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# Curvularin-Type Metabolites from the Fungus *Curvularia* sp. Isolated from a Marine Alga[‡]

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*Dedicated to Prof. Dr. András Lipták on the occasion of his 70th birthday*

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Two new curvularin-type macrolides, curvulone A (**1**) and B (**2**), and two known ones (**3a**, **4**) of the rare 15*R* series have been isolated from the fungus *Curvularia* sp., which has been isolated from the marine alga *Gracilaria folifera*. Their structures were determined by extensive 2D NMR experiments and supported by the single-crystal X-ray analysis of **1**. The structural elucidation of **1**, which has a benzo[*b*]furanone moiety as part of a 12-membered macrolactone, has led to a revision of the structure of the previously reported (11*S*,15*S*)-11β-hydroxy-12-oxocurvularin. The absolute configuration of curvulone A was established independently by the solid-state TD-DFT ECD method and by measuring the anomalous dispersion effect. The absolute configuration of curvulone B was determined by TD-DFT ECD calculations on the computed solution conformers. Two different solid-state conformers of **4** were identified by X-ray analysis of the single crystals obtained from different solvents; TD-DFT ECD calculations were performed to reproduce the experimental ECD spectra. All four metabolites were biologically active against fungal, bacterial and algal test organisms.

## Introduction

Marine fungi have proven to be a rich source of novel organic compounds with biological activity.[1,2] Curvularins with a 12-membered lactone ring are examples of polyketides, more specifically, fungal phenylacetic acid lactones. Curvularins elicit diverse biological effects such as phytotoxic and antifungal activities,[3–5] as well as cytotoxicity against human cancer cell lines.[6,7] These activities are responsible for the recent stereoselective synthesis of some derivatives.[8,9] The C-15 chiral centre adjacent to the lactone oxygen is a common feature of curvularins and all reports of their isolation, with one exception,[10] have described natural curvularins of the 15*S* series. In connection with our ongoing search for biologically active metabolites from fungi, we have investigated the constituents of the fungus *Curvularia* sp., strain 6540, which has been isolated from the marine red algae *Gracilaria folifera*. From the ethyl acetate extract we isolated two new curvularin-type metabolites (**1** and **2**) and two known ones (**3a** and **4**) with the 15*R* absolute configuration. In this report, we describe the isolation, structural elucidation using different spectroscopic techniques and biological activity of these metabolites.

## Results and Discussion

The fungus *Curvularia* sp., internal strain no. 6540, was obtained from the marine alga *Gracilaria*, isolated from the Gulf of Mexico at John's Pass, Madeira Beach, Florida, USA. Cultivation was carried out on a solid medium as described in the Exp. Sect. After growth for 28 d, the culture and medium were extracted with ethyl acetate and fil-

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tered. The filtrate was then evaporated under reduced pressure to afford 12.5 g of the crude extract. The extract was purified by column chromatography and preparative TLC to give four compounds.

Compound **1** (Figure 1) was obtained as white crystals with an optical rotation of –75.9 (EtOH, $c$ = 0.75) and its molecular formula was determined from the MS molecular peak ($[M + Na]^+$, $m/z$ = 327.083) as $C_{16}H_{16}O_6$, which implies nine elements of unsaturation. The IR spectrum shows a strong absorption for keto groups at 1723 $cm^{-1}$ and the 1219 $cm^{-1}$ control band suggests the presence of an ester group. The $^1H$ and $^{13}C$ NMR spectroscopic data (Table 1) together with the DEPT and HSQC spectra reveal a tetrasubstituted benzene ring with two *meta* aromatic protons (4-H and 6-H, $\delta_H$ = 6.67 and 6.36 ppm, respectively), an isolated methylene (C-2, $\delta_C$ = 38.80 ppm, $\delta_H$ = 3.49 and 4.31 ppm), two adjacent methylenes (C-13, $\delta_C$ = 42.23 ppm; C-14, $\delta_C$ = 26.29 ppm), a methylene next to a methine group (C-11, $\delta_C$ = 43.15 ppm), two oxygen-substituted methine groups (10-H, $\delta_H$ = 4.94 ppm; 15-H, $\delta_H$ = 4.73 ppm) and a methyl group (16-H, $\delta_H$ = 1.17 ppm). Moreover, resonance signals for seven $sp^2$ quaternary carbons were detected of which two have been attributed to keto groups (C-9, $\delta_C$ = 197.92 ppm; C-12, $\delta_C$ = 206.79 ppm), one to an ester carbonyl (C-1, $\delta_C$ = 175.88 ppm) and four to aromatic carbons.

The –(O)CHCH$_2$– and –CH$_2$CH$_2$CH(Me)O– spin systems were identified on the basis of $^1H$–$^1H$ COSY and TOCSY experiments (Figure 2). The deshielded $^{13}C$ NMR resonances for C-5 and C-7 ($\delta_C$ = 166.78 and 169.43 ppm) indicate their substitution by phenolic oxygens. The HMBC

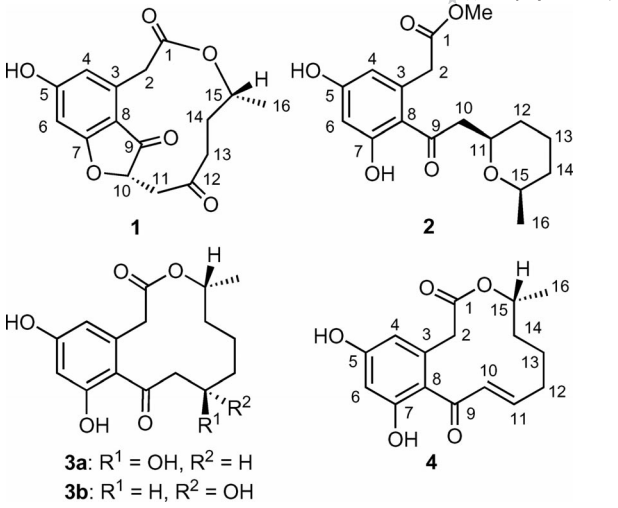


Figure 1. Compounds isolated from the fungus *Curvularia* sp.

correlations allowed the structure of **1** to be assembled from the subunits. The isolated methylene protons of C-2 (C-2, $\delta_H$ = 3.49 and 4.31 ppm) gave HMBC correlations with the aromatic C-3, C-4 and C-8 carbons and the ester carbonyl (C-1, $\delta_C$ = 175.88 ppm), which suggests that it is a benzylic methylene adjacent to the ester carbonyl. The C-9 proved to be a benzylic carbonyl group *ortho* to the isolated methylene showing weak HMBC crosspeaks to 4-H, 6-H and 2-H$_b$. In addition, the C-9 also has strong HMBC correlations with 10-H and 11-H$_b$, which indicates its connection with the –(O)CHCH$_2$– subunit, the C-11 methylene protons of which show correlations with the C-12 carbonyl and C-13

Table 1. NMR spectroscopic data of **1** and **2** in $CDCl_3$.

| Position | Curvulone A (**1**) | | Curvulone B (**2**) | |
|---|---|---|---|---|
| | $\delta_C$ [ppm] | $\delta_H$ [ppm] (mult., $J$ [Hz]) | $\delta_C$ [ppm] | $\delta_H$ [ppm] (mult., $J$ [Hz]) |
| 1 | 175.88 | – | 172.64 | – |
| 2 | 38.80 | a: 3.49 (d, 17.6)<br>b: 4.31 (d, 17.6) | 39.81 | a: 3.54 (d, 16.6)<br>b: 3.94 (d, 16.6) |
| 3 | 136.10 | – | 135.76 | – |
| 4 | 113.73 | 6.67 (d, 1.5) | 111.85 | 6.21 (d, 2.4) |
| 5 | 166.78 | – | 159.68 | – |
| 6 | 97.70 | 6.36 (d, 1.5) | 104.08 | 6.27 (d, 2.4) |
| 7 | 169.43 | – | 159.55 | – |
| 8 | 112.82 | – | 120.64 | – |
| 9 | 197.92 | – | 204.33 | – |
| 10 | 82.62 | 4.94 (t, 4.1, 4.6) | 49.07 | ax: 3.33 (dd, 14.3, 10.1)<br>eq: 2.58 (dd, 14.3, 3.2) |
| 11 | 43.15 | a: 3.25 (dd, 12.6, 4.1)<br>b: 2.94 (dd, 12.6, 4.6) | 77.85 | 4.15 (m, 10.1, 3.2) |
| 12 | 206.79 | – | 30.73 | ax: 1.43 (m, 13.5, 3.7)[a]<br>eq: 1.68 (m, 13.5) |
| 13 | 42.23 | ax: 2.49 (ddd, 20.1, 10.9, 3.1)<br>eq: 2.74 (m, 20.1, 6.2, 2.6) | 32.70 | ax: 1.59 (m)[a]<br>eq: 1.88 (m, 3.6, 3.7) |
| 14 | 26.29 | ax: 2.01 (m, 6.2, 10.9, 11.8)<br>eq: 1.48 (m, 3.1, 2.6, 2.6) | 23.11 | ax: 1.27 (m, 9.1, 3.6)[a]<br>eq: 1.62 (m, 2.1) |
| 15 | 73.14 | 4.73 (m, 11.8, 2.6, 6.1) | 74.91 | 3.58 (m, 6.1, 9.1, 2.1) |
| 16 | 20.22 | 1.71 (d, 6.1) | 21.49 | 1.19 (d, 6.1) |
| COOMe | – | – | 52.11 | 3.71 (s) |
| OH | – | – | – | 9.79 (b) |

[a] Other coupling constants are approximated from the TOCSY spectrum as follows; $^3J_{12e,13a} \approx {}^3J_{12e,13e} \approx {}^3J_{12e,11} \approx$ 2–3 Hz, $^3J_{12a,13e} \approx {}^3J_{12a,11} \approx$ 10–11 Hz, $^3J_{14e,13a} \approx {}^3J_{14e,13e} \approx$ 2–3 Hz, $^3J_{14e,13a} \approx$ 10–11 Hz.

  

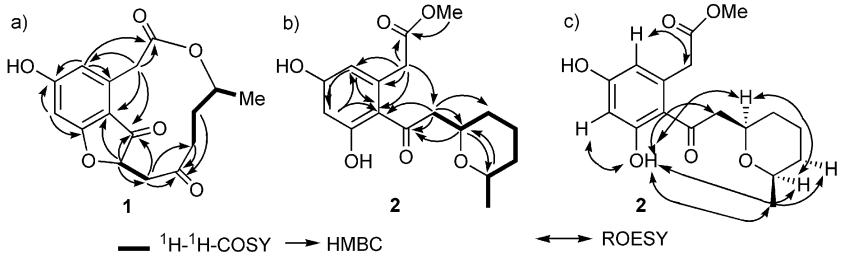


Figure 2. a) $^1$H–$^1$H COSY and selected $^1$H–$^{13}$C long-range correlations of **1**; b) $^1$H–$^1$H COSY and selected $^1$H–$^{13}$C long-range correlations of **2**; c) ROESY correlations of **2**.

of the $-CH_2CH_2CH(Me)O$ subunit. The substitution pattern of the aromatic ring was established by the HMBC crosspeaks of the 4-H,C and 6-H,C aromatic nuclei; correlations were found from both 2-H atoms and 6-H to C-4, from 4-H to C-1, C-3, C-5 and C-9 (weak) and from 6-H to C-5, C-7, C-4 and C-9 (weak).

At this stage molecule **1** could be assembled without the C-10–O ether and C-15–O lactone linkages. The free valences of the two methine groups and the remaining elements of unsaturation could only be rationalized by the formation of a 12-membered lactone and a benzo[*b*]furanone ring, as shown in the structure of **1**. Upon storing the NMR sample in a refrigerator, single crystals of **1** precipitated from the $CDCl_3$ solution making feasible the X-ray diffraction analysis that unequivocally confirmed the structure of **1** as deduced from the NMR study (Figure 3). It is noteworthy that a carbon skeleton incorporating a benzofuranone system is unprecedented in macrolide structures.

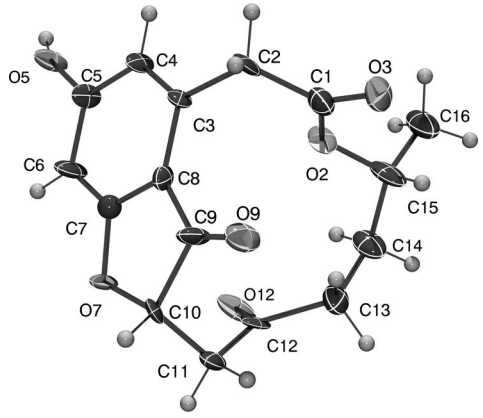


Figure 3. X-ray structure of curvulone A (**1**). ORTEP view at the 30% probability level. $CDCl_3$ solvent molecules have been omitted for clarity.

As a result of the benzene-condensed 12-membered lactone ring, **1** belongs to the group of curvularin-type macrolides and was named curvulone A. Curvulone A (**1**) contains two chiral centres, the relative configurations of which were determined as 10*S**,15*R** by X-ray diffraction analysis. The unambiguous determination of its absolute configuration was accomplished by two independent methods. First, the electronic circular dichroism (ECD) spectrum of **1** was measured on a microcrystalline solid sample and compared with the spectrum calculated by the TD-DFT method using the X-ray geometry.[11–13] This method, known as the solid-state ECD/TD-DFT approach, has been recently developed for the configurational assignment of natural products.[14–17] It is especially useful for flexible compounds because it does not require any conformational analysis.

ECD spectra of (–)-**1** were recorded in two different solvents (acetonitrile and methanol) and in the solid state as KCl pellet (Figure 4, a). The main features of the ECD spectrum were preserved in the different conditions, in particular, a negative Cotton effect (CE) at long wavelengths (320–350 nm), one or more positive CEs between 250–320 nm and a negative CE at 240 nm. Some differences, however, emerged between the three spectra that are possibly related to different conformations.

Following the usual procedure for the solid-state TD-DFT ECD approach,[11] the input structure for ECD calculations was generated from the X-ray geometry of curvulone A (**1**) with the 10*S*,15*R* absolute configuration upon DFT optimization of the hydrogen atoms. TD-DFT calculations[18] were then performed by using various hybrid functionals (B3LYP, BH&HLYP and CAM-B3LYP) and the triple-ζ split-valence basis set TZVP. All combinations resulted in similar computed ECD spectra, in particular, the combination of a positive CE at longer wavelengths and a negative CE at shorter wavelengths was reproduced in all cases. The spectrum best matching the solid-state experimental spectrum was obtained with CAM-B3LYP/TZVP and is shown in Figure 4 (b); it allows the assignment of the absolute configuration of curvulone A as (–)-(10*S*,15*R*)-**1**.

The other independent method used to determine the absolute configuration is based on measuring the anomalous dispersion effects by collecting Friedel pair reflections in the X-ray diffraction experiment.[19] Fortunately, **1** crystallized with two molecules of $CDCl_3$ in the NMR tube and the heavy chlorine atoms had a measurable anomalous dispersion effect. The Flack parameter[19] was 0.02(16) in the final refinement for all 5483 reflections with 533 Friedel pairs, which indicates the absolute configuration in **1** to be 10*S*,15*R*.

It is worth mentioning that Furukawa and co-workers described the isolation of (11*S*,15*S*)-11β-hydroxy-12-oxocurvularin (**5a**; Figure 5) from the mycelium of a hybrid

  

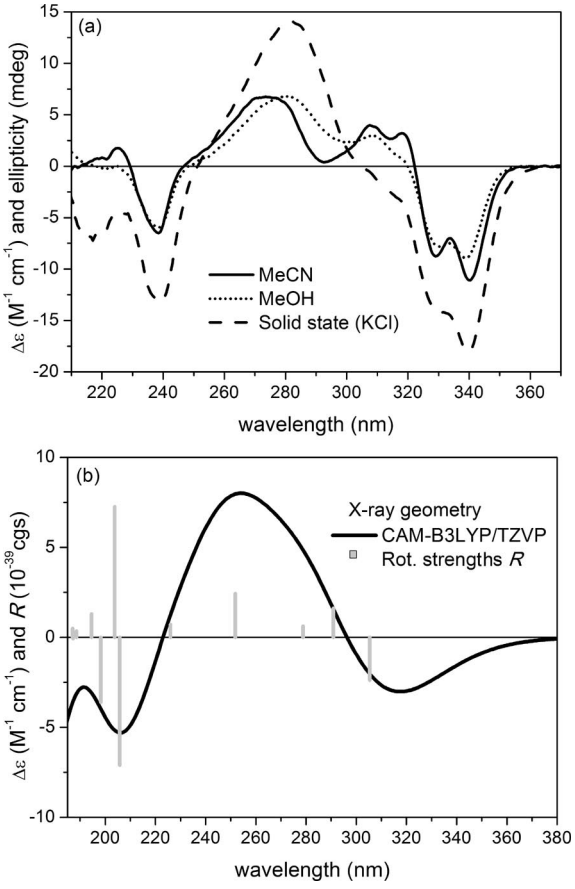


Figure 4. (a) Experimental ECD spectra of curvulone A [(–)-**1**] in acetonitrile, methanol and as KCl pellet. (b) CD spectrum calculated with CAM-B3LYP/TZVP using the X-ray structure of (10*S*,15*R*)-**1**. Vertical bars represent rotational strengths; the spectrum was obtained as the sum of Gaussians with variable bandwidth (amounting to 28 nm at 300 nm, see the computational section in the Exp. Sect.).

strain derived from *Penicillium citreo-viride* B together with other known curvularin-type secondary metabolites such as 12-oxocurvularin (**5b**), 11-β-hydroxycurvularin (**6a**) and 11-α-hydroxycurvularin (**6b**).[20] Compound **5a** was reported to give *m*/*z* = 304.0961 ($C_{16}H_{16}O_6$) due to $[M - H_2O]^+$ and its published $^1$H NMR spectroscopic data and coupling constants measured in $CDCl_3$ are identical to those observed for curvulone A (**1**).[20] The structure of **5a** was proposed on the basis of the co-occurrence with other curvularin derivatives of the 15*S* series, but the position of the 11β-hydroxy group was not confirmed by HMBC experiments. Later, the biosynthesis of **5a** was studied and the $^{13}$C NMR spectroscopic data obtained in $CD_3OD$[21] are practically identical to those we measured for curvulone A (**1**) in $CDCl_3$. More recently, the isolation of the known compound 11β-hydroxy-12-oxocurvularin (**5a**) was reported from the fungus *Aspergillus* sp.[22] On the basis of the identical NMR spectroscopic data we believe that the structure of curvulone A (**1**) had been erroneously determined earlier as 11β-hydroxy-12-oxocurvularin (**5a**) and thus the structure has to be revised to **1**. The previously reported curvulone A, which was isolated together with curvularin derivatives having the 15*S* configuration, has a positive specific rotation {$[\alpha]_D^{25}$ = +41 (EtOH)},[20] whereas our (10*S*,15*R*)-curvulone A (**1**) has a negative one {$[\alpha]_D^{25}$ = –76 (EtOH)} and identical spectroscopic data, which implies that they are probably enantiomers.

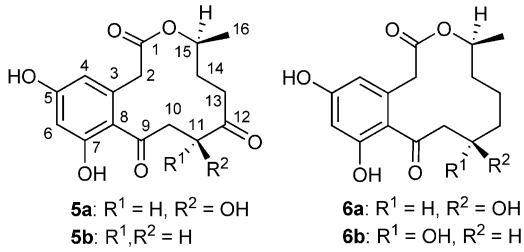


Figure 5. Structures of curvularin derivatives **5a**,**b** and **6a**,**b**.

The optically active compound **2** (Figure 1; $[\alpha]_D^{25}$ = –22) was isolated as a colourless oil with the molecular formula $C_{17}H_{22}O_6$, as deduced from the ESI-MS {*m*/*z* = 345.129 [M + Na]$^+$} and NMR data, which implies seven elements of unsaturation. The IR spectrum shows strong absorptions for hydroxy and ester carbonyls at $\tilde{\nu}$ = 3389, 1724 and 1283 cm$^{-1}$ and the $^1$H NMR spectroscopic data (Table 1) indicate a tetrasubstituted benzene ring with *meta* aromatic hydrogens ($\delta_H$ = 6.21 and 6.27 ppm, $^3J_{4H,6H}$ = 2.4 Hz), a chelated phenolic hydroxy group ($\delta_H$ = 9.79 ppm), a methoxy group ($\delta_H$ = 3.71 ppm), a methyl group ($\delta_H$ = 1.19 ppm, *J* = 6.1 Hz) and an oxygenated methine group ($\delta_H$ = 4.15 ppm, m). In contrast to curvulone A (**1**), the presence of a chelated hydroxy signal at $\delta$ = 9.79 ppm suggests a phenolic hydroxy *ortho* to a benzylic carbonyl group. The $^{13}$C DEPT spectrum of **2** displays 17 signals, that is, a benzylic carbonyl ($\delta_C$ = 204.33 ppm), an ester carbonyl ($\delta_C$ = 172.64 ppm), four quaternary aromatic carbons ($\delta_C$ = 159.68, 159.55, 135.76, 120.64 ppm), four methines including two oxygenated ones ($\delta_C$ = 77.85 and 74.91 ppm), two methyl groups ($\delta_C$ = 52.11 and 21.49 ppm) and five methylenes ($\delta_C$ = 49.07, 39.81, 32.70, 30.73, 23.11 ppm).

From the $^1$H–$^1$H COSY and HSQC spectra, a –$CH_2$CH(O)$CH_2CH_2CH_2$CH(O)$CH_3$ spin system and an isolated benzylic methylene group (C-2) could be identified. Based on the HMBC correlation map, the isolated methylene (C-2) is a benzylic one *ortho* to the C-9 carbonyl group and is connected to a methoxycarbonyl group as both 2-H atoms ($\delta_H$ = 3.54 and 3.94 ppm) display correlations with aromatic carbons C-3, C-4 and C-8 and the C-1 ester carbonyl. Moreover, a weak HMBC crosspeak was observed between the 2-H at $\delta$ = 3.94 ppm and the C-9 carbonyl group at the benzylic position.

Correlations of the C-10 methylene protons with the C-9 phenyl carbonyl, the C-8 aromatic carbon, C-11 methine and C-12 methylene establish the connectivity to the C-9 carbonyl. The crosspeaks between 15-H and C-11 and between 11-H and C-15 confirm that the C-11 and C-15 methine carbons are connected through an oxygen atom to form a 1,3-disubstituted tetrahydropyran ring with two chiral centres. With the combined use of HMBC and ROESY

  

spectra, the aromatic substitution pattern, characteristic for curvularin derivatives, was established as shown in Figure 2.

The relative configuration of the tetrahydropyran ring was deduced from the ROESY spectrum. The ROESY cross-speak observed between the axial methine protons 11-H and 15-H corroborates the *cis* orientation of the C-11 and C-15 substituents and hence the 11*R**,15*R** relative configuration. The large coupling constants of 11-H and 15-H with the adjacent 12-$H_{ax}$ and 14-$H_{ax}$, respectively ($^3J_{15H,14Hax}$ = 9.1 Hz and $^3J_{11H,12Hax} \approx$ 10–11 Hz, as determined from the TOCSY experiment, see Table 1), confirm that the C-11 and C-15 substituents adopt a diequatorial orientation, which was further supported by the results of molecular modelling.

To determine the absolute configuration of **2**, the ECD spectrum was measured in acetonitrile and compared with the Boltzmann-averaged spectrum calculated with TD-DFT for the solution conformers.[23] Because of the presence of several flexible torsions, the number of low-energy minima obtained by a preliminary MMFF conformational analysis for compound **2** was quite large. However, several structures, which did not fit the NMR spectroscopic data (especially the two different $^3J_{HH}$ coupling constants between protons 10-H and 11-H), could be discarded. The refined set of structures was optimized by the DFT method (see the Computational Details in the Exp. Sect.) and converged to 12 conformers within 10 kJ/mol, five of which were within 6 kJ/mol; these five conformers amounted to an overall 82% population at 300 K and were considered for CD calculations. Surprisingly, in the lowest-energy conformer, as well as in most of the low-energy minima, the 7-OH is bonded to the tetrahydropyran oxygen forming an eight-membered ring. The structure of the absolute lowest-energy conformer (shown in Figure 6) is also in agreement with the strong ROESY effect observed for 7-OH with the β-oriented 10-$H_a$ and with weaker effects to the three 16-H atoms, 11-H and 15-H.

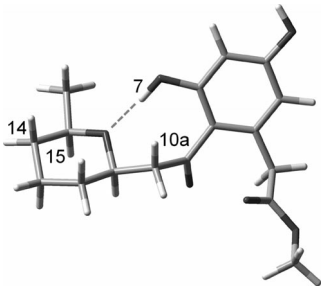


Figure 6. Lowest-energy structure for (11*R*,15*R*)-curvulone B (**2**) optimized by DFT [B3LYP/6-31(d)].

The 7-OH proton also shows a negative three-spin ROE effect[24,25] with 14-$H_{eq}$, which indicates a linear arrangement of the 7-OH, 15-H and 14-$H_{eq}$ hydrogen atoms. The six-membered cyclic conformer (7-OH hydrogen-bonded to C=O) is disfavoured by the steric crowding between the C-2 and C-10 methylene groups, which is relieved by rotation about the (C-8)–(C-9) single bond and formation of the eight-membered chelate.

In the solution ECD spectrum of **2**, an intense broad negative CE was observed at 327 nm with shoulders and three positive CEs at shorter wavelengths (Figure 7, a). ECD calculations were performed on the lowest-energy structure assuming the 11*R*,15*R* configuration using TD-DFT methods with various functionals and basis sets. The B3LYP/TZVP combination gave the best result and was applied to TD-DFT calculations of the remaining four lowest-energy minima. All the calculated CD spectra, as well as their weighted average [using Boltzmann populations at 300 K from B3LYP/6-311G+(d,p) internal energies], show a negative band at around 325 nm and a positive one centred around 270 nm; the latter is due to two distinct transitions both with positive rotational strengths in the 260–300 nm region (Figure 7, b). The above approach includes some simplifications, first, the neglect of solvent effects[26] and the use of non-corrected internal energies. However, in view of

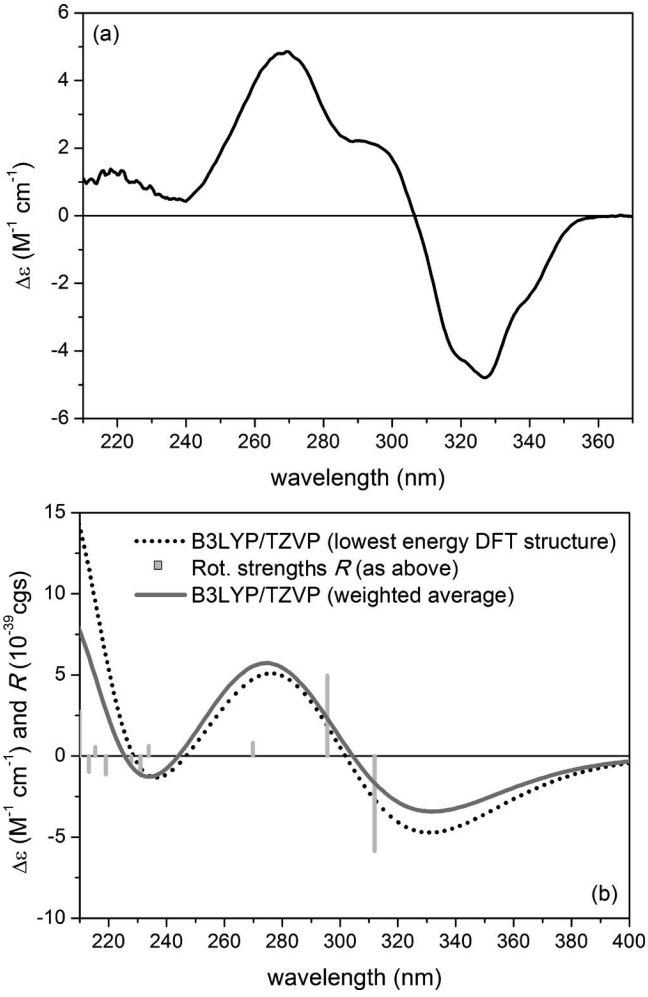


Figure 7. (a) Experimental ECD spectra of (11*R*,15*R*)-curvulone B (**2**) in acetonitrile. (b) B3LYP/TZVP-calculated ECD spectra for the DFT lowest-energy structure of (11*R*,15*R*)-**2** optimized with B3LYP/6-31G(d) (dotted line) and Boltzmann-weighted average of the ECD spectra calculated at the same level for the first five DFT energy minima [populations at 300 K estimated at the B3LYP/6-311G+(d,p) level; solid line]. Vertical bars represent rotational strengths calculated for the lowest-energy structure; spectra obtained as the sum of Gaussians with a 4000 $cm^{-1}$ bandwidth.

  

the consistency between all the calculated spectra, comparison of the calculated and experimental CD spectra led us to conclude that the absolute configuration of compound **2** is (–)-11*R*,15*R*. Thus, compound **2** belongs to the 15*R* series of curvularin derivatives such as curvulone A (**1**) and was named curvulone B.

Compound **3** (Figure 1) was isolated as a 7:1 mixture of the known (11*R*,15*R*)-11-hydroxycurvularin (**3a**) and its 11*S*,15*R* epimer **3b**.[10] Previously, all the 11-hydroxycurvularin epimers isolated from different *Penicillium* strains[3,7,20,27,28] had 15*S* absolute configuration (**6a**, **6b**). The absolute configuration of their C-11 had been assigned on the basis of chemical correlation with 10,11-epoxycurvularins of known stereochemistry.[27] There is a recent report on two 11-hydroxycurvularin epimers (**3a** and **3b**) with 15*R* configuration isolated from the marine fungus *Curvularia* sp.[10] The major component **3a** of our mixture was found to be the (11*R**,15*R**)-11-hydroxycurvularin epimer as it has $^1$H NMR chemical shifts for the 10-H, 11-H and 15-H protons identical to those reported for (11*S*,15*S*)-11-hydroxycurvularin[27,28] and (11*R*,15*R*)-11-hydroxycurvularin.[10] The positive specific rotation measured for the epimeric mixture **3** {$[\alpha]_D^{25}$ = +25 (EtOH)} in comparison to the reported negative values for (11*S*,15*S*)-11-hydroxycurvularin {$[\alpha]_D^{25}$ = –29.4 (EtOH)}[27] and (11*R*,15*S*)-11-hydroxycurvularin {$[\alpha]_D^{25}$ = –10.9 (EtOH)}[27] point to an 11*R*,15*R* absolute configuration for the major component. This was confirmed by the online HPLC–CD measurement of 11-hydroxycurvularin epimers, which gave an ECD curve for **3a** identical to that reported for (11*R*,15*R*)-11-hydroxycurvularin and for the minor component **3b** identical to that of (11*S*,15*R*)-11-hydroxycurvularin.[10]

Compound **4** (Figure 1) was identified as the *R* enantiomer of the known α,β-dehydrocurvularin. Although the isolation of (10*E*,15*S*)-α,β-dehydrocurvularin has been reported from a number of fungal species such as *Curvularia*,[29,30] *Stemphylium*,[31] *Penicillum*,[3,7,21,27,28,32] *Cochliobolus*,[33] *Aspergillus*[6,22] and *Alternaria*,[34,35] there is only a single example of the isolation of (10*E*,15*R*)-α,β-dehydrocurvularin from the marine fungus *Curvularia* sp.[10] Although the absolute configuration of (10*E*,15*S*)-α,β-dehydrocurvularin has been determined unambiguously by total synthesis[36] and chemical correlation,[32] we intended to test our solid-state TD-DFT ECD spectroscopic method for its configurational assignment. This was especially interesting as two solid-state X-ray geometries with different conformations of the 16-membered macrolide ring were available. One of the single crystals was obtained by the crystallization of **4** from a mixture of wet methanol and dichloromethane (Figure 8 and "type B" in Figure 9), whereas the other was obtained from a $CDCl_3$ solution of **3** upon storage ("type A" in Figure 9). The latter single crystal was studied with the prospect of obtaining the solid-state geometry of (11*R*,15*R*)-11-hydroxycurvularin. However, it proved to be its degradation product, (10*E*)-α,β-dehydrocurvularin. A search of the Cambridge Structural Database[37] (Ver. 5.31 Update February 2010) showed that the reported structure YEGGIA[38] is the same as **4** crystallized from $CDCl_3$, that is, (10*E*)-α,β-dehydrocurvularin, except for the configuration of C-15, which is *S* in YEGGIA. Although a complete fit of (15*S*)-**4** crystallized from $CDCl_3$ and YEGGIA could be reached, there is no heavy atom in the structure and determination of the absolute configuration using our experimental diffraction data was not possible for either of the two crystal structures. In this case the assignment could be made by using the solid-state TD-DFT ECD spectroscopic method, which established the 15*R* configuration. The change in the conformation (Figure 9) observed when (10*E*)-α,β-dehydrocurvularin was crystallized from wet methanol could be rationalized by the incorporation of a water molecule into the lattice, which forms strong hydrogen bonds with the O-9 and bridges it with the O-5 phenolic hydroxy group. In this case the phenyl ring and the C-9 carbonyl group are nearly coplanar with a strong intramolecular hydrogen bond between 7-OH and O-9. In the absence of water, O-9 is an acceptor of the O-5 and O-7 phenolic hydrogen atoms of symmetry-related molecules in the lattice and the carbonyl group and the C=C bond are planar, their plane being nearly perpendicular to the phenyl ring. Further details of structural data can be found in the Supporting Information.

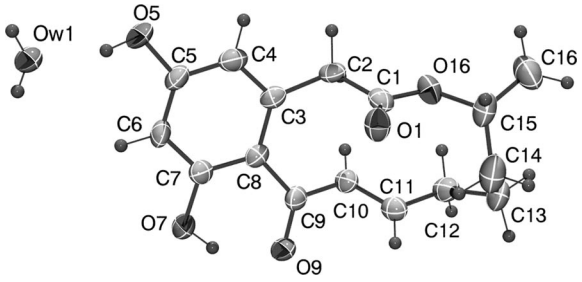


Figure 8. Molecular structure of **4** in the crystal containing a water solvent molecule. Anisotropic displacement ellipsoids drawn at the 50% probability level.

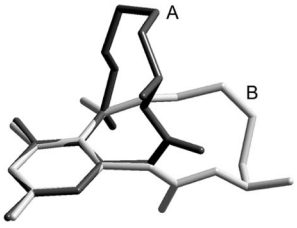


Figure 9. Overlapped solid-state X-ray geometries of (10*E*,15*R*)-α,β-dehydrocurvularin (**4**) recorded with single crystals from $CDCl_3$ (dark structure, "type A") and a mixture of wet MeOH and $CH_2Cl_2$ (light structure, "type B"). Hydrogen atoms have been omitted for clarity.

As shown in Figure 9, **4** adopts different conformations even in the solid state depending on the solvent used for crystallization. The conformational flexibility of the macrolide is also evident from the experimental ECD curves as the spectra recorded in methanol and acetonitrile show distinct differences, although the main bands are preserved (Figure 10). These include a negative CE at 335 nm and two positive ones at 300 and 230 nm. However, the ECD spec-

  

trum in acetonitrile shows a weak negative CE at 246 nm, which is absent in the ECD spectra recorded in methanol and KCl.

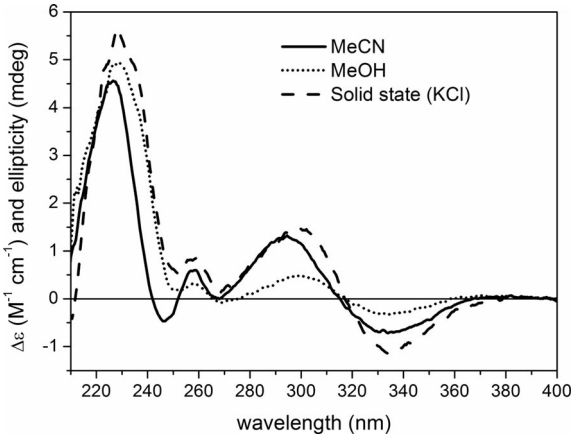


Figure 10. Experimental ECD spectra of (+)-(10*E*,15*R*)-10,11-dehydrocurvularin (**4**) in acetonitrile, methanol and as KCl pellet.

The solid-state TD-DFT ECD approach described above was applied to compound **4** using the X-ray geometry with the 15*R* configuration found in the crystals obtained from $CDCl_3$. TD-DFT calculations using different functionals and basis sets reproduced the three main bands of the experimental solid-state spectrum (part a of Figure 11 shows B3LYP/TZVP results). Thus, the absolute configuration of 10,11-dehydrocurvularin could be confirmed as (+)-(15*R*)-**4**. Very interestingly, when the alternative X-ray geometry (crystals obtained from a mixture of wet MeOH and $CH_2Cl_2$) with the same 15*R* configuration was employed as input for the TD-DFT calculations, the resulting spectra were in poor agreement with the experimental ones shown in Figure 10 regardless of the functional and basis set employed (see B3LYP/TZVP result in Figure 11, b). Unfortunately we could not measure the solid-state ECD spectrum for the second type of crystals. The above results suggest that the solid-state TD-DFT ECD approach can be perfectly employed for crystalline materials exhibiting polymorphism provided that the crystal form used for the solid-state ECD measurement is known and the corresponding molecular structure is determined.

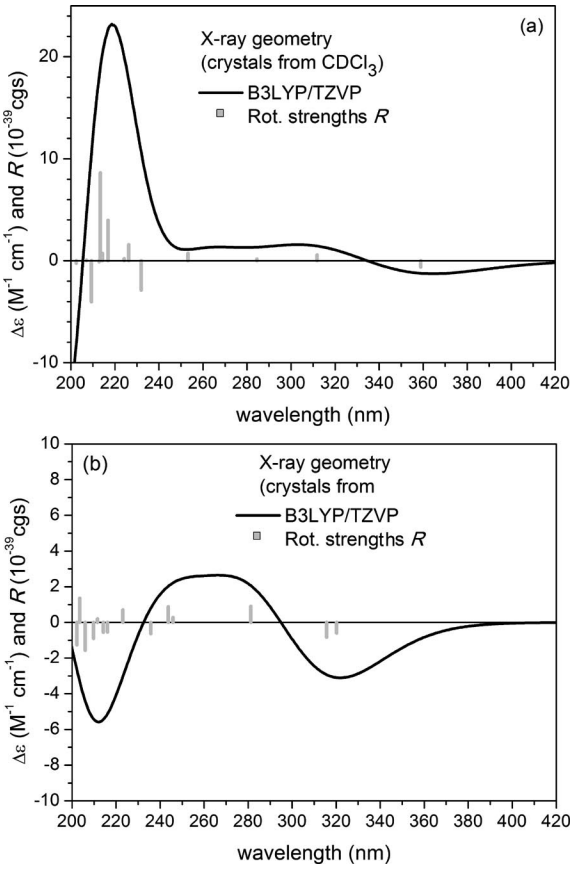


Figure 11. CD spectra calculated at the B3LYP/TZVP level using the X-ray structures of (+)-(15*R*)-**4** for the crystals isolated from (a) $CDCl_3$ and (b) a mixture of wet MeOH and $CH_2Cl_2$. Vertical bars represent rotational strengths; spectra obtained as the sum of Gaussians with variable bandwidth (amounting to 28 nm at 300 nm, see Computational Details in the Exp. Sect.).

All of the isolated metabolites were biologically active against all four of the test organisms (Table 2). Antibacterial activity against the Gram positive bacterium *Bacillus megaterium* was particularly strong, strain 6540 inhibiting 92% of the bacterial growth. The four metabolites also had pronounced antifungal activities against *Microbotryum violaceum* and *Septoria tritici* and were antialgal against *Chlorella fusca*. These antimicrobial activities concur well with those obtained for curvularin macrolides by other groups.[3–7]

Table 2. Biological activities in a microtiter assay.[a]

| | Inhibition [%] | | | |
|---|---|---|---|---|
| | *Bacillus megaterium* | *Microbotryum violaceum* | *Septoria tritici* | *Chlorella fusca* |
| **1** | 92 | 68 | 62 | 59 |
| **2** | 88 | 79 | 68 | 74 |
| **3** | 78 | 80 | 56 | 74 |
| **4** | 72 | 60 | 43 | 11 |
| Methanol/acetone | 0 | 0 | 0 | 0 |
| Control | 0 | 0 | 0 | 0 |

[a] Growth (cell count) was evaluated as % inhibition in comparison to the control, which was not inhibited. Each well contained 200 μL medium (NB for *B. megaterium*, MPY for *M. violaceum* and *S. tritici* and CP for *C. fusca*), 50 μL test organism, 10 μL substance (10 mg/mL dissolved in 1:1 methanol/acetone) yielding a final concentration of 0.4 mg/mL. Control: extracted medium.

## Conclusions

Four curvularin-type macrolides **1**–**4** have been isolated with the rare 15*R* absolute configuration of which curvulone A (**1**) and B (**2**) are new natural products. Curvulone A has a unique benzo[*b*]furanone moiety as part of the 12-membered macrolactone, which can be formed by an intramolecular oxa-Michael addition of the phenolic hydroxy group to the α,β-unsaturated ketone moiety. Similarly, the tetrahydropyran ring of curvulone B (**2**) is formed by cleavage of the lactone bond and oxa-Michael addition of the

  

secondary hydroxy group to the α,β-unsaturated ketone moiety. It is particularly interesting to note that these metabolites were isolated from a *Curvularia* sp. associated with a marine red alga, as were the curvularins isolated from another *Curvularia* sp. by the group of König et al.[10] Thus, one can speculate that these antialgal metabolites may play a role in the interaction of the fungus with its host.

## Experimental Section

NMR spectra were recorded with a Bruker DRX 500 ($^1$H: 500 MHz; $^{13}$C: 125 MHz) and WP 200 SY ($^1$H: 200 MHz; $^{13}$C: 50 MHz) spectrometers using TMS as the internal standard. Chemical shifts are reported in ppm. Optical rotations were determined with a Perkin–Elmer 241 polarimeter and concentrations are given as g/100 mL. CD spectra were recorded with a J-810 spectropolarimeter and concentrations are given as mol/dm$^3$. The CD spectra were measured in millidegrees and normalized to $\Delta\varepsilon_{max}$ [L/mol cm]/$\lambda$ [nm] units. For solid-state CD protocol see ref.[11] and for online LC–CD see ref.[39] IR spectra were recorded with a Perkin–Elmer 16 PC FTIR spectrometer and absorption bands are given in cm$^{-1}$. ESI-TOF MS was performed with a MicroTOF-Q instrument (Bruker Daltonik GmbH, Bremen, Germany). For microbiological methods and conditions of cultures, see ref.[40] Melting points were determined with a Büchi SMP-20 melting-point apparatus. Silica gel (70–230 mesh) was used for column chromatography. Spots were detected on TLC under UV or by heating after spraying with 0.5 mL of anisaldehyde in 50 mL of HOAc and 1 mL of $H_2SO_4$.

**Isolation:** *Curvularia* sp., internal strain 6540, was isolated from the marine red alga *Gracilaria folifera* that was growing on rocks at the mouth of John's Pass, Madeira Beach, Florida. It was cultivated at room temperature for 4 weeks on a biomalt agar medium. The cultures (media and fungi) were then extracted with ethyl acetate for 1 week to afford 12.5 g of a residue on removal of the solvent under reduced pressure. The extract was subjected to column chromatography with silica gel (200 g) and eluted with a gradient of dichloromethane/methanol to yield 20 mg of **1**, 15 mg of **2**, 80 mg of **4** and 70 mg of **3**.

**Biological Activity:** The metabolites were tested in a microtitre assay for antifungal (*Microbotryum violaceum, Septoria tritici*), antibacterial (*Bacillus megaterium*) and antiagal (*Chlorella fusca*) activities. The test organisms chosen had cells that could be counted enabling an exact evaluation of growth inhibition. Each well contained 200 μL of medium (NB for *B. megaterium*, MPY for *M. violaceum* and *S. tritici* and CP for *C. fusca*),[41] 50 μL of the test organism, 10 μL of substance (10 mg/mL dissolved in 1:1 methanol/acetone) yielding a final concentration of 0.4 mg/mL. Extracted culture medium served as the controls. The culture was maintained at 20 °C for the fungal and algal test organisms with a 12 h light regime and at 37 °C for the bacterium.

**(–)-(10*S*,15*R*)-Curvulone A (1):** White crystals, m.p. 63–65 °C, $[\alpha]_D^{25}$ = –76 (*c* = 0.75, EtOH). CD (MeCN, *c* = 5.96 × 10$^{-4}$ M): $\lambda$ ($\Delta\varepsilon$) = 340 (–11.09), 329 sh (–8.75), 318 sh (3.21), 307 (3.98), 273 (6.75), 253 sh (1.56), 238 (–6.50), 225 (1.75), 220 sh (0.83), 207 (–0.71), 197 (4.2 L/mol cm) nm. CD (MeOH, *c* = 6.38 × 10$^{-4}$ M): $\lambda$ ($\Delta\varepsilon$) = 339 (–8.95), 330 sh (–7.82), 317 sh (0.85), 308 (2.97), 280 (6.78), 253 sh (0.77), 238 (–5.99), 222 (–0.23), 201 (5.52 L/mol cm) nm. CD (KCl, 40 μg of **1** in 248 mg of KCl): $\lambda$ ($\phi$) = 339 (–17.67), 329 sh (–13.66), 312 sh (–1.33), 305 sh (0.81), 282 (14.43), 257 sh (3.77), 239 (–12.28), 216 (–6.32), 200 (9.30 mdeg) nm. IR (KBr): $\tilde{\nu}_{max}$ = 3389, 2958, 2929, 2858, 1723, 1613, 1446, 1348, 1266, 1219, 1074, 772 cm$^{-1}$. $^1$H and $^{13}$C NMR spectroscopic data are presented in Table 1. MS (ESI): calcd. for $C_{16}H_{16}O_6Na^+$ 327.0839; found 327.083 [M + Na]$^+$; calcd. for [Na + 2$C_{16}H_{16}O_6$]$^+$ 631.1786; found 631.178 [2M + Na]$^+$.

**Crystal Structure Determination of 1 Crystallized from $CHCl_3$:** Colourless prism crystals (0.3 × 0.2 × 0.07 mm) of $C_{16}H_{16}O_6 \cdot 2CHCl_3$, orthorhombic, *a* = 9.796(19), *b* = 10.4728(10), *c* = 22.8285(10) Å, $\tilde{v}$ = 2342(5) Å$^3$, *Z* = 4, space group $P2_12_12_1$, $\rho_{calcd.}$ = 1.54 g/cm$^3$. Data were collected at 293(1) K, Enraf–Nonius MACH3 diffractometer, Mo-$K_\alpha$ radiation, $\lambda$ = 0.71073 Å, $\omega$–2$\theta$ motion, $\theta_{max}$ = 25.6°, 5845 measured reflections of which 2940 were unique with $I > 2\sigma(I)$, decay 2%. The structure was solved by using the SIR-92 software[42] and refined on $F^2$ using the SHELX-97[43] program, material for publication was prepared with the WINGX-97 suite. Non-hydrogen atoms were refined anisotropically except for C-7, which was left isotropic to prevent it from acquiring a non-positive definition. Hydrogen atoms were placed in geometric positions with the exception of the O–H hydrogen atoms which could be found on the difference electron density map, although their distance to the oxygen atom had to be constrained. $R(F)$ = 0.082 and $wR(F^2)$ = 0.215, *S* = 1.05 for 5845 reflections, 270 parameters. Residual electron density = 0.47/–0.53 e/Å$^3$. Compound **1** crystallizes with two chloroform molecules and the heavy chlorine atoms made a significant anomalous scattering effect. The Flack parameter[19] is 0.02(16) for 533 measured Friedel pairs, which indicates that the correct configuration is (10*S*,15*R*)-**1**.

**(–)-(11*R*,15*R*)-Curvulone B (2):** Colourless oil, $[\alpha]_D^{25}$ = –22 (*c* = 0.27, EtOH). CD (MeCN, *c* = 3.84 × 10$^{-4}$ M): $\lambda$ ($\Delta\varepsilon$) = 338sh (–2.55), 327 (–4.79), 318 sh (–4.11), 290 sh (2.22), 269 (4.86), 218 (1.38 L/mol cm) nm. IR (KBr): $\tilde{\nu}_{max}$ = 3389, 2930, 2858, 1724, 1614, 1283, 1164, 1070, 771 cm$^{-1}$. $^1$H and $^{13}$C NMR spectroscopic data are presented in Table 1. MS (ESI): calcd. for $C_{17}H_{22}O_6Na^+$ 345.1309; found 345.129 [M + Na]$^+$; calcd. for [Na + 2$C_{16}H_{16}O_6$]$^+$ 667.2725; found 667.271 [2M + Na]$^+$.

**11-Hydroxycurvularin (3):** Formed as a 7:1 mixture of (11*R*,15*R*)- and (11*S*,15*R*)-epimers **3a** and **3b**, respectively,[10] white crystals, m.p. 154–156 °C. $[\alpha]_D^{25}$ = 25 (*c* = 0.55, EtOH). MS (ESI): calcd. for $C_{16}H_{20}O_6Na^+$ 331.1152; found 331.116 [M + Na]$^+$; calcd. for [Na + 2$C_{16}H_{20}O_6$]$^+$ 639.2412; found 639.241 [2M + Na]$^+$.

Compounds **3a** and **3b** were separated on a Chiralcel IA column (150 × 4.6 mm, 5 μm) using hexane/ethanol (9:1) as eluent (1 mL/min) with a retention time $t_r$ =12.92 min for **3a** and 9.17 min for **3b**.

**(11*R*,15*R*)-3a (Major Component):** Online LC–CD in hexane/ethanol (9:1): $\lambda_{max}$ ($\phi$) = 331 (8.59), 324 sh (7.63), 310 (–1.32), 298 (1.58), 279 (–2.43), 263 (1.69), 239 (–11.67), 225 (20.73 mdeg) nm. $^1$H and $^{13}$C NMR spectroscopic data are identical with those reported in ref.[10]

**(11*S*,15*R*)-3b (minor component):** Online LC–CD in hexane/ethanol (9:1): $\lambda_{max}$ ($\phi$) = 340 sh (0.68), 326 (1.55), 316 sh (0.52), 298 sh (–0.50), 265 sh (–1.85), 245 (3.92), 227 (15.91 mdeg) nm.

**(+)-(10*E*,15*R*)-10,11-Dehydrocurvularin (4):** White crystals. $[\alpha]_D^{25}$ = 37 (*c* = 0.15, EtOH). CD (MeCN, *c* = 3.92 × 10$^{-4}$ M): $\lambda$ ($\Delta\varepsilon$) = 334 (–0.72), 294 (1.32), 259 (0.60), 246 (–0.47), 226 (4.56), 199 (–3.58 L/mol cm) nm. CD (MeOH, *c* = 5.65 × 10$^{-4}$ M): $\lambda$ ($\Delta\varepsilon$) = 335 (–0.33), 299 (0.48), 269 (–0.09), 258 (0.31), 228 (4.94 L/mol cm) nm. CD (KCl, 44 μg of **1** crystallized from $CDCl_3$ in 250 mg of KCl): $\lambda$ ($\phi$) = 335 (–1.17), 299 (1.48), 259 (0.85), 228 (5.66 mdeg) nm.

**Crystal Structure Determination of 4 Crystallized from a Mixture of Methanol and $CH_2Cl_2$:**[44] $C_{16}H_{18}O_5 \cdot H_2O$, $M_r$ = 308.3, ortho-

  

rhombic, space group $P2_12_12_1$, $a$ = 10.286(3), $b$ = 11.263(3), $c$ = 12.800(4) Å, $V$ = 1482.8(8) Å$^3$, $Z$ = 4, $D_x$ = 1.381 g/cm$^3$, $F(000)$ = 656, $T$ = 120(2) K. Bruker AXS SMART APEX CCD diffractometer, graphite monochromator, Mo-$K_\alpha$ radiation, $\lambda$ = 0.71073 Å, $\mu$ = 0.106 mm$^{-1}$, colourless crystal, size 0.44 × 0.18 × 0.15 mm$^3$, 13355 intensities collected $2.4 < \theta < 28.2°$, $-13 < h < 13$, $-13 < k < 14$, $-17 < l < 17$. Structure solved by direct methods,[45] full-matrix least-squares refinement[36] with 2082 independent reflections based on $F^2$ and 209 parameters, all non-hydrogen atoms refined anisotropically, hydrogen atoms from difference Fourier maps refined with a riding model at idealized positions with $U = 1.5U_{iso}$ (O and methyl-C) or $1.2U_{iso}$ (C). Compound **4** crystallizes with one water solvent molecule per asymmetric unit in the non-centrosymmetric space group $P2_12_12_1$. However, in the absence of significant anomalous scattering effects, the Flack[19] parameter is essentially meaningless. Accordingly, Friedel pairs were merged. Refinement converged at $R_1[I > 2\sigma(I)]$ = 0.051, $wR_2$ (all data) = 0.119, $S$ = 0.93, max($\delta/\sigma$) < 0.001, min/max height in final $\Delta F$ map –0.19/0.30 e/Å$^3$. Figure 8 shows the molecular structure.

**Crystal Structure Determination of 4 Crystallized from $CDCl_3$:**[44] Colourless prism crystals (0.33 × 0.34 × 0.3 mm) of $C_{16}H_{18}O_5$, $M_r$ = 290.3, orthorhombic, $a$ = 8.0646(10), $b$ = 9.9968(10), $c$ = 17.7293(10) Å, $V$ = 1429.3(2) Å$^3$, $Z$ = 4, space group $P2_12_12_1$, $\rho_{calcd.}$ = 1.349 g/cm$^3$. Data were collected at 293(1) K, Enraf–Nonius MACH3 diffractometer, Mo-$K_\alpha$ radiation, $\lambda$ = 0.71073 Å, $\omega$–$2\theta$ motion, $\theta_{max}$ = 25.4°, 2449 measured reflections of which 2322 were unique with $I > 2\sigma(I)$, decay 2%. The structure was solved by using the SIR-92 software[41] and refined on $F^2$ using the SHELX-97[43] program, material for publication was prepared with the WINGX-97 suite. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed at geometric positions except for O–H hydrogen atoms, which could be found on the difference electron density map, although their distances to the oxygen atom had to be constrained. $R$(F) = 0.044 and $wR(F^2)$ = 0.112, $S$ = 1.17 for 2449 reflections, 197 parameters, 2 restraints. Residual electron density: 0.25/–0.19 e/Å$^3$.

**Computational Details:** MMFF (molecular Merck force field) and DFT calculations were executed with the Spartan08[46] software package. TD-DFT calculations were executed with Gaussian09.[47]

Conformational searches were carried out by employing the Monte–Carlo-based procedure implemented in Spartan09 using MMFF with standard parameters and convergence criteria. All MMFF minima found within 50 kJ/mol for compound **2** were optimized with DFT at the B3LYP/6-31G(d) level of theory. DFT-optimized structures were then screened for consistency with NMR spectroscopic data ($^3J_{10H,11H}$ coupling constants). Of the remaining structures, the five lowest-energy conformations (overall population 84%) were subdued to single-point calculations at the B3LYP/6-311G+(d,p) level to estimate the relative energies and used as input for ECD calculations. The input geometries of compounds **1** and **4** were obtained from the solid-state structures upon reoptimization of the hydrogen atoms using the DFT method at the B3LYP/6-31G(d) level.

TD-DFT calculations were performed by employing the B3LYP, CAM-B3LYP and BH&HLYP hybrid functionals with TZVP and SVP basis sets.[48] CD spectra were generated by using rotational strengths computed with a dipole–length gauge formulation to which a Gaussian band-shape was applied. For compound **2**, the Gaussian bands had a fixed half-height full-width of 4000 cm$^{-1}$, which corresponds to 36 nm at 300 nm. For compounds **1** and **4**, a variable width was employed using the relationship $\Gamma = \kappa\lambda^{1.5}$,[49] in which $\Gamma$ is the exponential half-bandwidth in nm, $\lambda$ the wavelength in nm and $\kappa$ an adjustable parameter that was set to 0.0033 (corresponding to a half-height full-width of 28 nm at 300 nm). Rotational strengths computed for all transitions with the dipole-velocity gauge formulation differed from dipole-length values by less than 5–10% (TZVP basis set).

**Supporting Information** (see also the footnote on the first page of this article): Data for X-ray structure analysis of **1**, **4** and **4**·$H_2O$.

## Acknowledgments

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