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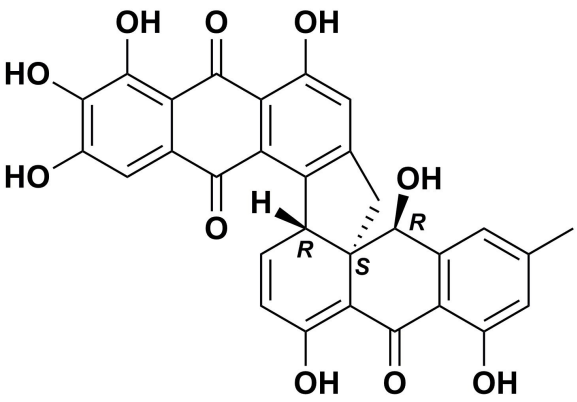
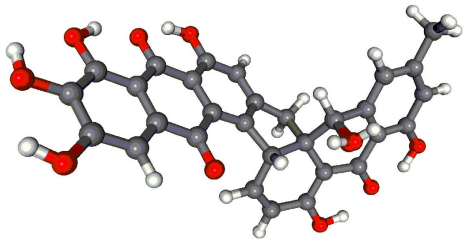
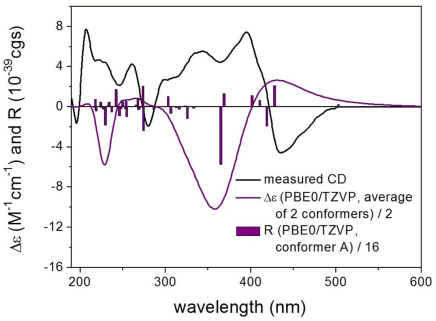
Graphical abstract



Torrubiellin B





Absolute configuration and anti-tumor activity of torrubiellin B

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Abstract

The dimeric anthracene derivative torrubiellin B (**1**) was isolated from the endophytic fungus *Acremonium* sp. that had been obtained from leaves of the Mangrove plant *Sonneratia caseolaris*. The absolute configuration of **1** was established as (5'*R*,10'*S*,10a'*R*) for the first time on the basis of its electronic circular dichroism (ECD) spectra aided with TDDFT-ECD calculations. Torrubiellin B (**1**) exhibited strong anti-tumor activity when tested *in vitro* against several solid cancer cell lines including cells that are resistant against the widely used cytostatic drug cisplatin. The IC_{50} values of **1** against cisplatin sensitive and cisplatin resistant cells were in the range of 0.2 - 2.6 µM depending on cell line investigated.

Keywords: Torrubiellin B, *Acremonium* sp., Absolute configuration, Anti-tumor activity

Introduction

Endophytic fungi are known as a rich source of structurally diverse bioactive compounds that are often unprecedented in other organisms.[1-7] Endophytes which inhabit plants that are subject to severe stress conditions are especially interesting for bioprospecting since the fungi are assumed to contribute to the survival of their hosts e.g. through accumulation of defense metabolites.[8-10] We and others have repeatedly shown that Mangrove associated endophytes are valuable sources of new bioactive metabolites such as the anti-tumor metabolite phomoxanthone A isolated from the Mangrove endophyte *Phomopsis longicolla* which was found to strongly inhibit tumor cells.[11] Mangroves are prone to numerous stress factors such as changing levels of salinity, periodical flooding and a high incidence of herbivores and of pathogenic microbes. Apparently these factors have shaped not only the secondary metabolites of the hosts but also that of their associated endophytes.[12,13]

In the course of ongoing studies on natural products from Mangrove derived endophytes we investigated the endophytic fungus *Acremonium* sp. that was isolated from leaves of the Mangrove plant *Sonneratia caseolaris* from the island of Hainan (P. R. China). Our interest in this fungus had been aroused by the strong anti-tumor activity of its crude EtOAc extract when tested *in vitro* against solid cancer cell lines including cells that are highly resistant against the widely used cytostatic drug cisplatin (severe growth inhibition of A2780sens cells observed at 10 µg/mL, and of A2780CisR cells at 100 µg/mL). Resistance of cancer cells that emerges frequently during chemotherapy is a serious problem for therapy of tumor patients and is responsible for the ultimate failure of this therapeutic approach in spite of reduction of tumor load during early phases of treatment.[14,15] Therefore, new therapeutics

that are able to break resistance against currently employed cytostatic drugs are urgently needed.

During bioassay guided fractionation of the extract of *Acremonium* sp. torrubiellin B (**1**)[16], an asymmetric anthracene derivative was isolated as the active ingredient that is responsible for the anti-tumor activity of the fungal extract. Torrubiellin B had first been reported from *Torrubiella* sp. BCC 28517 and the relative configuration of the compound had been determined through NOE experiments.[17] However, the absolute configuration of the compound was unknown so far. The determination of the absolute configuration of torrubiellin B (**1**) especially in light of its prominent activity against cancer cells is an important issue during structure determination. Here we report for the first time the absolute configuration of torrubiellin B (**1**) together with its pronounced anti-tumor activity against cisplatin sensitive and cisplatin resistant solid cancer cell lines.

Results and discussion

The ethyl acetate extract of the endophytic fungus *Acremonium* sp. grown on solid rice medium was submitted to a bioassay guided chromatographic separation using different stationary phases including silica gel, Sephadex LH-20 and semipreparative HPLC on a C_{18} reversed phase column. Torrubiellin B (**1**) was isolated as the major constituent of the extract (Figure 1). Compound **1** exhibited a UV/Vis spectrum with three absorption maxima at 206, 283, and 397 nm that is typical for a conjugated system. In the HRESIMS the pseudomolecular ion was detected at *m/z* 541.1138 $[M+H]^+$ and the molecular formula was determined as $C_{30}H_{20}O_{10}$. Comparison of the MS and NMR data of **1** with those previously reported for torrubiellin B suggested that they are identical.[17] The structure of **1** was finally confirmed as torrubiellin B by careful analysis of COSY, HMBC and ROESY correlations. Interpretation of the COSY and HMBC correlations shown in Figure 2 revealed two

anthracene moieties being fused via a cyclopentene ring. The HMBC correlations from H-5' to C-4, C-3, C-10a' and to C-11 established the connectivity of both monomers. The formation of this rare linkage between C-4 and C-5' and between C-3 and C-10a' through C-11 is a structural feature that is inherent to torrubiellins. The linkage of the two anthracene units was further corroborated through correlations from H_2-11 to H-2 and from H-11 to H-4' in the ROESY spectrum of **1**. The relative configuration of C-5' and C-10' was deduced from ROESY experiments (Figure 4). H-10' showed ROE correlations to H-5' and H-11_{eq} and furthermore H-11_{eq} had a correlation to H-2, thus indicating the (5'*S**,10'*R**,10a'*S**) relative configuration of **1** as shown in Figure 4, which is in agreement with that previously reported.[17]

Torrubiellin B may be viewed as a 4,5'-linked heterodimeric biaryl of 7-hydroxyemodin and dihydro-1,8,10-trihydroxyanthracene-9(4*H*)-one, in which the rotation along the biaryl axis is blocked by the methylene linker between C-3 and C-10a. In contrast to axially chiral biaryls of anthracene-9,10-dione monomers,[18] torrubiellin B has only central chirality elements due to the C-5', C-10' and C-10a' chirality centers and the projection angle between the planes of the two monomers is governed by the central chirality. The solution ECD spectra of torrubiellin B showed intense negative Cotton effect (CE) at 435 nm and a positive one at 395 nm accompanied by shoulders and quite a number of other overlapping high-energy ECD transitions (Figure 3). For the configurational assignment, solution conformational analysis and TDDFT-ECD calculations were performed. The initial MMFF conformational analysis of the arbitrarily selected (5'*S*,10'*R*,10a'*S*)-**1** resulted in 13 conformers, which were reoptimized at B3LYP/6-31G(d) level *in vacuo*. The reoptimization afforded 2 conformers (96.8% and 2.9% populations) above 1% Boltzmann-population, which differed only in the orientation of hydrogens of the 10'-OH and 3'-Me groups (Figure 4). The $\omega_{C\text{-}3,C\text{-}4,C\text{-}5',C\text{-}6'}$ dihedral angle, a parameter describing the relative orientation of the electric transition moments of the two subunits, was found +103.17°. TDDFT-ECD calculations of the two low-energy conformers

were carried out at 3 different levels of theory and the Boltzmann-averaged spectra were found mirror-image of the experimental ECD curve. The PBE0/TZVP method showed the best agreement, the result of which is shown in Figure 4. This allowed determining the absolute configuration of torrubiellin B (**1**) as (+)-(5'*R*,10'*S*,10a'*R*).

The putative biogenetic formation of **1** and of the structurally related 6,7-dideshydroxy derivative torrubiellin A (**2**) has been suggested to occur through oxidative coupling of emodin (**5**), aloe-emodin (**6**) or of chrysophanol (**7**) on the basis of the co-occurrence of these monomers in the respective investigated fungal extracts.[17] After thorough UV and LCMS analysis of the crude ethyl acetate extract of *Acremonium* sp. analyzed in this study typical UV absorption maxima at 223, 250, 273, 293 and 442 nm, as well as a prominent pseudomolecular ion peak at 269.5 $[M-H]^-$ were detected that account for emodin, but no signals were found for aloe-emodin or for chrysophanol. These findings suggest that **1** may biogenetically arise from two emodin (**5**) moieties through subsequent reduction and hydroxylation.

Compound **1** showed pronounced cytotoxic activity against several human solid cancer cell lines including Cal27 (head-neck cancer), Kyse510 (esophageal squamous cell carcinoma), HCC38 (breast cancer), A2780 (ovarian cancer) and MDA-MB-231 (breast cancer) (Table 1). The cell lines included pairs of cells that are either sensitive (sens) or resistant (CisR) towards the well known cytostatic drug cisplatin as indicated by the significantly different IC_{50} values for cisplatin. Torrubiellin B (**1**) proved to be strongly active against all cell lines investigated with IC_{50} values ranging from 0.3 - 1.5 μM against the cisplatin sensitive cells and from 0.2 - 2.6 μM against the cisplatin resistant cells, dependent on the cell line investigated. For all cell lines investigated the activity of torrubiellin B (**1**) was superior to that of cisplatin which makes compound **1** an interesting candidate for further studies.

Experimental

General

The optical rotation was determined with a PerkinElmer 241MC Polarimeter. The 1H, 13C and 2D NMR spectra were recorded in DMSO-d6 on a Bruker Avance III 600 NMR spectrometer. Mass spectra were measured with a HP110 Agilent Finnigan LCQ Deca XP Thermoquest and high- resolution mass spectrometry (HRESIMS) spectra were recorded using a UHR-TOF maxis 4G mass spectrometer. ECD spectra were recorded on a J-810 spectropolarimeter. HPLC analysis was performed with a HPLC system Dionex p580 coupled to a UVD340S photodiode array detector; routine detection was at 235, 254, 280, and 340 nm. The separation column was prefilled with Eurosphere-10 C18 using a linear gradient of 0.1% aqueous HCOOH and MeOH with a flow rate of 1mL/min. Final purification was performed via semi-preparative HPLC using a linea gradient of 0.1% aqueous TFA and MeOH over a Eurosphere-100 C18 RP column (5µm; 300x8mm) with a flow rate of 5mL/min using a Merck Hitachi HPLC system (I-7400 UV detector; L-7100 pump). Column chromatography included Silica gel 60M (Macherey.Nagel) and Sephadex LH20 (Sigma). Precoated TLC plates (Silica gel 60 F254) were used to monitor fractions; detection was under UV at 254 and 366 nm. Solvents were distilled before use, and spectral grade solvents were used for spectroscopic measurements.

Computational section

Mixed torsional/low mode conformational searches were carried out by means of the Macromodel 9.9.223[19] software using Merck Molecular Force Field (MMFF) with implicit solvent model for chloroform applying a 42 kJ/mol energy window. Geometry

reoptimizations of the resultant conformers [B3LYP/6-31G(d) level *in vacuo*] and TDDFT calculations were performed with Gaussian 09[20] using various functionals (B3LYP, BH&HLYP, PBE0) and TZVP basis set. ECD spectra were generated as the sum of Gaussians[21] with 2400 cm^{-1} half-height width (corresponding to ca. 29 nm at 350 nm), using dipole-velocity computed rotational strengths. Boltzmann distributions were estimated from the ZPVE corrected B3LYP/6-31G(d) energies. The MOLEKEL[22] software package was used for visualization of the results.

Fungal material

Acremonium sp. was isolated from leaves of the mangrove plant *Sonneratia caseolaris*, collected in October 2005 in Dong Zhai Gang Mangrove Garden, Hainan, P. R. China, and cultivated in large-scale following the respective protocol described in the literature.[23] The fungal strain could be identified after DNA extraction and amplification and after sequencing of ots ITS region according to the literature.[24] The sequence data was submitted to GenBank, accession number **FR822815.1**.

Extraction and isolation

After 4 weeks of cultivation the fungi were extracted with EtOAc. The dried crude extract (1g) was submitted to VLC separation. The last fraction (0.1% methanolic TFA) containing **1** was further separated over a Sephadex LH-20 column affording F1-F10 fractions. Fractions 6 and 7 were combined and purified using semipreparative HPLC with an eluting gradient of MeOH/0.1% aqueous TFA to yield **1** (20 mg).

Bioassay

The cytotoxicity was tested against the following human carcinoma cell lines obtained from DSMZ: A2780 (ovarian), Cal27 (tongue), Kyse510 (esophagus), HCC38 (triple-negative mamma carcinoma), and MDA-MB-231 (triple-negative mamma carcinoma). The CisR cell lines were generated as previously described.[25,26]

Acknowledgments

The financial support by grants from BMBF awarded to P. P. is gratefully acknowledged. We thank Prof. Hao-Fu Dai for his support during sample collection of *S. caseolaris* on Hainan, P. R. China. T. K. thanks the Hungarian National Research Foundation (OTKA K105871) for financial support and the National Information Infrastructure Development Institute (NIIFI 10038) for CPU time.

Supplementary data

The supplementary data contains the ^{13}C, ^{1}H, HMBC and ROESY NMR spectrum and the NMR table of **1**.

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16. Data for **1**: Orange amorphous powder. $[\alpha]_D^{27}$ +189 (*c*. 0.025, $CHCl_3$). UV λ_{max} (PDA): 458sh, 417sh, 397, 373sh, 307sh, 283, 206sh nm. HRESIMS *m/z* 541.1138 $[M+H]^+$ (calcd for $C_{30}H_{20}O_{10}$, 541.1138). ECD (MeCN, λ [nm] ($\Delta\varepsilon$), $c = 3.08\times10^{-4}$

M): 465sh (–2.12), 435 (–4.13), 415sh (2.50), 395 (6.85), 343 (4.96), 296 (2.30), 281 (–2.39), 260 (4.08), 231sh (4.56), 221sh (5.05), 206 (8.55), 195 (–2.51).

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Tables

Table 1. Cytotoxic activities of **1**

Compound	IC_{50} (μM) in various cell lines[a,b]									
	Cal27		Kyse510		HCC38		A2780		MDA-MB-231	
	sens	CisR	sens	CisR	sens	CisR	sens	CisR	sens	CisR
1	0.3	2.1	1.2	0.2	0.4	0.7	0.3	0.5	1.5	2.6
Cisplatin	2.9	6.7	1.8	6.8	3.5	27.9	1.5	11.7	13.9	38.1

[a] Incubation for 72 h.

[b] Abbreviations: sens, sensitive to cisplatin; CisR, cisplatin-resistant.

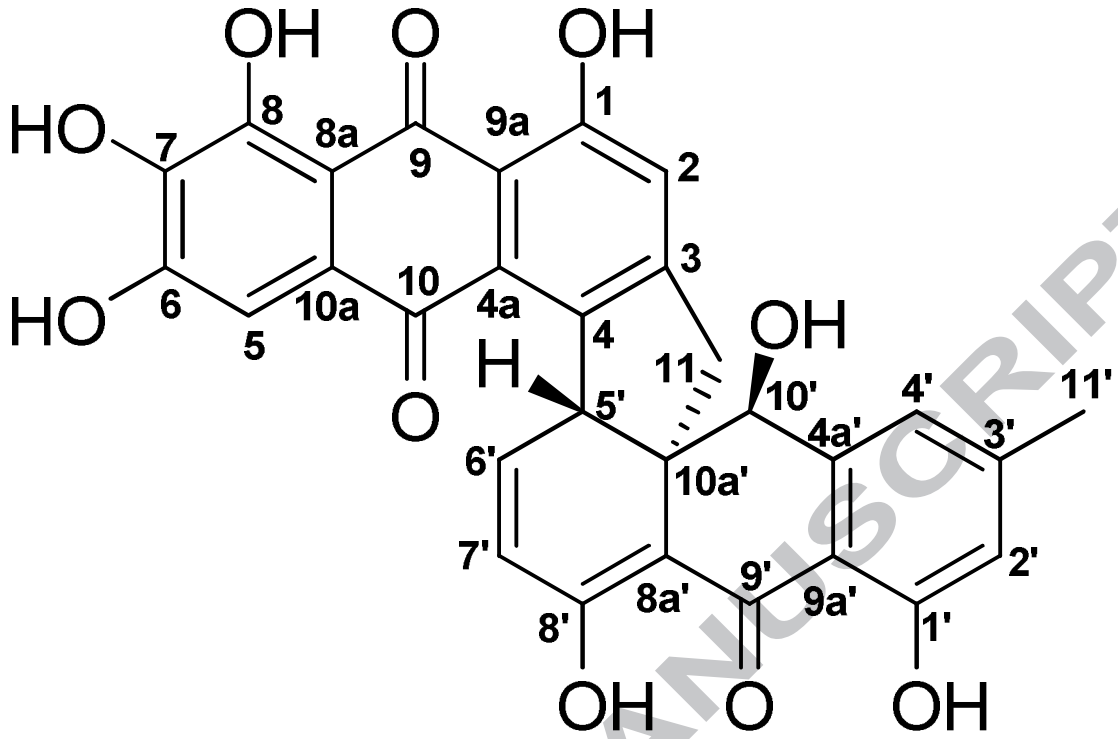
Figure legends

Figure 1. Structure of torrubiellin B (**1**).

Figure 2. Key COSY (bold lines) and HMBC (arrows) correlations of torrubiellin B (**1**).

Figure 3. Experimental spectrum of **1** in MeCN and PBE0/TZVP-calculated ECD spectrum of the two low-energy conformers (> 1%) of (5'*S*,10'*R*,10a'*S*)-**1** *in vacuo*. Bars represent the calculated rotational strength values of the lowest-energy conformer.

Figure 4. Key long-range ROE correlations shown on the overlapped lowest-energy computed conformers of (5'*R*,10'*S*,10a'*R*)-**1**.



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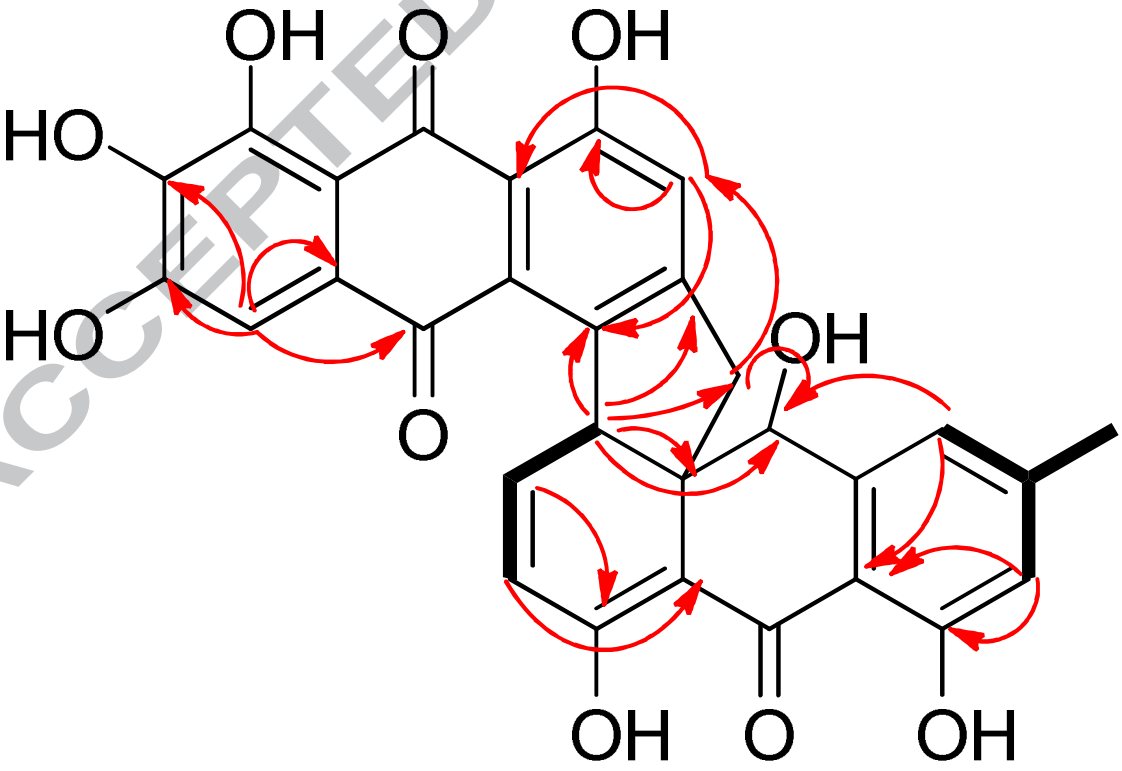

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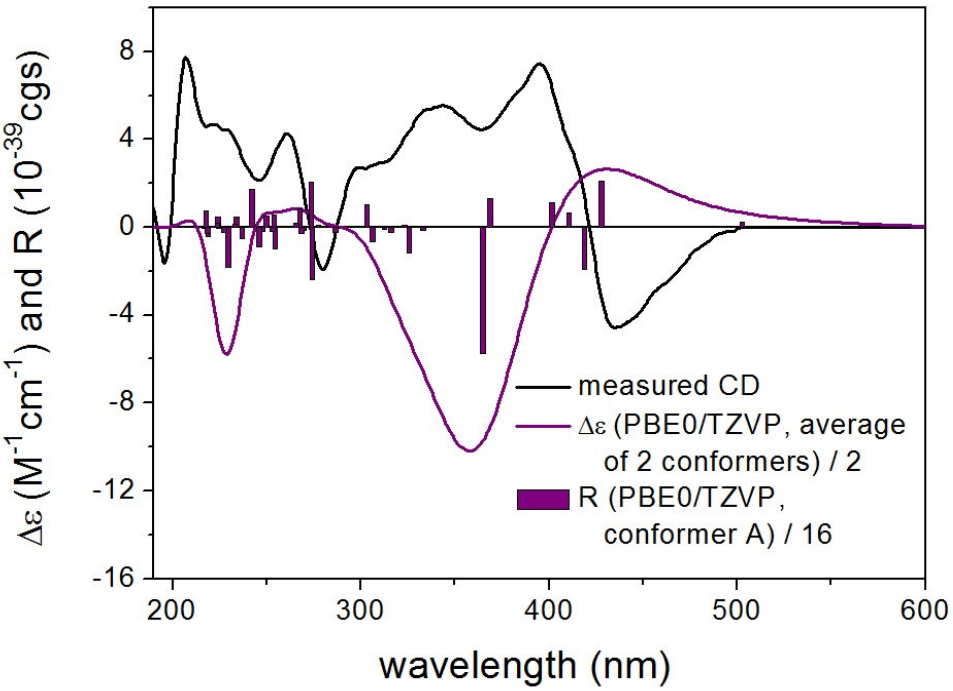

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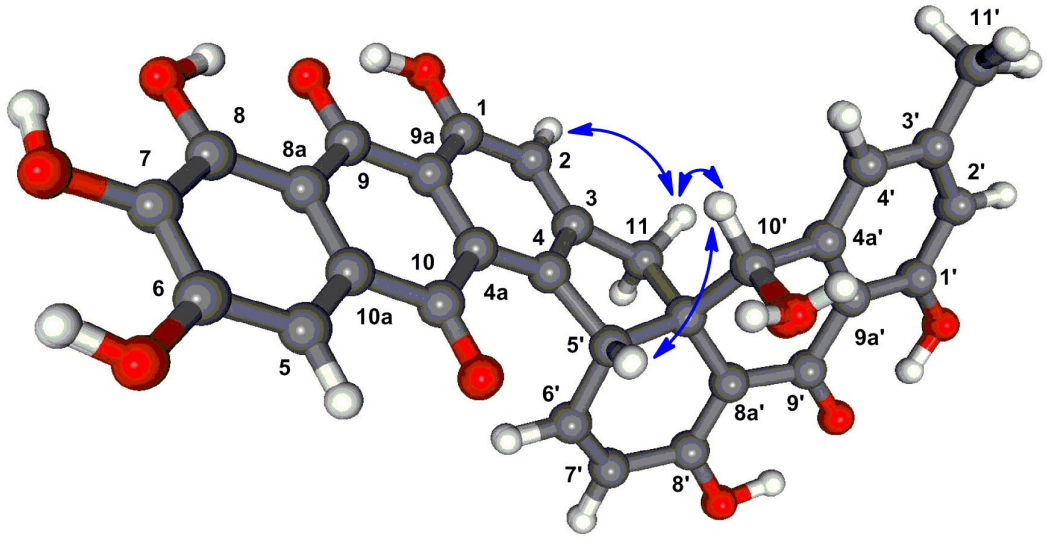

Figure 4. Key long-range ROE correlations shown on the overlapped low-energy computed conformers of (5'*R*,10'*S*,10a'*R*)-**1**.

Highlights

- A dimeric anthracene derivative was isolated from the endophyte *Acremonium* sp.
- The absolute configuration of torrubiellin B was established by ECD calculations.
- Torrubiellin B showed strong anti-tumor activity against several cancer cell lines.