obtained when using butanoyl chloride (22 µL, 0.204 mmol) and the same procedure. Spectroscopic data of **7** are as follows: HRMS, m/z (M^+) calcd for $C_7H_{10}O_4$ 157.0506, found 157.0505 $[M-H]^-$; IR $\tilde{\upsilon}_{max}$ 3372 (O-H), 2926 (C–H), 1682 (C=O) cm^{-1}, $[\alpha]^{20}_D$ = +65.7° (c = 0.03). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 6.68 (dq,*J* = 2.3, 1.4 Hz, 1H, H-3), 4.59 (ddd, *J* = 7.1, 2.3, 2.1 Hz, 1H, H-4), 4.57 (d, *J* = 3.5 Hz, 1H, H-6), 4.05 (d, *J* = 7.1, 3.5 Hz, 1H, H-5), 1.88 (dd, *J* = 2.1, 1.4 Hz, 3H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 190.6 (s, C-1), 144.1 (d, C-3), 134.3 (s, C-2), 74.6 (d, C-5), 69.3 (d, C-4), 61.4 (d, C-6), 15.7 (q, C-7). Spectroscopic data of **8** are as follows: HRMS, m/z (M^+) calcd for $C_7H_{10}O_4$ 157.0506, found 157.0540 $[M-H]^-$; IR $\tilde{\upsilon}_{max}$ 3370 (O-H), 2925 (C–H), 1691 (C=O) cm^{-1}, $[\alpha]^{20}_D$ = +28.2° (c = 0.03). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 6.70 (dq, *J* = 2.1, 1.5 Hz, 1H, H-3), 4.51 (ddd, *J* = 8.2, 2.1, 2.1 Hz, 1H, H-4), 4.38 (d, *J* = 11.3 Hz, 1H, H-6), 3.84 (d, *J* = 11.3, 8.2 Hz, 1H, H-5), 1.89 (dd, *J* = 2.1, 1.5 Hz, 3H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 189.7 (s, C-1), 144.2 (d, C-3), 135.1 (s, C-2), 78.3 (d, C-5), 71.4 (d, C-4), 66.9 (d, C-6), 15.8 (q, C-7).

(1*S*,2*R*,5*S*,6*R*)-3-methyl-7-oxabicyclo[4.1.0]hept-3-ene-2,5-diol (9). 9.8 mg of **1** (0.070 mmol) were solved in 2 mL of MeOH containing 0.4 M $CeCl_3$ (0.8 mmol) in a 10 mL pear-shaped flask. Then $NaBH_4$ (3.8 mg, 0.102 mmol) were added in small portions and stirred at rt. The reaction was quenched after 5 min. with 2 mL saturated aqueous NH_4Cl, evaporated the MeOH in a rotatory evaporator and extracted ×3 with EtOAc. The organic layers were combined, dried over anhydrous Na_2SO_4 and the solvent evaporated. Product was purified in the HPLC with hexane:acetone 7:3 observing a peak at 34 min., corresponding to **9**, with a total mass of 3.8 mg (39% yield) as an amorphous colorless solid. Spectroscopic data of **9** are as follows: HRMS, m/z (M^+) calcd for $C_7H_{11}O_3$ 143.0708, found 143.0681 $[M+H]^+$; IR $\tilde{\upsilon}_{max}$ 3234 (O-H), 2916 (C–H) cm^{-1}, $[\alpha]^{20}_D$ = +0.8° (c = 0.03). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 5.51 (dd, *J* = 5.1, 1.6 Hz, 1H, H-3), 4.46 (ddd, *J* = 5.1, 3.6, 0.9Hz, 1H, H-4), 4.25 (d, *J* = 3.5, 1.5 Hz, 1H, H-1), 3.36 (dd, *J* = 3.5, 1.5 Hz, 1H, H-6), 3.30 (dd, *J* = 3.6, 1.5 Hz, 1H, H-5), 1.83 (dd, *J* = 1.6, 0.9 Hz, 3H, H-7). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 135.2 (s, C-2), 121.6 (d, C-3), 66.3 (d, C-1), 63.1 (d, C-4), 53.0 (d, C-6), 51.9 (d, C-5), 21.2 (q, C-7).

3.5. Etiolated wheat coleoptile bioassay

The compounds (**1–9**) were tested for their bioactivity in an etiolated wheat coleoptile bioassay. The conditions for this bioassay were reported previously [29,30] and replicated in this study, using the same herbicide (Logran®) as positive control and the buffer solution as negative control. Wheat (*Triticum aestivum*) was the 'catervo' variety. All the samples were solved in a 0.5% of dimethyl sulfoxide and gave clear solutions at all the tested concentrations (10^{-3}–10^{-5} M). The results are shown in figure 2.

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3.6. Calculation of IC_{50} and logP

The bioactivity data were fitted to a sigmoidal dose-response modelusing the GraphPad Prism v.5.00 software package [31] to obtain the IC_{50} values shown in Table 1. The Alog*P* was calculated using the ALOGPS v.2.1 software, based on computed atom contributions [32–34].

4. Conclusions

(+)-*epi*-Epoformin (**1**) was successfully isolated from extracts of *D. quercivora* following the procedures in the literature. Then, eight derivatives of **1** were synthesized and tested altogether for their bioactivity in the etiolated wheat coleoptile bioassay. The derivatives were compared with the starting material to look for structure-activity relationships.

The α-β-unsaturated system in **1** proved to be essential to obtain active derivatives and the inclusion of a iodine and an extra hydroxyl in the molecules increased the activity, while the acetylation of the hydroxyl did not have a significant effect.

The results described here illustrate that natural products, especially those provided by fungus, are valuable resources which need to be investigated further as natural pesticides against weeds.

Supplementary Materials: Supplementary data to this article can be accessed online.

Acknowledgments: This research was carried out in the frame of Programme STAR 2017, financially supported by UniNA and Compagnia di San Paolo. This work was partly funded by the 'Ministerio de Economía, Industria y Competitividad' (MINEICO), Spain, Project AGL2017-88-083-R. A.C. (Spain) would like to thank the financial support of the University of Cádiz in the 'Plan Propio de Investigación 2017'. The authors are very grateful to Prof. L. Maddau, Dipartimento di Agraria, Università di Sassari, Sassari,Italy to providing the culture filtrates of the fungus *D. quercivora*. We also thank Fitó for providing the seeds for the etiolated wheat coleoptile bioassay free of charge. A.E. is associated to the Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy.

Author Contribution: All the authors contributed in the design of the study, the analysis of the data and writing of the manuscript. A. C. (Spain) and M. M. performed the experiments. A. C. (Italy) purified the *epi*-epoformin. J. M. G. M., F. A. M. and A. E. revised and finalized the manuscript. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare no conflict of interest. The founding sponsors had no role in design the study, analyses, or interpretation of the data, in the writing of the manuscript, and in the decision to publish the results.

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Sample Availability: Samples of the compounds **1**-**9** are available from the authors.