Structure Elucidation

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Unprecedented Immunosuppressive Polyketides from *Daldinia eschscholzii*, a Mantis-Associated Fungus**

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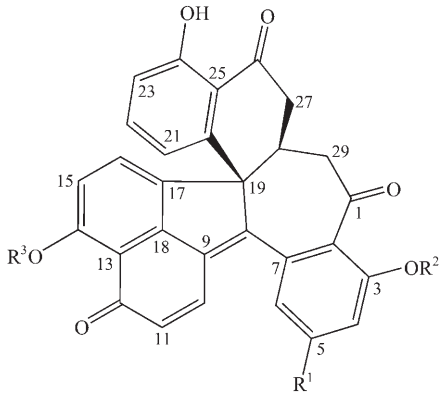
Immunosuppressants are required for an array of medical purposes, such as organ transplantations and the treatment of autoimmune-associated diseases.[1–2] However, most of the currently available immunosuppressive drugs have been shown to inevitably possess severe adverse effects, such as hepatotoxicity, nephrotoxicity, and hypertension induction.[2] Therefore, there is an urgent need for new therapeutic agents for modulating the autoimmune response. Some microorganisms are a reliable source of immunocompromising compounds, as exemplified by the discovery of cyclosporin A (CsA),[3] rapamycin,[4] and FK506.[5] Symbionts, a diverse microbial community present in plants, insects, and mammals without the generation of any detectable symptoms, are receiving renewed attention for their production of chemically inspiring and biologically potent metabolites, presumably as a result of their long coevolution with hosts.[6] In particular, some insect-associated fungi might have acquired instinctlike capabilities for synthesizing immunoalleviating metabolites from their initial microbe–host interaction through to the final colonization.[7–9]

In continuation of our characterization of new bioactive metabolites from endophyte cultures,[10–12] this observation tempted us to explore novel immunosuppressive metabolites that could be produced by fungi inhabiting healthy insect organs, such as the mantis gut, which is clearly an important entrance and shelter for symbionts (including quiescent pathogens[13]) and meal-carried "foreign" microbes.[14] As expected, a preliminary screen recognized the presence of an immunosuppressive substance or immunosuppressive substances in a culture of *Daldinia eschscholzii* IFB-TL01 residing in the gut of the mantis species *Tenodera aridifolia*, a common predator of many insects that feed on plants harboring endophyte. Subsequent bioassay-guided fractionation of the extract derived from the scaled-up fermentation of the fungus afforded two polyketides, dalesconols A (**1**) and B (**2**), which share an unprecedented carbon skeleton, together with the biosynthetically related intermediates **5**[15] and **8**.[16] Optical resolution of **1** and **2** by HPLC on a chiral phase gave the corresponding enantiomers (+)-dalesconol A ((+)-**1**) and (−)-dalesconol A ((−)-**1**), (+)-dalesconol B ((+)-**2**) and (−)-dalesconol B ((−)-**2**). We report herein the isolation, structural and stereochemical elucidation, and immunosuppressive activity of the enantiomeric polyketides, as well as details of their biosynthesis.



	R^1	R^2	R^3
1:	H	H	H
2:	OH	H	H
1':	H	*p*-$BrC_6H_4SO_2$	*p*-$BrC_6H_4SO_2$

The first isolate, dalesconol A (**1**), was obtained as red crystals. A protonated molecular ion at *m*/*z* 463.1180 in its high-resolution ESI mass spectrum indicated a molecular formula that was in accordance with the ^1H and ^{13}C NMR spectroscopic data (*m*/*z* calcd for $C_{29}H_{19}O_6$: 463.1176; see Tables S1 and S3 in the Supporting Information). The ^1H NMR spectrum of **1** suggested the presence of an α,β-unsaturated carbonyl group as well as one 1,2,3,4-tetrasubstituted and two 1,2,3-trisubstituted benzene nuclei. This assumption was confirmed by the ^{13}C NMR spectrum, which revealed additionally the existence of two further ketone groups and a fully substituted vinyl group, as well as a quaternary, a methine, and two methylene carbon atoms. These assigned fragments accounted in total for seventeen indices of unsaturation, with the rest having to be edited into four more rings in the molecule. Detailed interpretation of the 2D NMR spectra of **1** allowed the construction of the most likely planar structure of dalesconol A (**1**).

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The red crystals of **1** were suitable for single-crystal X-ray diffraction, which revealed the relative configuration of the metabolite (Figure 1).[17] However, the crystal of **1** had the space group $P2_1/c$, which was indicative of its racemic nature, as also evidenced by circular dichroism (CD) and its lack of optical activity. Subsequent HPLC of **1** on a chiral phase led to the separation of the two enantiomers, (+)-**1** and (−)-**1**,

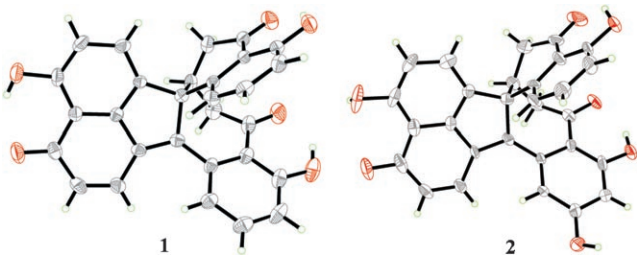


Figure 1. X-ray crystal structures of dalesconols A (**1**) and B (**2**).

which were opposite in terms of their CD curve and optical rotation but with identical ^{1}H and ^{13}C NMR spectra. Strangely, we failed to obtain single crystals of either of the two optically pure enantiomers in several solvent systems.

Electronic circular dichroism (ECD) spectra derived from quantum-chemical calculations have been used successfully for the stereochemical assignment of small- and medium-sized molecules.[18] To determine the absolute configuration of the enantiomers of **1**, a comparison was made between the experimental and calculated ECD spectra of (−)-dalesconol A ((−)-**1**) by using the time-dependent DFT method.[18] The single-crystal X-ray diffraction data showed that **1** contained two stereogenic carbon atoms of opposite configuration; thus we only needed to optimize the structures for a pair of enantiomers, (19*R*,28*S*)-**1** and (19*S*,28*R*)-**1**. The calculated ECD spectra of these two species are depicted in Figure 2. The ECD spectrum calculated for (19*R*,28*S*)-**1** agreed well with that measured for (−)-**1** (Figure 2a), whereas that calculated for (19*S*,28*R*)-**1** showed an opposite CD curve (Figure 2b). The first calculated Cotton effect was a negative minimum at 327 nm. Many excitations contributed to this broad absorption band, among which the excitation from a π-type orbital (MO 112) to a π*-type orbital (MO 121, LUMO) played a dominant role (see Figure S20 in the Supporting Information). This band could be correlated with the experimentally observed broad Cotton effect at 320 nm. The next calculated intense positive Cotton effect at 270 nm could be assigned to the experimental Cotton effect at 265 nm. Two excitation types contributed to this absorption: an excitation from a π-type orbital (MO 120, HOMO) to a π*-type orbital (MO 125) of a ring, and a transition from a π-type orbital (MO 117) to a π*-type orbital of a carbonyl group (MOs 122 and 123). The two experimental bands at 233 and 216 nm were also reproduced by our calculations, despite difficulties in the unambiguous assignment of particular calculated states. Thus, the most likely absolute configuration of (−)-dalesconol A ((−)-**1**) was established to be 19*R*,28*S*, and that of its enantiomer (+)-**1** to be 19*S*,28*R*.

To test the ability of dalesconol A to undergo racemization during fractionation, an extract of the freshly harvested broth was subjected directly to HPLC on a normal reversed-phase column to give **1** as a red powder. Analysis of this sample by HPLC on a chiral phase showed it to consist of (+)-**1** and (−)-**1** in an approximate ratio of 1:2 (see Figure S15 in the Supporting Information). Both enantiomers were then added separately to the extract left over following the aforementioned removal of dalesconol A by HPLC, and the original fractionation procedure was repeated, including chromatography on silica gel and sephadex LH-20, and the thermal process required for solvent evaporation. The recovered pure enantiomers of dalesconol A retained their optical properties; thus, the possibility of the racemization of **1** during separation was excluded.

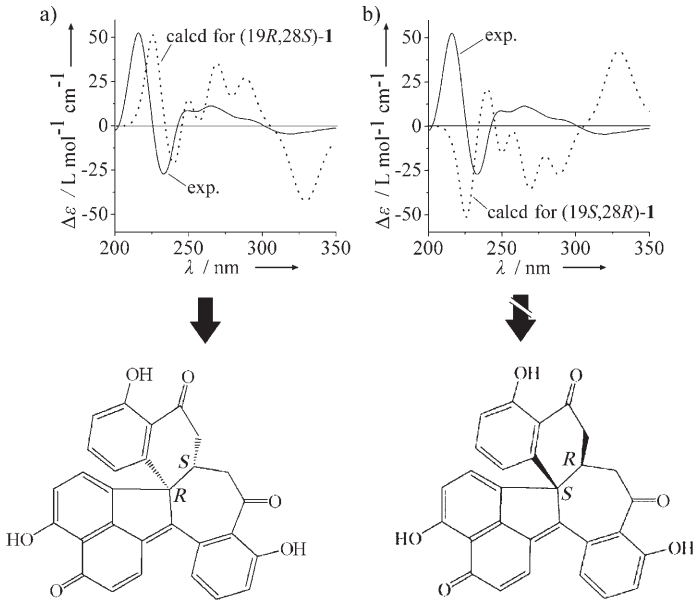


Figure 2. Attribution of the absolute configuration of the enantiomers of dalesconol A (**1**) as 19*R*,28*S* and 19*S*,28*R* by comparing the experimental (solid line) with the calculated (dotted line) ECD spectra.

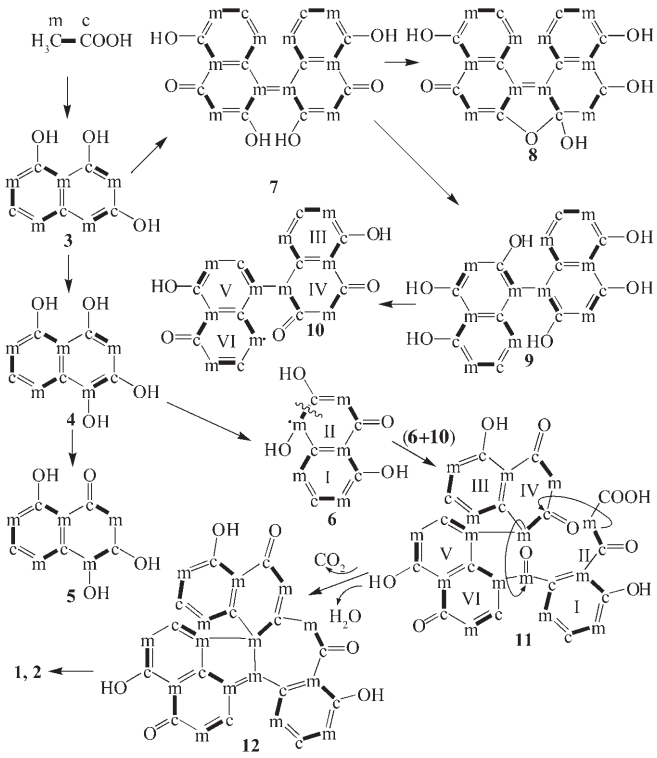
The second isolate, dalesconol B (**2**), recrystallized as red needles. A molecular formula of $C_{29}H_{18}O_7$ (with one more oxygen atom than in **1**) was determined from its ^{1}H NMR, ^{13}C NMR, and HREIMS spectral data, which were very similar to those of **1**. Subsequent 2D NMR analysis of **2** indicated the presence of a 5-hydroxy group, the existence of which was reinforced by X-ray crystallographic diffraction (Figure 1).[17] The CD spectrum of (−)-**2** resembled that of (−)-**1** and thus indicated the absolute configuration 19*R*,28*R* (28*R* owing to the presence of the 5-hydroxy group) for (−)-dalesconol B ((−)-**2**) and 19*S*,28*S* for its enantiomer (+)-**2**. As in the case of **1**, compound **2** was obtained by HPLC directly from the freshly harvested broth as an approximately 1:2 mixture of (+)-**2** and (−)-**2** (see Figure S17 in the Supporting Information). Each single enantiomer of **2** was very reluctant

to form any crystals in solvent systems from which the racemate crystallized readily.

Our failure to obtain crystals of the pure enantiomers of **1** and **2** could be related to special interactions resembling molecular recognition between the two enantiomers. Inspection of the unit cells of **1** and **2** revealed weak and clear, respectively, offset π–π interactions between the acenaphthylen-5(1*H*)-one residues in the two pairs of racemic isomers (see Figures S21 and S22 in the Supporting Information). To confirm this assumption, both the racemate and the single enantiomers of **1** were transformed into the bis(bromobenzenesulfonate)s **1′**, in which the contribution of hydrogen bonding to the interenantiomeric interaction is eliminated or at least minimized. As anticipated, after careful purification and confirmation of the structure of the products by MS(ESI+) and ^{1}H NMR spectroscopy, the enantiomeric dibromobenzenesulfonates failed to crystallize from any solvent tested, whereas the corresponding racemate yielded crystals suitable for the X-ray diffraction analysis, which confirmed the anticipated offset π–π interaction (see Figure S23 in the Supporting Information).[17]

Metabolites **1** and **2** could be postulated to be acetate-derived polyketides with a unique framework. This hypothesis was confirmed by ^{13}C-labeling experiments (see Tables S3 and S4 in the Supporting Information). Thus, the addition of sodium [1-^{13}C]acetate led to the enrichment of the ^{13}C isotope at C1, C3, C5, C7, C10, C12, C14, C16, C18, C20, C22, C24, C26, and C28 of both **1** and **2** to give the labeled products **1a** and **2a**. Enrichment of the ^{13}C isotope was observed at C2, C4, C6, C8, C9, C11, C13, C15, C17, C19, C21, C23, C25, C27, and C29 in **1b** and **2b**, which resulted from a [2-^{13}C]acetate feeding experiment. Thus, all carbon atoms in dalesconols A (**1**) and B (**2**) were labeled, either by [1-^{13}C]acetate or by [2-^{13}C]acetate (Scheme 1). A further feeding experiment with [1,2-$^{13}C_2$]acetate was also performed to determine the distribution of the acetate unit. The pairs of carbon atoms C1–C29, C2–C3, C4–C5, C6–C7, C9–C18, C10–C11, C12–C13, C14–C15, C16–C17, C19–C28, C20–C21, C22–C23, C24–C25, and C26–C27 were observed to be derived from intact acetate units, as they showed detectable ^{13}C–^{13}C spin couplings.

The feeding experiments with [1-^{13}C] and [2-^{13}C]acetate led to 14 and 15 ^{13}C labels, respectively, which confirms the polyketide nature of both metabolites. The general architecture of **1** and **2** suggested that the common building block could be 1,3,8-trihydroxynaphthalene (**3**), a compound that was presumed previously to be related to the generation of **5** and **8** (Scheme 1),[16,19] both of which were isolated by the fractionation (monitored by ^{1}H NMR spectroscopy) of the fungal extract. Thus, compound **3** was oxidized to **4**, which could be oxidized further to the radical **6**. This radical is stabilized presumably by conjugation with the adjacent phenyl and vinyl groups. The dimerization of **3**, perhaps also via radical states, would give **7**, which could be converted via **9** into the radical **10** by keto–enol tautomerism. The subsequent combination of radicals **6** and **10** followed by the glycol cleavage of ring II might provide **11**, which could undergo aldol-type cyclization, decarboxylation, and dehydration to afford **12**. The reduction of **12** would result in the formation of



Scheme 1. Postulated biosynthesis of dalesconols A (**1**) and B (**2**). c = carbon atom labeled by [1-^{13}C]acetate; m = carbon atom labeled by [2-^{13}C]acetate; — = intact acetate unit.

1 and **2** with the relevant geometry, whereby (−)-**1** and (−)-**2** are produced more readily than the enantiomeric products (Scheme 1).

We tested both single enantiomers of dalesconols A (**1**) and B (**2**), as well as **5** and **8**, for immunosuppressive activity by using a T-cell-proliferation assay to gain an understanding of the dependence of this activity on the steric architecture and the oligomerization of the tetralone unit. All dalesconols inhibited the proliferative response of mouse spleen cells to concanavalin A (2.5 μg mL^{-1}), whereby their IC_{50} values ranged from 0.16 to 0.58 μg mL^{-1} and were therefore comparable to that of cyclosporin A (CsA; 0.06 μg mL^{-1}), which was coassayed as a positive reference (see Table S5 in the Supporting Information). No significant difference in activity was detected between the two pairs of single enantiomers of dalesconols A and B. The selective index (SI) values were compared to provide a measure of the noncytotoxic immunosuppressive activity for each substance. The SI values of the enantiomer (+)-**1** and the natural compounds dalesconols A (**1**) and B (**2**) were better (SI > 276) than that of CsA (SI = 187). The enantiomers (−)-**1**, (+)-**2**, and (−)-**2** were less potent than the natural compounds but exhibited comparable SI values (SI > 138) to that of CsA. These IC_{50} and SI values suggest that tetralone trimers, such as dalesconols A and B, may be promising leads for the development of novel therapeutic agents.

In summary, dalesconols A (**1**) and B (**2**) are potent polyketides with a unique carbon skeleton. These immunosuppressive agents are produced by a mantis-associated

fungus, which highlights the possibility that neglected or poorly investigated fungal flora, such as the title strain, could be a big reservoir for novel bioactive natural products. The interenantiomeric interaction discerned with dalesconols A and B may provide useful hints both for the understanding of the ability of certain compounds to crystallize and for the design or optimization of molecular-recognition systems for chiral compounds with a similar geometry. Dalesconols A (**1**) and B (**2**) collected directly by HPLC were both shown to be enriched with one enantiomer; a similar phenomenon was encountered previously.[20] This observation highlights the possibility that some reported natural products whose chirality has been addressed previously could in fact be partially racemized mixtures with a CD spectrum and optical rotation that arise from the excess amount of one particular enantiomer. Finally, the novel architecture and biological profiles of **1** and **2** make the dalesconols attractive lead compounds for the development of a valuable class of immunosuppressive agents.

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