Bipolenins K–N: New sesquiterpenoids from the fungal plant pathogen Bipolaris sorokiniana

Chin-Soon Phan¹, Hang Li¹, Simon Kessler¹, Peter S. Solomon², Andrew M. Piggott*³ and Yit-Heng Chooi*¹

Abstract

Chemical investigation of the barley and wheat fungal pathogen Bipolaris sorokiniana BRIP10943 yielded four new sativene-type sesquiterpenoid natural products, bipolenins K–N (1–4), together with seven related known analogues (5–11), and a sesterterpenoid (12). Their structures were determined by detailed analysis of spectroscopic data, supported by TDDFT calculations and comparison with previously reported analogues. These compounds were evaluated for their phytotoxic activity against wheat seedlings and wheat seed germination. The putative biosynthetic relationships between the isolated sesquiterpenoids were also explored.

Introduction

Fungi belonging to the genus Bipolaris (teleomorph: Cochliobolus) have been reported to produce a diverse array of secondary metabolites, including sesquiterpenes [1-7], sesquiterpene-xanthones [8], diterpenes [9], sesterterpenes [10], cochlioquinones and peptides [11]. Moreover, several of these secondary metabolites are known to play important roles in mediating the virulence of these fungi against plant hosts [12]. Well-known examples include the host-specific toxins victorin and T-toxin and other non-host-specific toxins such as the ophiobolins [11]. Bipolaris sorokiniana (syn. Cochliobolus sativus) has been identified as the causative agent of multiple diseases on wheat and barley and is a major threat to yield improvement and food security in Central Asia [13]. Recent genome sequencing of 35 Australian strains of B. sorokiniana identified a known proteinaceous necrotrophic effector, ToxA, which confers host-specific virulence proteins and is proposed to be acquired through horizontal gene transfer [14]. To date, only three studies have explored phytotoxins from B. sorokiniana [2,7,10]. Therefore, in the framework of furthering our understanding of the roles of B. sorokiniana sec-
ondary metabolites in crop disease, we investigated the compounds produced by the ToxA-containing strain BRIP10943 (CS10) [14] and their phytotoxicity. This led to the isolation of four new sativene-type sesquiterpenoid natural products along with seven related known analogues and one sesterterpenoid. Herein, the isolation, structure elucidation and phytotoxic activities of these compounds are presented.

Results and Discussion

*B. sorokiniana* was cultivated for 22 days in Fries medium supplemented with rolled oats. The resulting broth and mycelia were extracted with methanol and the extracts were partitioned against EtOAc/MeOH/acetic acid (89.9:10:0.1 ratio). The combined organic layer was chromatographed repeatedly with silica gel and RP-HPLC to afford four new sativene-type sesquiterpenoids, bipolenins K–N (1–4), along with eight previously reported compounds (5–12), which were identified as sativene-type sesquiterpenoids prehelminthosporol lactone (5) [1], helminthosporic acid (6) [1], helminthosporol (7) [15], bipolenin A (8) [3], secolongifolene diol (9) [15], dihydrophehelminthosporol (10) [1] and sorokinianin (11) [2], and the cytotoxic sesterterpenoid, terpestacin (12) [16,17] (Figure 1).

Bipolenin K (1) was isolated as a colourless oil. Its molecular formula was determined as C_{15}H_{22}O_{3} from the HRESIMS [M + H]^+ ion at m/z 251.1646 (calcd for C_{15}H_{23}O_{3}^+, 251.1642), corresponding to five degrees of unsaturation. The IR absorption bands at 3445 and 1729 cm\(^{-1}\) revealed the presence of hydroxy and ester moieties, respectively. The \(^{13}\)C NMR spectrum (Figure S2, Supporting Information File 1) showed 15 distinct carbon signals, while \(^{13}\)C and \(^{1}\)H NMR data (Table 1) indicated an isopropyl unit (\(\delta_{C} 28.3, 29.2 \text{ and } 73.1; \delta_{H} 1.20 \text{ and } 1.24\)), one tertiary methyl (\(\delta_{C} 20.1; \delta_{H} 1.25\)), a disubstituted olefin (\(\delta_{C} 105.7 \text{ and } 155.2; \delta_{H} 5.12 \text{ and } 4.89\)) and four methines (\(\delta_{C} 39.6, 51.1, 51.6 \text{ and } 54.0; \delta_{H} 1.73, 1.88, 2.65 \text{ and } 3.77\)), which are typical resonances for sativene-type sesquiterpenoids. The NMR data for 1 were very similar to those for prehelminthosporol lactone (5) except for the replacement of a methine group (\(\delta_{C} 32.1; \delta_{H} 1.42\)) at C-9 in 5 with a hydroxylated quaternary carbon (\(\delta_{C} 73.1\)) in 1. This suggested that 1 was the 9-hydroxy analogue of 5, which was further confirmed by detailed analysis of key 2D NMR correlations (Figure 2). Compound 1 was previously reported in 1970 as a semi-synthetic analogue of 9-hydroxyprehelminthosporol [18], but has not been previously isolated and characterised from a natural source.

The relative configuration of 1 was established based on NOESY correlations (Figure 3) of H-1 to H-2-5 and H-3-10; and H-2-14 to H-7, H-8 and H-13. Due to the constrained bicyclo[3.2.1]octane ring system, these NOESY correlations indicated that H-1 was \(\beta\)-oriented, while H-6, H-7, H-8 and H-13

![Figure 1: Structures of compounds 1–12 isolated from *B. sorokiniana.*](image-url)
Table 1: $^1$H and $^{13}$C NMR data for bipolenins K–N (1–4).

<table>
<thead>
<tr>
<th>No.</th>
<th>Bipolenin K (1)$^a$</th>
<th>Bipolenin L (2)$^b$</th>
<th>Bipolenin M (3)$^b$</th>
<th>Bipolenin N (4)$^c$</th>
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<tr>
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$^a$Recorded at 500/125 MHz for $^1$H/$^{13}$C in CD$_3$OD; $^b$Recorded at 600/150 MHz for $^1$H/$^{13}$C in CD$_3$OD; $^c$Recorded at 600/150 MHz for $^1$H/$^{13}$C in CDCl$_3$.

Figure 2: Key 2D NMR correlations of bipolenins K–N (1–4).

were α-oriented. The absolute configuration of 1 was determined to be $1R,3R,6S,7R,13S$ by comparison of the experimental electronic circular dichroism (ECD) spectrum with time-dependent density functional theory (TDDFT)-calculated ECD spectra of the two possible enantiomers of 1 (Figure 4).

Bipolenin L (2) was isolated as a colourless oil. The HRESIMS [M + H]$^+$ ion at $m/z$ 251.1649 corresponded to a molecular formula C$_{15}$H$_{22}$O$_3$ (calcd for C$_{15}$H$_{23}$O$_3$ $^+$, 251.1642), which is isomeric with 1. The $^1$H and $^{13}$C NMR data for 2 (Table 1) were also very similar to those for 5, with the only significant difference being the presence of a hydroxymethylene group ($\delta_C$ 65.8; $\delta_H$ 3.58 and 3.44) in place of the methyl group at C-11. Thus, the structure of 2 was assigned as the 11-hydroxy analogue of 5. The absolute configurations of the chiral centres in 2 were established to be the same as for 1 after investigation of the proton coupling constants (Table 1), NOESY correlations (Figure 3) and ECD spectra (Figure 4). The configuration at C-9...
Figure 3: Key NOESY correlations of bipolenins K–N (1–4).

Figure 4: (a) Experimental ECD spectrum of 1 (MeOH) compared to TDDFT-calculated spectra (B3LYP-D3/def2-TZVPP) for the two possible enantiomers of 1, which were blue-shifted by 9 nm. (b) Comparison of experimental ECD spectra of 1–3 and 5 (MeOH).

was not determined. Hence, structure 2 was determined as shown in Figure 1.

Bipolenin M (3) was purified as a colourless oil. The molecular formula C_{15}H_{22}O_{3} was based on a HRESIMS [M + H]^+ ion at m/z 251.1647 (calcld for C_{15}H_{23}O_{3}, 251.1642), and is isomeric with 1 and 2. The $^1$H and $^{13}$C NMR data for 3 (Table 1) were very similar to those for 5, with the only significant difference being the replacement of the methine group ($\delta_C$ 47.1; $\delta_H$ 1.40) at C-6 in 5 with a hydroxylated quaternary carbon ($\delta_C$ 74.8) in 3. This suggested that 3 was the 6-hydroxy analogue of 5, which was further confirmed by detailed analysis of key 2D NMR correlations (Figure 2). The absolute configurations of the stereocentres in 3 were established to be identical to 1 and 2 based on the analysis of proton coupling constants (Table 1). NOESY correlations (Figure 3) and ECD spectra (Figure 4).

Bipolenin N (4) was acquired as a colourless oil. Its molecular formula was determined to be C_{15}H_{28}O_{4} from the HRESIMS [M + H − 2H_{2}O]^+ ion at m/z 237.1859 (calcld for C_{15}H_{25}O_{2}, 237.1849). The UV–vis spectra of 1–3 (Figure S37, Supporting Information File 1) and previously reported congener 5 were almost identical, while 4 displayed no significant UV–vis absorptions, suggesting the absence of both the ester and alkene moieties. This was confirmed by the analysis of the $^1$H and $^{13}$C NMR data for 4 (Table 1), which revealed the absence of ester and alkene resonances and the presence of three hydroxylated methylenes at C-12 ($\delta_C$ 62.2; $\delta_H$ 3.79 and 3.65), C-14 ($\delta_C$ 69.8; $\delta_H$ 3.90 and 3.36) and C-15 ($\delta_C$ 62.9; $\delta_H$ 3.77 and
3.47), and one hydroxylated quaternary carbon at C-2 (δc 88.1). This suggested 4 was related to 5, but with reduction of the lactone ring to the dialcohol and dihydroxylation of the Δ2,12 double bond. Detailed analysis of the 2D NMR data for 4 (Figure 2) confirmed the seco-sativene-type scaffold. The relative configurations at C-1, C-3, C-6, C-7 and C-13 were determined to be the same as those of 1–3 and other reported analogues based on NOESY correlations (Figure 3), while the configuration at C-2 was not determined. The ECD spectrum of 4 (Figure S38, Supporting Information File 1) was measured, but no significant Cotton effect was observed. Therefore, the structure of 4 was determined as shown in Figure 1.

Equipped with the compounds, we tested 1–12 for phytotoxic activity against wheat seedlings. The compounds all showed negligible activities at 200 ppm, although 6 and 10 showed signs of necrosis at 500 ppm (Figure S40, Supporting Information File 1). In addition, the activities of 1, 6–10 and 12 against wheat seed germination were also tested, with 7 inhibiting germination at 100 ppm (Figure S41, Supporting Information File 1). This corresponds to a previous report of the inhibitory effects of 7 on lettuce seed germination [19]. This activity could be due to the presence of an aldehyde moiety in 7. Interestingly, an earlier study showed that 7 promoted the elongation of the shoots of rice seedlings [20]. Compound 12 was reported to have a broad spectrum of biological activities, including phytotoxicity on juvenile plant Bromus tectorum [21], synctium formation inhibitory effects on cells infected with respiratory syncytial virus [22,23], induction of aerial mycelium formation in Fusarium culmorum [24], and as an inhibitor of ubiquinol-cytochrome c reductase binding protein, blocking mitochondrial ROS-mediated vascular endothelial growth factor receptor type 2 signalling pathways in endothelial cells [25]. However, 12 showed no activity against wheat seedlings or wheat seed germination in this study.

The sativene-type sesquerpenoids contain a bicyclo[3.2.1]octane backbone and are related to seco-sativene and isosativene scaffolds [15] (Figure 5a). They were also proposed to be related to the bicyclo[4.2.1]nonane-containing longifolene and seco-longifolene sesquerpenoids, as they were often co-isolated [3,4,6,15,26,27]. A closer examination of the biosynthetic relationship between sativene and longifolene scaffolds suggests that the two pathways branched early at the nerolidyl cation (Figure 5b) [28-31]. The biosynthesis of 1–8 and 10–11 are likely to be derived from sativene with a key oxidation at C-15 followed by a Baeyer–Villiger oxidation to break the C-14-C-15 bond (Figure 5c). Based on an isotope labelling study, the γ-butyrolactone moiety on 11 has been proposed to be derived from oxaloacetic acid or similar TCA-cycle intermediates [32]. Compound 9, which contains the seco-longifolene scaffold, is likely to be derived from longifolene via a similar Baeyer–Villiger mechanism proposed above for 1–8 and 10 and 11.

Several sativene-type sesquerpenoids have been previously reported from fungi, including from Bipolaris sp. [1]. B. sorokiniana [2], B. eleusines [3-6,8], Cochliobolus sp. [26], Cochliobolus sativus [18], Helminthosporium sativum [20,33,34], Drechslera sp. [27], Drechslera dematioides [15], and Veronaea sp. [35], most of which are Dothideomycetes. Significantly, this is the first report pertaining to sativene-type sesquerpenoids from B. sorokiniana in 25 years, since the first and only literature account was published in 1994 [2]. Furthermore, structure 4 has a seco-sativene type scaffold without an olefin unit at C-1/C-2 or C-2/C-12. In contrast, all the previously known seco-sativene-type sesquerpenoids possessed a double bond either at C-1/C-2 or C-2/C-12 [1,2,15,18,20,33], except drechslerine C, which contains a decarboxylated seco-sativene-type scaffold [15]. To the best of our knowledge, the previously reported sativene-type sesquerpenoids 5 (isolated from B. eleusines and Cochliobolus sp., and from semi-synthetic analogue of prehelminthosporol with pyridinium chlorochromate or chromic acid) [1,4,18,26], 8 (isolated from B. eleusines) [3,6], and 10 (isolated from Bipolaris sp. and Cochliobolus sp.) [1,26], were reported in B. sorokiniana for the first time, while, known metabolites 6 and 7 [20], 9 [34], 11 [2], and 12 [16] were previously reported from B. sorokiniana (syn. C. sativus and H. sativum).

The terpene synthase responsible for the biosynthesis of the sativene/longifolene backbone of 1–11 remains unknown. Given that the genome of B. sorokiniana BRIP10943 has been sequenced [14], we surveyed the genome for potential terpene synthases that may be responsible for the biosynthesis of these compounds. Four putative sesquerpenene synthases were found, corresponding to the genes COCSADRAFT_31812, COCSADRAFT_346586, COCSADRAFT_83129 and COCSADRAFT_26102 annotated in the published genome B. sorokiniana ND90Pr in GenBank. However, it is difficult to determine which sesquerpenene synthase is responsible for biosynthesis of the sativene-type sesquerpenene backbone at this stage.

The biosynthetic gene cluster (tpc) for terpestacin (12) has been recently identified from Bipolaris maydis [36]. A didomain sesterterpene synthase (tpcA) with a terpene cyclase domain and polyene synthase domain was demonstrated to be responsible for the production of the sesterterpene backbone of 12. A BLASTp search using tpcA as query against the genome of B. sorokiniana ND90Pr and BRIP10943 identified COCSADRAFT_342920 in ND90Pr (and its homolog in
BRIP10943), which shares 96% identity to tpcA. In the vicinity of the sesterterpene synthase gene, we also identified homologs for the two P450 oxygenases (tpcB and tpcC) encoded in the tpc cluster as COCSADRAFT_342924 (92% identity) and COCSADRAFT_146541 (98% identity), respectively, while the tpcD homolog in B. sorokiniana corresponded to COCSADRAFT_94398 (91% identity). This suggests that the homologous gene cluster in B. sorokiniana is likely responsible for the biosynthesis of sesquiterpenoids.
for production of 12. We are currently investigating the genetic basis for the biosynthesis of the sativene-type terpenoid compounds identified from *B. sorokiniana*.

**Conclusion**

Following the first and only reported isolation of a sativene-type sesquiterpenoid, sorokinianin (11), from *B. sorokiniana* in 1994 [2], we have expanded the number of reported analogues to eleven. These include the new sesquiterpenoid natural products, bipolenins K–N (1–4), as well as the previously reported sesquiterpenoids prehelminthosporol lactone (5), helminthosporic acid (6), helminthosporol (7), bipolenin A (8), secolongifolene diol (9), dihydrohelminthosporol (10) and sorokinianin (11), together with a sesterterpenoid, terpestacin (12). We demonstrated that 6 and 10 have weak necrotic activity against wheat leaves, while 7 inhibited wheat seed germination at 100 ppm. These compounds served as markers for identifying putative sesquiterpene synthase genes in the genome of *B. sorokiniana* BRIP10943, allowing the molecular genetic evaluation of putative sesquiterpene synthase genes in the genome of *B. sorokiniana* BRIP10943, allowing the molecular genetic basis for their biosynthesis and their roles in mediating the virulence of *B. sorokiniana* against wheat to be explored.

**Experimental**

**General experimental procedures**

Optical rotations were measured on an A. Krüss Optrogon P8000 polarimeter. The IR spectra were collected on a Perkin Elmer Spectrum One FTIR spectrometer. The HR-ESIMS spectra were recorded on a Waters LCT Premier XE mass spectrometer. The ESIMS spectra were recorded on an Agilent 1260 LC system equipped with a DAD detector and coupled to an Agilent 6130 Quadrupole MS with an ESI source. The NMR spectra were recorded on Bruker Avance III HD 500 or AV600 spectrometers. The ECD spectra were recorded on a Jasco J-810 spectropolarimeter with MeOH as solvent. Flash cartridge (Reveleris, HP-silica, 12 g, 20 μm), Kinetex C18 (Phenomenex, 2.6 μm, 2.1 × 100 mm), and semi-preparative C18 (Grace, 5 μm, 10 × 250 mm) were used. All solvents used for extraction were analytical grade, and solvents for HPLC were HPLC grade.

**Biological material**

The fungal strain *B. sorokiniana* BRIP10943 was obtained from Queensland Plant Pathology Herbarium (BRIP). It was isolated from a wheat field at Hermitage, QLD, Australia. The fungus was maintained on potato dextrose agar (PDA).

**Extraction and isolation**

*B. sorokiniana* was cultured on 16 plates of V8PDA at 25 °C for 14 days, then inoculated in 4 L shake-flask culture (25 °C, 180 rpm for 22 days) in Fries medium supplemented with oat. The Fries medium was filtered and extracted by partition with EtOAc/MeOH/acetic acid at 89:9:10:0.1 ratio. The cells were extracted with MeOH and partition with EtOAc/MeOH/acetic acid at 89:7:10:0.3 ratio. Both organic partitioned layers were combined to obtain a light-yellow crude extract (205 mg), which was fractionated on a Reveleris flash chromatography (Grace) using gradient mode of H2O/MeOH equipped with the flash cartridge, UV and evaporative light scattering detector. The resulting fractions were further purified by RP-HPLC on gradient mode of H2O/MeCN equipped with the C18 column, and DAD detector to yield 1 (1.5 mg), 2 (0.4 mg), 3 (0.4 mg), 4 (0.4 mg), 5 (0.5 mg), 6 (1.0 mg), 7 (4.0 mg), 8 (1.8 mg), 9 (2.8 mg), 10 (3.0 mg), 11 (0.4 mg), and 12 (1.3 mg). The Kinetex C18 on RP-HPLC (Phenomenex, 2.6 μm, 2.1 × 100 mm, 0.75 mL/min, DAD detection 200–800 nm, gradient: 0–10 min 5–95% MeCN with 0.1% formic acid, 10–15 min 95% MeCN with 0.1% formic acid) eluted 1 (tR 5.10 min), 2 (tR 4.79 min), 3 (tR 5.28 min), 4 (tR 5.96 min), 5 (tR 7.64 min), 6 (tR 6.00 min), 7 (tR 6.45 min), 8 (tR 4.85 min), 9 (tR 5.69 min), 10 (tR 6.37 min), 11 (tR 6.48 min) and 12 (tR 6.88 min).

Bipolenin K (1): Colourless oil; [α]D 20°: 50 (c 0.15, MeOH); IR (KBr) max 3445, 2926 and 1729 cm⁻¹; 1H and 13C NMR data, see Table 1; HRESIMS m/z: [M + H]+ calcd for C15H23O3+, 251.1642; found, 251.1646, and [M + H − H2O]+ calcd for C15H21O2+, 233.1536; found, 233.1531.

Bipolenin L (2): Colourless oil; [α]D 20°: 63 (c 0.04, MeOH); IR (KBr) max 3419, 2920 and 1730 cm⁻¹; 1H and 13C NMR data, see Table 1; HRESIMS m/z: [M + H]+ calcd for C14H23O3+, 251.1642; found, 251.1649, and [M + H − H2O]+ calcd for C14H21O2+, 233.1536; found, 233.1535.

Bipolenin M (3): Colourless oil; [α]D 20°: 57 (c 0.04, MeOH); IR (KBr) max 3418, 2927 and 1720 cm⁻¹; 1H and 13C NMR data, see Table 1; HRESIMS m/z: [M + H]+ calcd for C14H23O3+, 251.1642; found, 251.1647, and [M + H − H2O]+ calcd for C14H21O2+, 233.1536; found, 233.1545.

Bipolenin N (4): Colourless oil; [α]D 20°: 38 (c 0.04, MeOH); IR (KBr) max 3334, 2927 and 1045 cm⁻¹; 1H and 13C NMR data, see Table 1; HRESIMS m/z: [M + H − 2H2O]+ calcd for C15H25O2+, 237.1849; found, 237.1859.

**Phytotoxicity assays**

The phytotoxicity assays on wheat leaves and seeds were carried out as previously reported [37]. Briefly, the leaves of 17-days-old wheat seedlings in 10 cm planting pots were grown at 20 °C under a 16 h/8 h light/dark cycle regime. Compounds were dissolved in 0.2% MeOH/H2O and 30 μL of dissolved solution was infiltrated on the adaxial face of leaves at concen-
trations of 100, 200 and 500 ppm (serial dilution) using a 1 mL syringe. The leaves were examined for the presence of necrosis or chlorosis after 24 h and 48 h. The control consisted of 30 μL of 0.2% MeOH/H2O without dissolved compound. Two wheat seeds (sterilised by 10% EtOH) were placed on top of the agar (1.5% agar in 1 mL of tap water) containing 100 ppm of compound. The control was agar containing 30 μL of MeOH. The seeds were monitored for the progress of germination on day 5 and day 7.

Calculation of ECD spectra

Structures were initially subjected to a LowModeMD conformational search using the Molecular Operating Environment 2019.0101 package. The lowest energy geometry for each molecule was further optimised by DFT at the B3LYP-D3/def2-TZVPP level of theory using Turbomole 7.1 [38] and ECD spectra were calculated in Turbomole using TDDFT (B3LYP-D3/def2-TZVPP).

Supporting Information

Supporting Information File 1
NMR, IR and MS spectra of compounds 1–4. [https://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-15-198-S1.pdf]

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ORCID® IDs

Peter S. Solomon - https://orcid.org/0000-0002-5130-7307
Andrew M. Piggott - https://orcid.org/0000-0002-5308-5314
Yit-Heng Chooi - https://orcid.org/0000-0001-7719-7524

References


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