

New Bioactive 2,3-Epooxycyclohexenes and Isocoumarins from the Endophytic Fungus *Phomopsis* sp. from *Laurus Azorica*^[‡]

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Six new metabolites, namely cycloepoxylactone (**1a**), cycloepoxytriol A (**2**), cycloepoxytriol B (**3**) and phomolactones A–C (**4–6**), were isolated from two cultures of the ethyl acetate soluble fraction of the endophytic fungus *Phomopsis* sp. (internal strain no. 7233). Their structures were determined by means of spectroscopic data including HREIMS, ¹H NMR, ¹³C NMR, and 2D NMR (HMQC, HMBC, and NOESY). The absolute configurations of **1a**, **4** and **5** were determined by circular dichroism and comparison of solution CD spectra

with TDDFT-calculated ones. Preliminary studies showed that cycloepoxylactone (**1a**) has good antibacterial, antifungal, and algicidal activity against *Bacillus megaterium*, *Microbotryum violaceum*, and *Chlorella fusca*, respectively, whereas cycloepoxytriol B (**3**) has good algicidal activity against *Chlorella fusca*.

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Introduction

The genus *Phomopsis* is known to be a rich source of bioactive secondary metabolites of diverse structures such as xanthenes,^[2,3] antifungal biaryl ethers (phomosines),^[4,5] cycochalisines,^[6,7] convolvulanic acids,^[8] or mycotoxins like phomopsisin A.^[9] Some of the compounds isolated from *Phomopsis* sp. exhibited significant in vitro antimalarial, antitubercular and cytotoxic activities.^[3] However, many of these bioactive compounds with antitumor and antibiotic activities have not yet found application due to toxicity problems.^[10] In connection with our ongoing screening for biologically active secondary metabolites from endophytic fungi, we investigated *Phomopsis* sp. (internal strain no. 7233), isolated from the plant *Laurus azorica*. In a series of preliminary screenings, the culture extract of the *Phomopsis*

sp. displayed fungicidal, antibacterial, algicidal, and herbicidal activities. In this paper, we report on the isolation, structure elucidation, and preliminary bioactivity of five new compounds from two fermentations of the fungus.

Results and Discussion

The endophytic fungus *Phomopsis* sp. (internal strain no. 7233) was isolated from the leaves of *Laurus azorica*, growing in Gomera, Spain. The fungus was cultured on biomalt solid agar medium for 28 d, and the culture medium was then extracted with ethyl acetate to afford 5.6 g of a crude extract. The ethyl acetate extract was subjected to column chromatography resulting in the isolation of three new metabolites, cycloepoxylactone (**1a**), cycloepoxytriol A (**2**), and cycloepoxytriol B (**3**). In order to also obtain the minor components, the same fungus was recultivated, and three additional new metabolites, named phomolactones A–C (**4–6**), along with cycloepoxylactone (**1a**) from the first cultivation, were isolated (Figure 1). Their structures, and in particular their stereochemistry, were elucidated by a thorough spectroscopic analysis in comparison to related hydroxycyclohexene epoxides and isocoumarins from the literature (Figure 2) and to calculation results.

The molecular formula of cycloepoxylactone (**1a**) was assigned C₁₂H₁₆O₅ on the basis of HREIMS with a molecular peak at *m/z* = 240.0989 and ¹H and ¹³C NMR spectral analyses (see Experimental Section). IR absorption bands at 3460 and 1655 cm^{−1} indicated the presence of hydroxy

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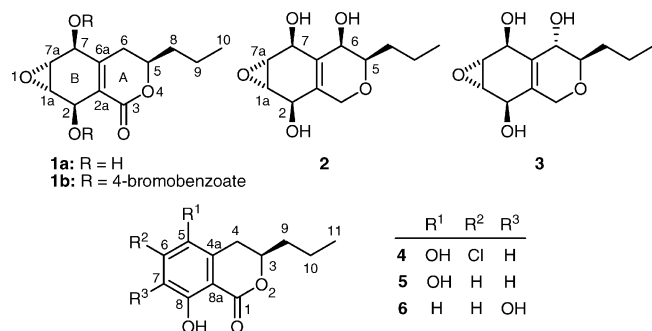


Figure 1. Structures of compounds **1a**, **2–6** isolated from *Phomopsis* sp.

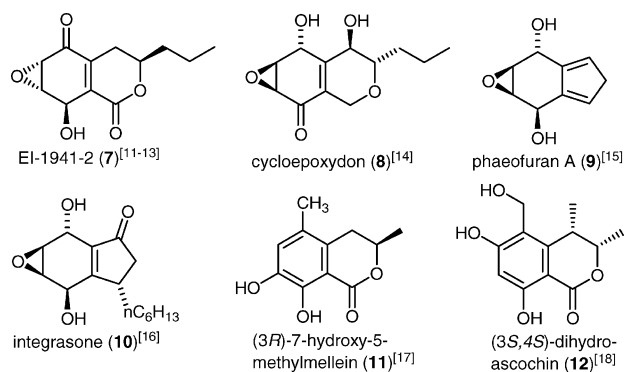


Figure 2. Structures of related hydroxycyclohexene epoxides (**7–10**) and dihydroisocoumarins (**11**, **12**) from the literature.

and carboxyl groups. The ^1H NMR spectrum in CDCl_3 exhibited a methine proton signal at $\delta = 4.47$ ppm and a propyl group signal [$\delta = 1.80$ (m, 1 H, 8a-H), 1.63 (m, 1 H, 8b-H), 1.50 (m, 2 H, 9-H), 0.95 (t, $J = 7.3$ Hz, 3 H, 10-H) ppm]. The signals of the methylene protons 6-H₂ at $\delta = 2.22$ (dd, $J = 3.5, 17.0$ Hz) and 2.73 (dd, $J = 11.0, 17.0$ Hz) ppm were similar to those found in EI-1941-2 (**7**)^[11–13] and cycloepoxydon (**8**)^[14] (Figure 2). The NMR shifts for two of the oxymethine units ($\delta_{\text{C}} = 55.7$ and 52.2 ppm, $\delta_{\text{H}} = 3.39$ and 3.46 ppm) were diagnostic of the presence of a 1,2-disubstituted epoxide.^[14] Each of the epoxide protons was coupled to one of the other two oxymethine groups [$\delta = 4.42$ (m, 7-H) and 4.85 (m, 2-H) ppm] with a small coupling constant, enabling construction of a substructure corresponding to the C2–C7 unit in **1a**. According to the molecular formula ($\text{C}_{12}\text{H}_{16}\text{O}_5$), the unsaturation index of cycloepoxylactone (**1a**) was 5, and as the ^{13}C NMR spectroscopic data suggested the presence of one lactone carbonyl group ($\delta = 166.0$ ppm) and one double bond [$\delta = 148.1$ (C-6a) and 122.7 (C-2a) ppm], the molecule has to be tricyclic. The structure of **1a** was definitely determined by 2D NMR experiments, giving pertinent COSY and HMBC correlations (Figure 3).

The acylation of compound **1a** with 4-bromobenzoyl chloride was carried out in the hope of confirming the structure by single-crystal X-ray analysis with a heavy atom incorporated in the bis(4-bromobenzoate) **1b**. Unfortunately, the compound did not afford suitable crystals for X-

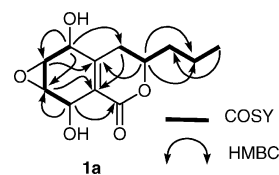


Figure 3. Summary of ^1H - ^1H COSY and ^1H - ^{13}C HMBC for **1a**.

ray analysis. However, NMR spectra of bis(4-bromobenzoate) **1b** were in total agreement with the proposed structure and thus reconfirmed the presence of two hydroxy groups in compound **1a**.

The relative stereochemistry of **1a** was determined by NOESY correlations (see Figure 4) of its benzoate derivative **1b**, and by ^1H - ^1H coupling constants and comparison of data from related compounds (Figure 2) and with estimated values. The large coupling constant (12.0 Hz) between 6-H_{ax} and 5-H as well as the correlation observed between 6-H_{eq} and 5-H in the NOESY spectrum of **1b** indicated that 6-H_{ax} and 5-H are *trans* and axial, and thus the propyl side-chain adopts an equatorial position. The link between the relative configuration of C-5 in ring A to the C-7 stereogenic center in ring B was based on the strong correlation of the equatorial 6-H_{eq} with 7-H, clearly establishing the equatorial position of that hydrogen atom and thus the pseudo-axial position of 7-O.

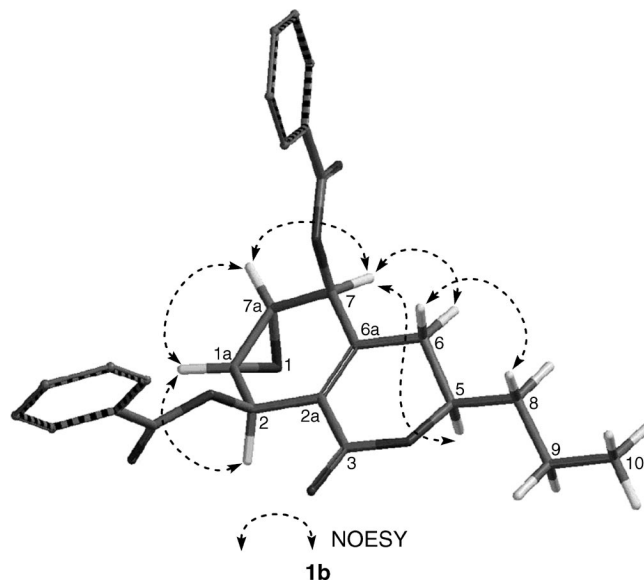


Figure 4. Summary of ^1H - ^1H NOESY data for **1b**.

The two 1a-H and 7a-H *cis*-hydrogen atoms of the epoxide show the expected coupling constant of $J = 3.5$ Hz. To establish the relative configuration of the epoxide and hydroxy groups, comparison of the coupling constants $J_{1a,2}$ and $J_{7a,7}$ of **1a** and **1b** with closely related α -hydroxy epoxides were helpful. Usually, a *trans* relationship in these systems shows a smaller coupling constant than a *cis* one. For example, the relevant coupling constants for the *trans* protons in EI-1941-2 (**7**)^[11–13] (1.7 Hz), in cycloepoxydon (**8**)^[14] (1.4 Hz), in phaeofuran A (**9**)^[15] (1.8 Hz), or in integ-

rasone (**10**)^[16] (1.2 Hz) indicate a similar *trans* orientation in **1a** and **1b** ($J_{1a,2}$ and $J_{7a,7} = 1.5$ Hz). By contrast, the corresponding *cis* couplings were found to be 2.5 Hz in phaeofuran A (**9**)^[15] and 3.3 Hz in integrasone (**10**).^[16] This trend is also supported by DFT calculations. The lowest-energy B3LYP/6-31G(d)-computed structure for the *trans* isomer of **1a** has dihedral angles $\omega_{1a-H,C-1a,C-2,2-H} = 74^\circ$ and $\omega_{7a-H,C-7a,C-7,7-H} = 69^\circ$; in the *cis* isomer, they are 50° and 32° , respectively. Therefore, according to Karplus' relationship (see Computational Section), a larger J value is expected for the *cis* isomer. The identical coupling constants of $J_{1a,2}$ and $J_{7a,7}$ of $J = 1.5$ Hz in **1a** and **1b** confirmed the symmetrical environment at positions 2 and 7 of the cyclohexane ring.

All 1,4-dioxy-2,3-epoxycyclohexene derivatives isolated from natural sources show interesting bioactivity, and these α -hydroxy epoxides seem to be part of a "privileged structure". For example, compound EI-1941-2 (**7**) (Figure 2) is a novel interleukin-1 β -converting enzyme inhibitor,^[11] cycloepoxidone (**8**) is an inhibitor of eukaryotic signal transduction,^[14] and integrasone (**10**) is a recently isolated HIV-integrase inhibitor.^[16] A closely related 1,4-dioxy-2,3-epoxycyclohexene derivative is phaeofuran A (**9**), isolated from a fungicolous *Phaeoacremonium* species.^[15]

The absolute configuration of **1a** was determined from the comparison of its experimental solution CD spectrum with the TDDFT-calculated one^[19] on DFT-optimized geometries.^[20] Cycloepoxylactone contains an α,β -unsaturated lactone chromophore which gave rise to three Cotton effects with alternating signs [265 ($\Delta\epsilon = -1.84$), 230 (2.80) and 203 (-3.55) nm] in the measured CD spectrum (Figure 5). It may arise in principle from a combination of the intrinsic chirality of the lactone chromophore,^[21] which is not planar (computed dihedral angle $\omega_{C-6a,C-2a,C-3,O} = 160^\circ$), with elements of chirality of the second (chromophore embedded into a chiral cycle) and third sphere (more remote stereogenic centers).^[22] However, analysis of Kohn–Sham orbitals from DFT calculations revealed that both enone n - and π -type orbitals are mixed with orbitals of similar character on the epoxy and hydroxy groups, therefore a division of **1a** into chiral spheres is inappropriate. Apart from the rotations of the alkyl chain at C-5, the rest of the structure of **1a** is quite rigid. Ring B is almost planar due to the presence of the unsaturation and of the epoxide ring; the two hydroxy groups also have strongly favoured conformations dictated by electrostatic O $\cdots$ H–O interactions, as seen in the lowest-energy DFT [B3LYP/6-31G(d)] structure of **1a** (Figure 5, inset). The propyl substituent at C-5 preferentially occupies an equatorial position (as also found experimentally), which controls the conformation of ring A. This lies in a sofa-like S(5) arrangement,^[23] with M helicity (negative $\omega_{C-2a,C-3,O-4,C-5}$ dihedral angle) for the (5*R*) configuration. Other structures with ring substituents (hydroxy and propyl groups) adopting different conformations are highly unfavoured (>2.3 kcal/mol, DFT energy). As for the possible rotamers of the propyl chain, we assumed that they would lead to almost identical CD spectra (a proof of that is given below for dihydroisocoumarins). Therefore, CD cal-

culations were performed on the lowest-energy DFT structure for **1a**; different combinations of functionals (B3LYP, BH&HLYP) and basis sets (TZVP, ADZP) led to consistent results. In particular, for (1*a*S,2*R*,5*R*,7*S*,7*a*R)-**1a**, three bands of alternating signs $-/+/-$ (from the low- to the high-energy region) were obtained, in keeping with the experimental spectrum (Figure 5). They are due to five transitions of $n\text{-}\pi^*$ (I, II and V) and $\pi\text{-}\pi^*$ (III and IV) character. In particular, the most intense bands II–IV arise from transitions from n - and π -bonding orbitals delocalized on the whole molecule (involving enone, hydroxy and epoxide groups). The agreement between experimental and calculated spectra shown in Figure 5 allows us to establish the absolute configuration of cycloepoxylactone as (1*a*S,2*R*,5*R*,7*S*,7*a*R)-**1a**.

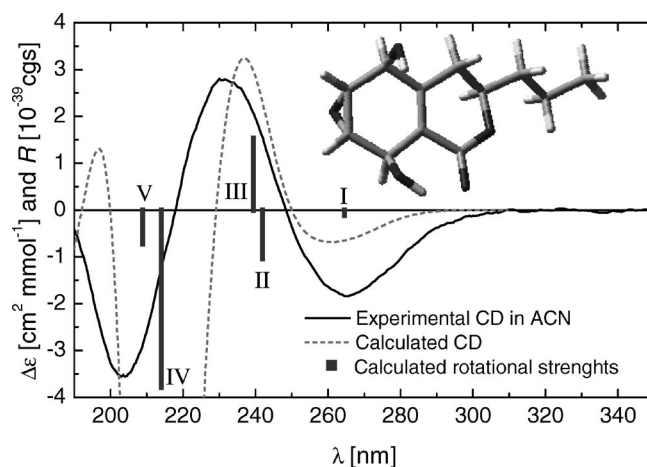


Figure 5. Measured (MeCN, solid line) and TDB3LYP/TZVP-calculated (dotted line) CD spectra of (1*a*S,2*R*,5*R*,7*S*,7*a*R)-**1a** by using the lowest-energy DFT structure drawn in the inset. Vertical bars represent computed rotational strengths R .

Cycloepoxytriol A (**2**) was found to have a molecular formula of $C_{12}H_{18}O_5$ due to its molecular ion peak at $m/z = 263$ [$M + Na$] $^+$ in positive FABMS. The structure of **2** was determined by the comparison of its NMR spectroscopic data with those of **1a**. However, two main differences were apparent in the spectrum of compound **2**. Firstly, the appearance of a methine proton ($\delta = 4.01$ ppm) instead of two methylene protons at C-6 [$\delta = 2.22$ (dd, $J = 3.5, 17.0$ Hz) and 2.73 (dd, $J = 11.0, 17.0$ Hz) ppm] as found in compound **1a**. The ^{13}C NMR spectrum showed the disappearance of the methylene carbon atom [$\delta = 32.5$ (C-6) ppm in **1a**] and appearance of a methine carbon atom ($\delta = 65.3$ ppm) at C-6. Secondly, the carbonyl signal in the ^{13}C NMR spectrum of compound **1a** for C-3 ($\delta = 166.0$ ppm) was missing, and instead a methylene signal was observed at $\delta = 65.9$ (C-3) ppm in **2**, confirmed by the appearance of two signals for methylene protons at C-3 ($\delta = 4.33$ and 3.95 ppm) in the 1H NMR spectrum. The planar structure of **2** shown in Figure 1 was also in agreement with the entire set of 1H - 1H COSY and 1H - ^{13}C HMBC correlations.

The relative stereochemistry of ring B of **2** was estimated by 1H - 1H coupling constants and comparison with those of **1a**. For instance, the 1H - 1H coupling constants between 1*a*-

H and 7a-H (3.5 Hz) and between 1a-H and 2-H (1.5 Hz) were very close to those observed for **1a**. These data demonstrated that 1a-H and 7a-H are *cis* as required for epoxide protons, and the hydroxy group should be oriented *trans* to the epoxide moiety as in **1a** (Figure 1). This was confirmed by the observed NOE between 1a-H and 2-H, 7a-H and 7-H (Figure 6). The NOE correlation of 5-H to 6-H disclosed the spatial proximity between these hydrogen atoms and therefore their *cis* relationship. This finding was in agreement with the coupling constant value of $J_{5,6} = 3.5$ Hz observed in the ^1H NMR spectrum, consistent with an axial-equatorial and incompatible with a *trans*-diaxial coupling.^[24] The link between the relative configuration of C-5 and C-6 in ring A to the stereocenter at C-7 in ring B was made by the NOE correlation of the equatorial 6-H^{eq} with 7-H, clearly establishing the equatorial position of that hydrogen atom and thus the pseudo-axial position of 6- and 7-OH.

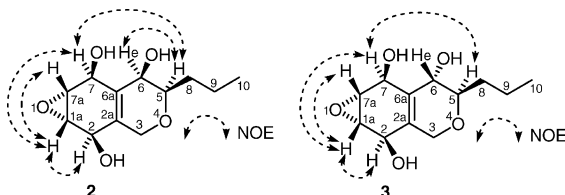


Figure 6. Selected NOE correlations for cycloepoxytriol A (**2**) and cycloepoxytriol B (**3**).

Analysis of the chemical ionization mass spectrum (CI-MS) of cycloepoxytriol B (**3**) gave a molecular ion peak at $m/z = 243.1$ [$M + 1$]⁺, corresponding to the molecular formula $\text{C}_{12}\text{H}_{18}\text{O}_5$, supported by ^1H and ^{13}C NMR analysis. Compound **3** showed similar spectroscopic data (1D NMR and 2D NMR) to those of cycloepoxytriol A (**2**), in accordance with the structure for cycloepoxytriol B (**3**) shown in Figure 1. The relative stereochemistry of ring A of **3** was determined by NOE correlations and by ^1H - ^1H coupling constants. The large coupling constant (7.0 Hz) between 5-H and 6-H as well as NOE correlation of 5-H to 8-H, but not to 6-H, indicate that 5-H and 6-H are *trans* and axial.^[14] The relative stereochemistry of ring B could be determined from the NOE correlations of 1a-H, which were the same as for compound **2**. The link between the relative configuration of C-5 in ring A and the stereocenter at C-7 in ring B was based on the NOE correlation of the equatorial 5-H^{eq} with 7-H, clearly establishing the axial position of that hydrogen atom and thus the pseudo-axial position of 7-OH.

The isocoumarin derivatives **4–6** (phomolactones A–C, Figure 1) show characteristic similarities in the IR, UV and NMR spectra (see Experimental Section). All compounds have absorption bands at 1640–1660 and 3450–3460 cm^{-1} in the IR spectra, typical for aromatic chelated carbonyl and hydroxy groups, respectively.^[17,25] The chelation of the phenolic hydroxy group was confirmed by low-field signals in the ^1H NMR spectra at $\delta = 10.6$ ppm and by signals at $\delta = 170$ ppm in the ^{13}C NMR spectra, indicating a lactone group. Further typical ^1H NMR signals could be detected as spin systems of a methine proton at $\delta = 4.4$ – 4.8 ppm and

a propyl group [$\delta = 1.80$ – 1.90 (m, 1 H, 9a-H), 1.65 – 1.75 (m, 1 H, 9b-H), 1.45 – 1.60 (m, 2 H, 10-H), 0.90 – 0.98 (t, $J = 7.3$ Hz, 3 H, 11-H) ppm] and methylene protons at C-4 ($\delta = 2.58$ – 2.73 and 3.06 – 3.20 ppm). For phomolactone A (**4**), ^{13}C NMR spectroscopic data revealed six resonances for sp^2 -carbon atoms as part of a benzene ring, two of them characterized by a downfield shift due to the presence of two hydroxy groups at C-5 and C-8. This combined information suggested a dihydroisocoumarin skeleton. The structural details of the lactone ring were elucidated by using ^1H - ^1H COSY in which couplings between 4-H₂ and 3-H as well as between 3-H and 9-H were visible. HMBC correlations of 4-H₂ to C-8a, C-5, and C-4a as well as of 3-H to C-1 proved the connection between the two rings. HMBC correlations of an aromatic proton [$\delta = 6.49$ (s) ppm] to C-5, C-6, and C-8 and of 8-OH to C-7 and C-8, clarified the substitution pattern on ring A. Based on the elemental composition and the characteristic molecular ion pattern in the mass spectrum, the remaining substituent at C-5 had to be a chlorine atom.

The ^1H and ^{13}C NMR spectroscopic data (see Experimental Section) of phomolactone B (**5**) were very similar to those of compound **4**, except that **5** had one aromatic proton signal at C-6 with *ortho* coupling [$\delta = 6.93$ (d, $J = 8.5$ Hz, 6-H) ppm] instead of a chlorine atom. The absence of the characteristic molecular ion pattern for chlorine in the mass spectrum and one additional methine signal at $\delta = 123.9$ ppm for C-7 was compatible with the proposed structure **5** (Figure 1). HMBC correlations of 6-H to C-5, C-7, and C-8 and of 7-H to C-5, C-6 and C-8 clarified the substitution pattern on ring A of phomolactone B (**5**).

Similarly, ^1H and ^{13}C NMR spectroscopic data of phomolactone C (**6**) were very similar to those recorded for phomolactone B (**5**), although with some differences. The ^1H NMR signal for 6-H [$\delta = 6.79$ (dd, $J = 0.5$, 8.5 Hz, 6-H) ppm] showed an allylic coupling ($J = 0.5$ Hz) with 4b-H [$\delta = 2.70$ (ddd, $J = 0.5$, 11.0 , 17.0 Hz, 4b-H) ppm], which was absent in compounds **4** and **5**. All these data suggested that the second hydroxy group is attached at C-7. HMBC correlations of 5-H to C-4a, C-4, C-6, and C-7 and of 6-H to C-5, C-7 and C-8 supported the substitution pattern on ring A of phomolactone C (**6**). The ^{13}C NMR values for C-7 and C-8 ($\delta = 143.5$ and 146.2 ppm) were compatible with the proposed regiochemistry.^[17,26]

The equatorial orientation of the C-3-attached propyl group in compounds **4–6** was established by the ^1H - ^1H coupling constants in the ^1H NMR spectra. The large coupling constant ($J = 11.0$ Hz) between 3-H and 4-H_{ax} indicates that 3-H and 4-H_{ax} are *trans* and axial.^[14] Similarly, the coupling constant of $J = 3.5$ Hz between 3-H and 4-H_{eq} suggested a *cis* relationship between these protons in phomolactone A–C (**4–6**). Compounds **4–6** contain a substituted dihydroisocoumarin chromophore whose $n\text{-}\pi^*$ CD transition, independently of the aromatic substitution pattern, can be used to determine the helicity of the heterocyclic ring and thus the C-3 absolute configuration. *P* helicity of the dihydroisocoumarin heteroring ($\omega_{\text{C-8a,C-1,O,C-3}} > 0^\circ$) was correlated with a positive $n\text{-}\pi^*$ Cotton effect (CE)

at around 260 nm,^[27] and the rule was then applied to the configurational assignment of synthetic^[28,29] and natural derivatives.^[25] For example, (3*S*,4*S*)-dihydroascochin (**12**) (Figure 2), recently prepared by reduction of the natural product ascochin, had a positive $n\text{-}\pi^*$ CE at 267 nm, which derives from *P* helicity of its heteroring (Figure 7).^[18] The $n\text{-}\pi^*$ CE of **4** at 262 nm is negative, and its high-energy CD transitions are also opposite to those of **12**, which suggests that **4** has a heteroring with *M* helicity and hence (3*R*) absolute configuration (Figure 7, inset). Dihydroisocoumarin **5** showed the same $-/+/-/+$ CD pattern from the low-energy ($n\text{-}\pi^*$ CE) to the high-energy region as **4**, which allowed its assignment as (3*R*) as well (Figure 8).

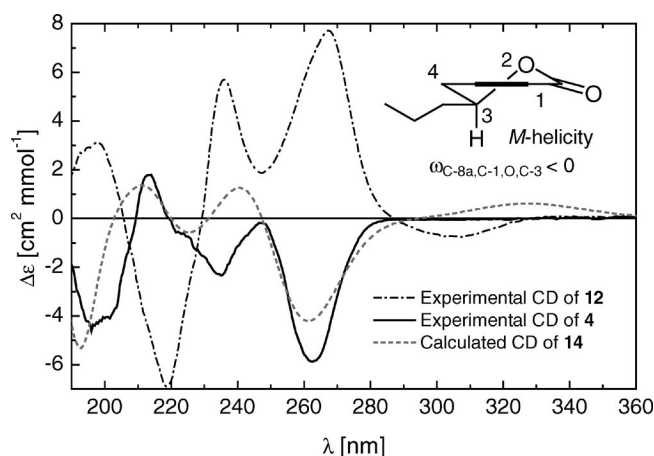


Figure 7. Measured (MeCN) CD spectrum of **4** (solid line) compared with TDDFT-calculated CD of (*R*)-**14** (grey dotted line, Boltzmann-weighted average over 2 DFT minima) and experimental CD of **12** (dashed-dotted line). Inset: (*M*) helicity of the dihydroisocoumarin heteroring with equatorial C-3 *n*-propyl group and (3*R*) absolute configuration.

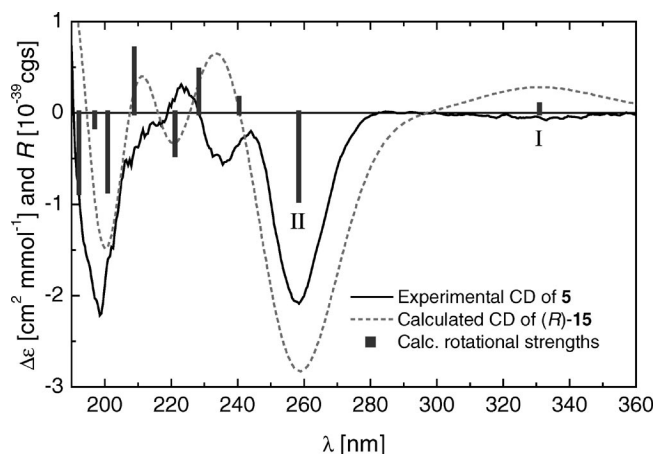


Figure 8. Measured (MeCN) CD spectrum of **5** (solid line) compared with TDDFT-calculated CD of (*R*)-**15** (grey dotted line, Boltzmann-weighted average over 4 DFT minima). Vertical bars represent computed rotational strengths *R*.

CD TDDFT calculations were employed to confirm the configurational assignment and support the CD correlation discussed above. A preliminary TDDFT//DFT study was executed on a series of 3-alkyl-3,4-dihydroisocoumarins

(**13a–c**, Figure 9) having an unsubstituted phenyl ring. First, we verified the strong preference for the equatorial position of the C-3 substituent, with respect to the axial one, regardless of its exact nature. For **13a–c**, in fact, DFT optimizations [B3LYP/6-31G(d)] led to equatorial structures with an overall population between 91 and 94% at 300 K. Second, all low-energy DFT structures of **13a–c** with equatorial C-3 substituent and (3*R*) configuration led to almost superimposable calculated CD spectra (TDDFT method, B3LYP/TZVP). On the contrary, structures with axial C-3 substituents led to almost mirror-image calculated CD spectra with respect to the equatorial ones. In other words, neither the length of the alkyl chain nor its conformation affects the shape of CD bands allied with the dihydroisocoumarin chromophore. Their sign is entirely determined by the ring A chirality, which is in turn dictated by the absolute configuration of C-3. This finding corroborated the CD correlation for $n\text{-}\pi^*$ CE of dihydroisocoumarin discussed above, and also simplified the treatment of compounds **4** and **5**, by considering the methyl analogs **14** and **15** (Figure 9). DFT geometry optimizations of **14** led to the two C-3 methyl equatorial/axial conformers, with the equatorial one more stable by 1.45 kcal/mol [92% population at 300 K, B3LYP/6-31G(d)]. For **15**, there was a second degree of freedom due to the rotation of 5-OH; the two conformers with equatorial C-3 methyl were again largely favoured over the axial ones (overall 91% population at 300 K). Figures 7 and 8 show TDDFT-calculated CD spectra (B3LYP/TZVP) as Boltzmann-weighted average for compounds (*R*)-**14** and (*R*)-**15**. There is a generally very good agreement with the experimental CD spectra of **4** and **5** below 300 nm, which confirms the absolute configuration of the two natural products established above as (*R*)-**4** and (*R*)-**5**. The strongest band around 260 nm (II in Figure 8) is mainly of $n\text{-}\pi^*$ character, although it is sizeably mixed with a $\pi\text{-}\pi^*$ -type transition. Interestingly enough, TDDFT calculations predict a further red-shifted band (I) around 330 nm where the experimental spectrum is very noisy. This is due to an electric-dipole-allowed $\pi\text{-}\pi^*$ transition with computed *g* factor ($\Delta\epsilon/\epsilon$ ratio) around 7×10^{-5} , i.e., close to the sensitivity limits of the CD instrument. At higher energies, a series of bands of alternating signs is predicted for both **14** and **15**, which reproduces the series of positive/negative maxima found in the experimental spectra of **4** and **5**.

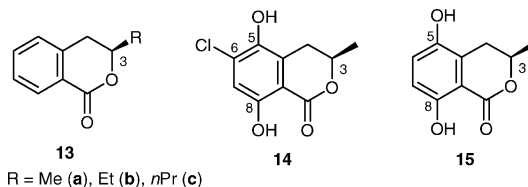


Figure 9. Compounds considered in the calculations.

Cycloepoxylactone (**1a**), cycloepoxytriol A and B (**2** and **3**) were tested for algicidal, antibacterial, and antifungal activities (Table 1). Cycloepoxylactone (**1a**) showed good antibacterial, antifungal, and algicidal activities against *Bacillus*

megaterium, *Microbotryum violaceum*, and *Chlorella fusca*, respectively, whereas cycloepoxytriol B (**3**) had good algicidal activity against *Chlorella fusca*. Cycloepoxytriol A (**2**) was inactive in these tests.

Table 1. Biological activity of the pure compounds in an agar diffusion test.

Compound (50 µg)	Algicidal Chl ^[a]	Antifungal Mb	Antibacterial Bm
Cycloepoxylactone (1a)	0	10	5
Cycloepoxytriol B (3)	9	6	5

[a] *Chlorella fusca* (Chl), *Microbotryum violaceum* (Mb) and *Bacillus megaterium* (Bm). The radius of zone of inhibition was measured in mm.

Conclusions

The stereochemistry of 1,4-dihydroxylated cycloepoxylactones of type **1a** is notoriously difficult to elucidate due to the similarity of the coupling constants of *cis* and *trans* configurations.^[15] Therefore, intensive NMR, CD, and calculation methods were required to unambiguously elucidate the relative and absolute configuration of this type of compounds. Their absolute configuration was determined for the first time, in addition to those of the dihydroisocoumarins **4–6**.

Experimental Section

General Experimental Procedures: For microbiological methods and culture conditions see refs.^[30,31] Melting points were determined with a Gallenkamp melting point apparatus. Optical rotations were measured with a Perkin–Elmer 241 MC polarimeter. The IR spectra were recorded with a Nicolet-510P spectrometer. NMR spectra were recorded with a Bruker Avance-500 NMR spectrometer with TMS as internal standard. EI mass spectra were obtained with an MAT 8200 mass spectrometer. CD spectra were recorded with a J-810 spectropolarimeter. Silica gel (70–230 mesh) was used for column chromatography.

Extraction and Isolation: The endophytic fungus *Phomopsis* sp., internal strain no. 7233, was isolated from the leaves of *Laurus azorica* from Gomera and was cultivated on 12 L of 5% w/v biomalt solid agar medium at room temperature for 28 d.^[32] The culture media were then extracted with ethyl acetate to afford 5.6 g of a residue after removal of the solvent under reduced pressure. The extract was separated into several fractions by column chromatography (CC) on silica gel by using gradients of hexane/ethyl acetate (85:15, 50:50, 0:100). The polar fraction was separated by repeated CC with hexane/ethyl acetate (7:3) to give crude compounds **1a**, **2**, and **3**. Subsequently, each crude fraction was further purified by CC with hexane/ethyl acetate (7:3) to give compounds **1a** (15.5 mg), **2** (15.0 mg), and **3** (8.0 mg). To obtain the minor compounds, the same fungus was recultivated under the previous conditions and extracted with ethyl acetate to give 4.5 g of a residue after removal of the solvent under reduced pressure. The extract was separated into two fractions by column chromatography (CC) on silica gel by using gradients of hexane/ethyl acetate (85:15, 50:50, 0:100). The less polar fraction 1 (2.1 g) contained mainly fatty acids and lipids. The polar fraction was separated by CC with hexane/ethyl acetate

(7.5:2.5) to give two subfractions A and B. Fraction A was separated by CC on silica gel with hexane/ethyl acetate (7.5:2.5) to give pure compounds **4** (5 mg), **5** (5.3 mg) and **6** (7.5 mg). Fraction B was separated by CC on silica gel with hexane/ethyl acetate (7:3) to give pure compound **1** (15.4 mg).

Cycloepoxylactone (1a): White solid; m.p. 108–110 °C. $[\alpha]_D^{20} = -119$ ($c = 0.1$, CD₂Cl₂). CD {MeCN, λ [nm] ($\Delta\epsilon$), $c = 1.25 \times 10^{-3}$ }: 265 (–1.84), 230 (2.80), 203 (–3.55). ¹H NMR (500 MHz, CDCl₃): δ = 4.85 (br. s, 1 H, 2-H), 4.47 (m, 1 H, 5-H), 4.42 (m, 1 H, 7-H), 3.46 (ddd, $J = 3.5, 1.5, 1.0$ Hz, 1a-H), 3.38 (ddd, $J = 3.5, 1.5, 1.0$ Hz, 7a-H), 2.73 (dd, $J = 17.0, 11.0$ Hz, 1 H, 6a-H), 2.22 (dd, $J = 17.0, 3.5$ Hz, 1 H, 6b-H), 1.80 (m, 1 H, 8a-H), 1.62 (m, 1 H, 8b-H), 1.50 (m, 2 H, 9-H), 0.95 (t, $J = 7.3$ Hz, 3 H, 10-H) ppm. ¹³C NMR (125 MHz, CDCl₃): δ = 166.0 (C-3), 148.1 (C-6a), 122.7 (C-2a), 77.4 (C-5), 66.5 (C-7), 61.2 (C-2), 52.2 (C-7a), 51.7 (C-1a), 36.6 (C-8), 32.5 (C-6), 17.9 (C-19), 13.7 (C-10) ppm. IR (CHCl₃): $\tilde{\nu}_{\max} = 3460, 2940, 1660$ (COO), 1250, 1130 cm^{–1}. HREIMS: m/z = 240.0989 (calcd. 240.0997 for C₁₂H₁₆O₅). EI-MS: m/z (%) = 242 (5), 224 (7), 206 (35), 188 (62), 170 (15), 152 (96), 123 (100), 121 (121), 95 (88), 71 (87), 55 (90), 41 (89).

4-Bromobenzoylation of 1a to 1b: A solution of phenol **1a** (8 mg, 0.5 mmol) in pyridine (2 mL) was treated with 4-bromobenzoyl chloride (0.5 mg, 1 mmol) and 4-(dimethylamino)pyridine (DMAP) (2 mg). The mixture was stirred at 21 °C for 10 h and then poured into cold 2 N HCl (5 mL) and stirred for 1 h to hydrolyze the excess 4-bromobenzoyl chloride. The aqueous phase was extracted three times with CH₂Cl₂ (5 mL); the organic phase was washed with 2 N NaHCO₃ (5 mL) to remove the 4-bromobenzoic acid, and dried with Na₂SO₄. The solvent was removed at reduced pressure followed by chromatography on silica gel (solvent EtOAc/petroleum ether, 4:6 to 6:4) to afford **1b**. White solid; m.p. 108–110 °C. ¹H NMR (500 MHz, CDCl₃): δ = 7.93 (d, $J = 8.1$ Hz, 2 H, 2'-H, 6'-H), 7.89 (d, $J = 8.1$ Hz, 2 H, 2''-H, 6''-H), 7.64 (d, $J = 8.1$ Hz, 2 H, 3'-H, 5'-H), 7.58 (d, $J = 8.1$ Hz, 2 H, 3''-H, 5''-H), 6.33 (m, 1 H, 2-H), 6.00 (m, 1 H, 7-H), 4.55 (m, 1 H, 5-H), 3.68 (ddd, $J = 3.5, 1.5, 1.0$ Hz, 1a-H), 3.52 (ddd, $J = 3.5, 1.5, 1.0$ Hz, 7a-H), 2.60 (dd, $J = 17.0, 12.0$ Hz, 1 H, 6a-H), 2.43 (dd, $J = 17.0, 3.5$ Hz, 1 H, 6b-H), 1.77 (m, 1 H, 8a-H), 1.62 (m, 1 H, 8b-H), 1.50 (m, 2 H, 9-H), 0.93 (t, $J = 7.3$ Hz, 3 H, 10-H) ppm. ¹³C NMR (125 MHz, CDCl₃): δ = 165.0 (COO), 164.9 (COO), 162.8 (C-3), 146.2 (C-6a), 128.1 (C-4'), 128.7 (C-1'), 131.5 (C-2', C-6'), 131.8 (C-3', C-5'), 122.5 (C-2a), 76.4 (C-5), 67.5 (C-7), 63.1 (C-2), 49.9 (C-7a), 49.3 (C-1a), 36.6 (C-8), 32.5 (C-6), 17.9 (C-9), 13.7 (C-10) ppm. IR (CHCl₃): $\tilde{\nu}_{\max} = 1660$ (COO), 1240, 1120 cm^{–1}.

Cycloepoxytriol A (2): White solid; m.p. 146–148 °C. $[\alpha]_D^{20} = +46.5$ ($c = 2.2$, MeOH). ¹H NMR (500 MHz, CD₃OD): δ = 4.73 (br. s, 1 H, 2-H), 4.33 (td, $J = 17.0, 2.0$ Hz, 1 H, 3a-H), 4.15 (br. s, 1 H, 7-H), 4.07 (d, $J = 7.0$ Hz, 1 H, 6-H), 3.95 (br. d, $J = 17.0$ Hz, 1 H, 3b-H), 3.52 (m, 1 H, 1a-H), 3.36 (m, 1 H, 7a-H), 3.33 (ddd, $J = 9.0, 7.0, 3.0$ Hz, 1 H, 5-H), 1.75 (m, 1 H, 8a-H), 1.60 (m, 1 H, 8b-H), 1.45 (m, 2 H, 9-H), 0.97 (t, $J = 7.3$ Hz, 3 H, 10-H) ppm. ¹³C NMR (125 MHz, CDCl₃): δ = 131.2 (C-6a), 130.1 (C-2a), 78.6 (C-5), 65.9 (C-3), 65.3 (C-6), 63.8 (C-7), 62.5 (C-2), 54.5 (C-7a), 54.3 (C-1a), 33.7 (C-8), 18.7 (C-9), 13.6 (C-10) ppm. IR (CHCl₃): $\tilde{\nu}_{\max} = 3460, 2940, 1250, 1130$ cm^{–1}. FABMS (+): m/z (%) = 263.1 [M + Na]⁺. EI-MS: m/z (%) = 242 (5), 224 (7), 206 (35), 188 (62), 170 (15), 152 (96), 123 (100), 121 (121), 95 (88), 71 (87), 55 (90), 41 (89).

Cycloepoxytriol B (3): White solid. $[\alpha]_D^{20} = -46.5$ ($c = 2.0$, MeOH). ¹H NMR (500 MHz, CD₃OD): δ = 4.92 (m, 1 H, 6-H), 4.83 (dd, $J = 17.0, 2.0$ Hz, 1 H, 3a-H), 4.62 (br. s, 1 H, 2-H), 4.50 (td, $J = 17.0, 2.0$ Hz, 1 H, 3b-H), 4.40 (br. s, 1 H, 7-H), 3.75 (ddd, $J = 9.0,$

7.5, 3.5 Hz, 1 H, 5-H), 3.50 (ddd, $J = 3.5, 1.5, 1.0$ Hz, 1 H, 7a-H), 3.44 (dd, $J = 3.5, 1.5$ Hz, 1 H, 1a-H), 1.63 (m, 1 H, 8a-H), 1.55 (m, 1 H, 8b-H), 1.40 (m, 2 H, 9-H), 0.97 (t, $J = 7.3$ Hz, 3 H, 10-H) ppm. ^{13}C NMR (125 MHz, CDCl_3): $\delta = 133.4$ (C-6a), 132.0 (C-2a), 89.3 (C-6), 75.2 (C-3), 73.3 (C-5), 63.0 (C-2), 60.3 (C-7), 54.9 (C-7a), 54.3 (C-1a), 33.5 (C-8), 18.7 (C-9), 13.0 (C-10) ppm. IR (CHCl_3): $\tilde{\nu}_{\text{max}} = 3460, 2940, 1250, 1130\text{ cm}^{-1}$. CI-MS (CH_4): $m/z = 243.1$ [$\text{M} + 1$]. EI-MS: m/z (%) = 242, (5), 224 (5), 204 (8), 187.1 (20), 170 (100), 170 (90), 151 (85), 123 (90), 124 (80), 109 (46), 81 (55).

Phomolactone A (4): White solid; m.p. 187 °C. $[\alpha]_{\text{D}}^{20} = -58.1$ ($c = 0.92$, CHCl_3). CD {MeCN, λ [nm] ($\Delta\epsilon$), $c = 3.89 \times 10^{-4}$ }: 262 (−5.84), 235 (−2.35), 214 (1.78), 196 (−4.61). ^1H NMR (500 MHz, CDCl_3): $\delta = 10.66$ (s, 1 H, OH), 6.49 (s, 1 H, 7-H), 4.55 (m, 1 H, 3-H), 3.20 (dd, $J = 17.0, 3.5$ Hz, 1 H, 4a-H), 2.73 (dd, $J = 17.0, 11.0$ Hz, 1 H, 4b-H), 1.90 (m, 1 H, 7-H), 1.75 (m, 1 H, 9b-H), 1.60 (m, 2 H, 10-H), 0.98 (t, $J = 7.3$ Hz, 3 H, 11-H) ppm. ^{13}C NMR (125 MHz, CDCl_3): $\delta = 169.3$ (C-1), 155.7 (C-8), 140.2 (C-5), 128.0 (C-4a), 125.2 (C-6), 115.6 (C-7), 107.8 (C-8a), 79.2 (C-3), 36.8 (C-9), 27.2 (C-4), 18.1 (C-10), 13.7 (C-11) ppm. IR (CHCl_3): $\tilde{\nu}_{\text{max}} = 3460, 2940, 1657$ (C=O), 1624 (Ar), 1250, 1130 cm^{-1} . UV (CHCl_3): $\lambda_{\text{max}} = 260$ (3.21), 315 (3.11) nm. EI-MS: m/z (%) = 256 (43) [$\text{M}^+ (^{35}\text{Cl})^+$], 258 (13) [$\text{M} (^{37}\text{Cl})^+$], 215 (10), 212 (36) [$\text{M}^+ - \text{CO}_2$], 172 (75), 171 (100), 69 (15). HREIMS: $m/z = 256.0511$ (calcd. 256.0502 for $\text{C}_{12}\text{H}_{13}\text{ClO}_4$).

Phomolactone B (5): White solid; m.p. 181–182 °C. $[\alpha]_{\text{D}}^{20} = -51.1$ ($c = 0.85$, $\text{CHCl}_3 + \text{CD}_3\text{OD}$). CD {MeCN, λ [nm] ($\Delta\epsilon$), $c = 8.99 \times 10^{-4}$ }: 258 (−2.08), 236 (−0.53), 223 (0.31), 198 (−2.21). ^1H NMR (500 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$): $\delta = 6.93$ (d, $J = 8.5$ Hz, 1 H, 6-H), 6.65 (d, $J = 8.5$ Hz, 1 H, 7-H), 4.48 (m, 1 H, 3-H), 3.06 (dd, $J = 17.0, 3.5$ Hz, 1 H, 4a-H), 2.58 (dd, $J = 17.0, 11.0$ Hz, 1 H, 4b-H), 1.80 (m, 1 H, 9a-H), 1.65 (m, 1 H, 9b-H), 1.45 (m, 2 H, 10-H), 0.90 (t, $J = 7.3$ Hz, 3 H, 11-H) ppm. ^{13}C NMR (125 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$): $\delta = 170.3$ (C-1), 155.0 (C-8), 145.2 (C-5), 124.3 (C-4a), 123.9 (C-6), 115.4 (C-7), 108.2 (C-8a), 79.6 (C-3), 36.8 (C-9), 26.6 (C-4), 18.0 (C-10), 13.6 (C-11) ppm. IR ($\text{CDCl}_3 + \text{CD}_3\text{OD}$): $\tilde{\nu}_{\text{max}} = 3450, 2935, 1660$ (C=O), 1620 (Ar), 1250, 1125 cm^{-1} . UV ($\text{CDCl}_3 + \text{CD}_3\text{OD}$): $\lambda_{\text{max}} = 260$ (3.00), 315 (3.32) nm. EI-MS: m/z (%) = 222.1 (45), 204 (31) [$\text{M}^+ - \text{H}_2\text{O}$] $^+$, 178 (33) [$\text{M}^+ - \text{CO}_2$], 171 (100), 69 (12). HREIMS: $m/z = 222.0881$ (calcd. 222.0892 for $\text{C}_{12}\text{H}_{14}\text{O}_4$).

Phomolactone C (6): White solid; m.p. 172 °C. $[\alpha]_{\text{D}}^{20} = -47.1$ ($c = 0.76$, CHCl_3). ^1H NMR (500 MHz, CDCl_3): $\delta = 10.66$ (s, 1 H, OH), 6.98 (d, $J = 8.5$ Hz, 1 H, 5-H), 6.79 (dd, $J = 8.5, 0.5$ Hz, 1 H, 6-H), 4.56 (m, 1 H, 3-H), 3.13 (dd, $J = 17.0, 3.5$ Hz, 1 H, 4a-H), 2.70 (ddd, $J = 17.0, 11.0, 0.5$ Hz, 1 H, 4b-H), 1.90 (m, 1 H, 9a-H), 1.72 (m, 1 H, 9b-H), 1.60 (m, 2 H, 10-H), 0.98 (t, $J = 7.3$ Hz, 3 H, 11-H) ppm. ^{13}C NMR (125 MHz, CDCl_3): $\delta = 169.3$ (C-1), 146.2 (C-8), 143.5 (C-7), 124.5 (C-4a), 124.0 (C-6), 116.0 (C-5), 107.2 (C-8a), 79.3 (C-3), 36.9 (C-9), 26.7 (C-4), 18.1 (C-10), 13.7 (C-11) ppm. IR (CDCl_3): $\tilde{\nu}_{\text{max}} = 3480, 2950, 1655$ (C=O), 1620 (Ar), 1250, 1125 cm^{-1} . UV (CDCl_3): $\lambda_{\text{max}} = 260$ (2.90), 315 (3.22) nm. EI-MS: m/z (%) = 222.1 (43), 204 (32) [$\text{M}^+ - \text{H}_2\text{O}$] $^+$, 178 (30) [$\text{M}^+ - \text{CO}_2$], 171 (100), 69 (14). HREIMS: $m/z = 222.0881$ (calcd. 222.0892 for $\text{C}_{12}\text{H}_{14}\text{O}_4$).

Bioactivity Tests. Agar Diffusion Test: The tested compounds were dissolved in acetone at a concentration of 1 mg/mL. 50 μL of the solution was pipetted onto a sterile filter disc (50 μg /disc), which was placed onto an appropriate agar growth medium for the respective test organism and subsequently sprayed with a suspension of the test organism.^[32] The test organisms were the Gram-positive bacterium *Bacillus megaterium* (NB medium), the fungus *Micro-*

botryum violaceum and the alga *Chlorella fusca* (both on MPY medium). The radius of zone of inhibition was measured in mm.

Computational Section: DFT calculations were performed with Spartan'06 (Wavefunction Inc., Irvine CA) by using standard parameters and convergence criteria. Geometry optimizations were run at B3LYP/6-31G(d) level. Lowest-energy DFT structures for **1a** and for its *cis* isomer were used for *J*-coupling estimations executed with the Mestre-J program (MestreLab Research, Santiago de Compostela) by using the Haasnoot-de Leeuw-Altona equation for chemical groups.^[33] Predicted $^3J_{1\text{a-H}/2\text{-H}}$ and $^3J_{7\text{-aH}/7\text{-H}}$ values were ca. 1 Hz for **1a**, and ca. 3.5 Hz for its *cis,cis* isomer. TDDFT-CD calculations were performed with Gaussian'03W, Revision D.01 (Gaussian, Inc., Pittsburgh PA) by using various functionals (B3LYP, BH&HLYP) and basis sets (TZVP, ADZP)^[34] for **1a** (all the functionals/basis sets combinations giving consistent results), and B3LYP/TZVP for other compounds. CD spectra were generated as sums of Gaussians with 1500 or 2000 cm^{-1} half-height widths by using dipole-velocity-computed rotational strengths. Dipole-length-computed values differed from dipole-velocity-computed ones by less than 10% for all relevant bands for all compounds. The latter ones always had energies well below the estimated ionizations potentials and involved virtual orbitals with negative eigenvalues.^[35]

Supporting Information (see footnote on the first page of this article): Supporting Information with ^1H and ^{13}C NMR spectra of compounds **1a**, **2–5**.

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