**Cyathane Diterpenes From Cultures of the Bird's Nest Fungus *Cyathus hookeri*
and Their Neurotrophic and Anti-neuroinflammatory Activities**

Dan Tang[†], Yuan-Zhen Xu[†], Wei-Wei Wang[†], Zhi Yang[†], Bo Liu[†], Marc Stadler[‡] ,
Ling-Li Liu^{*†} , and Jin-Ming Gao^{*†}

[†]Shaanxi Key Laboratory of Natural Products & Chemical Biology, College of Chemistry & Pharmacy, Northwest A&F University, Yangling 712100, Shaanxi, People's Republic of China

[‡]Department Microbial Drugs, Helmholtz Centre for Infection Research GmbH, Inhoffenstraße 7, 38124 Braunschweig, Germany

^{*}Co-corresponding authors

Six new cyathane diterpenoids, cyahookerins A–F (**1–6**), as well as nine known analogues (**7–15**), were isolated from the liquid culture of the basidiomycete *Cyathus hookeri* Berk. Their structures were elucidated on the basis of extensive spectroscopic analyses (1D and 2D NMR, HR-ESIMS, and ECD), and the absolute configurations of compounds **1** and **4** were determined by single-crystal X-ray crystallography. Compounds **1** and **2** represent the first unusual cyathane acetals featuring a dioxolane ring. Compounds **1–6** displayed differential nerve growth factor (NGF)-induced neurite outgrowth-promoting activity in PC-12 cells at concentrations of 10 μ M. In addition, cyahookerin B (**2**), cyathin E (**9**), cyathin B₂ (**12**) and cyathin Q (**13**) showed significant nitric oxide production inhibition in Lipopolysaccharide (LPS)-activated BV-2 microglial cells with IC₅₀ values of 12.0, 6.9, 10.9 and 9.1 μ M, respectively. Similar binding modes of the four compounds were indicated by molecular docking studies and structure-activity relationships are discussed.

Neurodegenerative diseases, such as Parkinson's disease (PD), and Alzheimer's disease (AD), affect millions of people worldwide.¹ In general, neuronal differentiation involves the growth, elongation and bifurcation of neuronal branches out of the neuronal cell body and this process is essential for the maintenance of the nervous system.² Neurodegenerative diseases occur when cells in the brain or peripheral nervous system lose function over time and ultimately die.³ The molecules collectively referred to as neurotrophins that promote the survival, maintenance and regeneration of neuronal populations can be used as a remedy against neurodegenerative diseases.⁴⁻⁷ In recent years, there has been an increasing interest in natural products with the potential for treating neurodegenerative disorders.⁸⁻¹¹

Cyathus species belong to the family Nidulariaceae (bird's nest fungi) among the Basidiomycota. They have been shown previously to constitute a prolific source of biologically active cyathane diterpenoids, which are characterized by a unique 5/6/7 tricyclic ring skeleton with a *trans* 6-7 ring junction.¹²⁻¹⁵ Several cyathane compounds possess high neurotrophic potency and might effect neuronal functions within the brain¹⁶⁻²⁰ as well as showing anti-inflammatory activity.²¹⁻²³ Hence, they might serve as useful leads in the search for small molecules having neurotrophic and neuroprotective activities with potential application in combating neurodegenerative disorders.

As part of our search for new bioactive cyathane-type diterpenes, we investigated the chemical constituents in the EtOAc extract of basidiomycete *Cyathus hookeri* Berk, resulting in the discovery of six new cyathane diterpenes (**1-6**) and nine known compounds (**7-15**). This report describes the isolation and structural elucidation of compounds **1-15**, together with the NGF-induced neurite-outgrowth and the anti-inflammatory activity of these isolated compounds.

RESULTS AND DISCUSSION

In order to exploit the chemical diversity of the fungus *C. hookeri* Berk., we optimized the culture conditions by adding azacitidine, a known DNA methyltransferase inhibitor. The diversity of secondary metabolites in cultures of *C. hookeri* growing with azacitidine was much higher compared to those of cultures growing without azacitidine (Figure S1, Supporting information). Thus, the fermentation of *C. hookeri* under optimized condition was carried out on a larger scale and the culture filtrates were extracted and fractionated as reported in detail in the Experimental Section, leading to the isolation of compounds **1–15**.

Cyahookerin A (**1**) was isolated as a colorless crystal. The molecular formula was established as $C_{24}H_{38}O_5$ on the basis of HRESIMS, with six degrees of unsaturation. The 1H NMR data (Table 1) indicated the presence of six methyls [δ_H 1.08 (3H, s, H-16); 1.09 (3H, s, H-17); 0.98 (3H, d, $J = 6.8$ Hz, H-19); 1.07 (3H, d, $J = 6.8$ Hz, H-20); 1.20 (3H, d, $J = 6.5$ Hz, H-22); 1.22 (3H, d, $J = 6.5$ Hz, H-24)], and five oxymethines [δ_H 4.41 (1H, m, H-11); 4.48 (1H, d, $J = 7.5$ Hz, H-13); 5.08 (1H, d, $J = 6.5$ Hz, H-15); 4.23 (1H, m, H-21); 4.18 (1H, m, H-23)]. The ^{13}C NMR data of **1** (Table 1) revealed 24 carbons including six methyls, five methylenes, eight methines, two quaternary carbons, one ketal quaternary carbon and two olefinic quaternary carbons. The HMBC correlations from H₃-16 to C-5 (δ_C 39.0), C-7 (δ_C 30.7), and C-14 (δ_C 107.7), from H₃-17 to C-1 (δ_C 41.0), C-4 (δ_C 136.9), C-8 (δ_C 38.1) and C-9 (δ_C 49.1), and from H₃-19 to C-2 (δ_C 29.5), C-3 (δ_C 141.6) and C-20 (δ_C 22.6), together with COSY correlations observed between H-10 (δ_H 2.20), H-11 (δ_H 4.41), H-12 (δ_H 2.28), H-13 (δ_H 4.48), confirmed the presence of a cyathane skeleton (Figure 1). The 4, 5-dimethyl-1, 3-dioxolane moiety, namely, an acetal type five-membered ring, was deduced from the COSY correlations between H-21 (δ_H 4.23), H-22 (δ_H

1.20), H-23 (δ_{H} 4.18) and H-24 (δ_{H} 1.22), and was also supported by HMBC correlations from H-15 (δ_{H} 5.08) to C-21 (δ_{C} 76.0), and C-23 (δ_{C} 75.4). The attachment of this moiety to the C-12 position was established by the HMBC correlations from H-12 to C-15 (δ_{C} 102.9) and from H-15 to C-11 (δ_{C} 75.0). Thus the planar structure of **1** was elucidated as shown. The relative stereochemistry of **1** was established based on NOESY correlations (Figure 2). H-5 showed NOE correlations to H₃-17, H-10 β , H-12, and H-13, indicating the same orientation of H-5, H-12, H-13 and H₃-17. Furthermore, H-10 α showed a NOE correlation to H₃-16, demonstrating the opposite orientation of H₃-16 and H₃-17. The single-crystal X-ray crystallographic analysis of **1** finally confirmed the above deduction (Figure 3). The absolute configuration of 5*S*, 6*R*, 9*S*, 11*S*, 12*S*, 13*R*, 14*S*, 21*R* and 23*S* was determined by the comparison of experimental and calculated CD spectra (Figure 6). This indicates that the butanediol unit present in the molecule is a 2*S*, 3*R*-mesomer. Thus the structure of **1** was established as shown and named cyahookerin A.

Cyahookerin B (**2**) was purified as a white powder and its molecular formula was established as C₂₄H₃₆O₄ by HRESIMS with seven degrees of unsaturation. The ¹H NMR data (Table 1) revealed signals due to six methyls [δ_{H} 1.04 (3H, s, H-16); 1.00 (3H, s, H-17); 0.96 (3H, d, *J* = 7.0 Hz, H-19); 1.09 (3H, d, *J* = 7.0 Hz, H-20); 1.17 (3H, d, *J* = 6.2 Hz, H-22); 1.16 (3H, d, *J* = 6.2 Hz, H-24)], four oxymethines [δ_{H} 4.83 (1H, m, H-11); 5.55 (1H, s, H-15); 4.24 (1H, m, H-21); 4.21 (1H, m, H-23)], and an olefinic proton at δ_{H} 6.31 (1H, s, H-13). The above data resembled those of compound **1**. However, compound **2** possessed one more double bond than **1**. The HMBC correlations (Figure 1) from the only olefinic proton H-13 to C-12 and C-15 indicated the location of this double bond at C-12 and C-13. Detailed analysis of the 2D NMR data demonstrated the remaining structure of **2** to be identical to that of compound **1**.

The relative stereochemistry at C-5, C-11 and C-9 was determined by NOE correlations of H-5 with H₃-17, H-11 and H-10 β , while the relative configuration at C-6 in contrary to C-9 was confirmed by NOE correlation of H₃-16 with H-10 α . Thus, compound **2** was determined as a new cyathane diterpene, named cyahookerin B.

Biogenetically, the compounds **1** and **2** could be formed by acetalization of the aldehyde group at C-12 of cyathane diterpene with *meso*-2*S*, 3*R*-butanediol produced by fermentation of sugar as carbon source. This is the first report of the cyathane natural products possessing the dioxolane ring.

Cyahookerin C (**3**) was obtained as a white powder. The molecular formula of **3** was determined to be C₂₃H₃₄O₅ by HRESIMS, implying six degrees of unsaturation. The ¹H NMR spectrum presented five methyls [δ_{H} 1.04 (3H, s, H-16); 1.00 (3H, s, H-17); 0.96 (3H, d, *J* = 7.0 Hz, H-19); 1.06 (3H, d, *J* = 7.0 Hz, H-20); 1.42 (3H, d, *J* = 6.9 Hz, H-23)], an olefinic proton at δ_{H} 6.17 (1H, s, H-13), two oxymethines [δ_{H} 4.73 (1H, m, H-11); 4.32 (1H, q, *J* = 6.9, H-22)], and an oxymethylene proton [δ_{H} 4.80 (1H, d, *J* = 14.0 Hz, H-15 α); 4.94 (1H, d, *J* = 14.0 Hz, H-15 β)]. The ¹³C NMR data (Table 1) revealed 23 carbons resonances ascribable for five methyls, six methylenes (one oxygenated), five methines, two quaternary carbons, one ketal quaternary carbon, three olefinic quaternary carbons and one carboxylic carbon. The ¹H and ¹³C NMR spectra of **3** displayed features similar to those of erinacine I,²⁴ except for the presence of an oxymethine. The HMBC correlations (Figure 1) from H-13 (δ_{H} 6.17) to C-15 (δ_{C} 61.3), from H-15 (δ_{H} 4.94) to C-11 (δ_{C} 80.6) and the carbonyl C-21 (δ_{C} 175.8) and from the methyl group H₃-23 (δ_{H} 1.42) to C-21 and C-22 (δ_{C} 67.9) indicated that the acetate moiety in erinacine I was replaced by the lactate moiety in **3**. The relative stereochemistry was determined from the NOESY correlations of H-5 with H₃-17, H-11 and H-10 β and H₃-16 with H-10 α as shown in Figure 2. Attempts to assign the

absolute configuration of the lactic acid residue were carried out using the hydrolysis method but unfortunately failed due to the limited amount of the isolated compound. Thus, compound **3** was elucidated as a new lactate-containing cyathane diterpene, named cyahookerin C.

Cyahookerin D (**4**) isolated as white crystal, has the molecular formula $C_{20}H_{28}O_4$, with seven degrees unsaturation, on the basis of HRESIMS. The 1H NMR spectroscopic data (Table 2) revealed the presence of four methyls [δ_H 0.92 (3H, d, $J = 7.0$ Hz, H-19); 0.96 (3H, s, H-17); 1.02 (3H, s, H-16); 1.03 (3H, d, $J = 7.0$ Hz, H-20)], an olefinic proton [δ_H 6.98 (1H, s, H-13)], an oxymethine proton [δ_H 4.90 (1H, m, H-11)]. The ^{13}C NMR spectrum of **4** showed 20 carbons including four methyls, five methylenes, four methines, and two quaternary carbons, one ketal quaternary carbon, three olefinic quaternary carbons and one carboxylic carbon. The 1D NMR spectra of **4** resemble those of cyathin A₃,²⁵ except for the absence of the oxymethylene group at C-12 and the presence of a carboxylic group. The HMBC correlation (Figure 1) from H-13 to C-12 (δ_C 140.4), C-14 (δ_C 111.6) and C-15 (δ_C 166.2) confirmed the attachment of a carboxylic group at C-12. The relative configuration of **4** was determined to be identical with that of cyathin A₃ from the NOESY correlations as described (Figure 2). Accordingly, the structure of **4** was established as depicted with a trivial name of cyahookerin D. The proposed structure of **4** was further confirmed by the single-crystal X-ray crystallographic analysis (Figure 4) and the absolute configuration of 5*S*, 6*R*, 9*S*, 11*S* and 14*S* was assigned based on the Flack parameter 0.18 (4).

The absolute configurations of compounds **2** and **3** (except lactic acid) were determined by biogenetic consideration and specific rotations. Their relative configurations were in agreement with that of **4**, combined with the comparison of the

specific rotations for **2** ($[\alpha]_D^{20} - 73$ (c 0.25, MeOH), **3** ($[\alpha]_D^{20} - 96$ (c 0.25, MeOH), and **4** ($[\alpha]_D^{20} - 84$ (c 0.25, MeOH). The experimental and calculated CD spectra of **2-4** were compared according to our previous reported protocol.²⁶ and the results were showed in Figure 6. Thus, the absolute configurations of 5*S*, 6*R*, 9*S*, 11*S*, 14*S*, 21*R* and 23*S*, and 5*S*, 6*R*, 9*S*, 11*S* and 14*S* (Figure 1) were established for both **2** and **3**, respectively.

Cyahookerin E (**5**) was obtained as a white powder. The molecular formula of **5** was determined to be C₂₃H₃₈O₄ (five degrees of unsaturation) by HRESIMS and NMR data. The ¹H NMR data (Table 2) showed the presence of four methyls [δ_H 0.81 (3H, s, H-16); 1.15 (3H, s, H-17); 1.03 (3H, d, $J = 7.0$ Hz, H-19); 1.04 (3H, d, $J = 7.0$ Hz, H-20)], three methoxyl groups [δ_H 3.42 (3H, s, H-21); 3.41 (3H, s, H-22); 3.39 (3H, s, H-23)], an olefinic proton at δ_H 6.30 (1H, d, $J = 7.5$ Hz, H-13), and two oxymethines at δ_H 3.54 (1H, d, $J = 8.0$ Hz, H-14), and at δ_H 4.57 (1H, s, H-15). The ¹³C NMR of **4** with the aid of HSQC spectrum revealed 23 carbons including four methyls, five methylenes, six methines (one acetal at δ_C 108.6), two quaternary carbons, three olefinic quaternary carbons and three methoxy carbons. The above data are very similar to those of cyathin E (**9**)²². However, comparison of NMR data of **5** with those of cyathin E (**9**) revealed the loss of aldehyde carbonyl signal (δ_C ca.195) in **9** and the appearance of additional dimethoxyl signals ($\delta_{C/H}$ 55.0/3.41; $\delta_{C/H}$ 54.6/3.39) and of one methine signal ($\delta_{C/H}$ 108.6/4.57), indicating that **5** contains an acetal [-CH(OCH₃)₂] moiety. The HMBC correlations (Figure 1) from H-11 to C-13, C-15 and C-21 and from H-15 to C-13, C-22 and C-23 confirmed the location of one methoxyl at C-11 and two methoxyls at C-15. In combination with COSY correlations of H-2–H-3, H-5–H-10–H-11, H-7–H-8 and H-18–H-19–H-20, this supported the planar structure of **5**. The hydrolysis²⁷ of **5** (Figure 5) was conducted with

p-toluenesulfonic acid (TsOH) affording compound **5a**, which has the molecular formula of C₂₁H₃₂O₃ deduced from the HRESIMS ion at *m/z* 355.2240 (calcd. for C₂₁H₃₂O₃Na, 355.2244) (Figure S23). The hydrolysis product **5a** displayed the exact same molecular formula of cyathin E (**9**), which confirmed the planar structure of **5** with the acetal group. However, further NMR analysis was limited by the small amount of **5a**. The NOESY spectrum of **5** showed NOE correlations (Figure 3) of H-5 with H-10 β , H-11 and H₃-17, indicating the β orientation of H-5, 9-CH₃, and H-11. While NOE correlations of H₃-16 with H-10 α and H-14, confirmed the α orientation of H-14 and 6-CH₃. Compared with the relative configuration of cyathin E, compound **5** showed the opposite orientation at H-11. The CD spectrum of **5** was obtained in acetonitrile and the absolute configuration of **5** was determined by the comparison of the experimental and calculated CD spectra (Figure 6). Accordingly, the structure of compound **5** was determined as described, and named cyahookerin E.

Cyahookerin F (**6**) was isolated as a white powder. The molecular formula was established as C₂₁H₃₀O₃ based on HRESIMS, requiring seven degrees of unsaturation. The molecular weight of **6** was 30 mass units more than that of cyathin B₂ (**12**),²⁸ suggesting the present of an additional methoxy group. The 1D NMR data (Table 2) showed the presence of five methyls (one methoxy at δ_C 58.7), five methylenes, five methines (one sp² carbon at δ_C 162.0, one aldehyde group at δ_C 194.7), and two quaternary carbons, three olefinic quaternary carbons, one carbonylic carbon and one ketone group at δ_C 212.4. The ¹H and ¹³C NMR data of **6** were in close correspondence to those of cyathin B₂, except for the presence of the methoxy group. The HMBC correlation (Figure 1) from H-13 (δ_H 4.79) to C-14 (δ_C 212.4) and C-21 (δ_C 58.7) confirmed the location of methoxy group at C-13. The relative configurations at C-5, C-9 and C-13 of compound **6** were determined on the basis of

NOESY correlations of H-5 to H-13 and H₃-17. Therefore, the structure of **6** (cyahookerin F) was established as shown. The known compound, cyathin Q (**13**), originally isolated from the fungus *Cyathus africanus*,²⁹ and compound **6** showed the same relative configuration at C-5, C-6, C-9, and C-13. The absolute configuration of **6** was defined as 5*S*, 6*R*, 9*S*, and 13*R* by comparing the experimental CD spectrum with the calculated one (Figure 6).

The ten known compounds (**7–15**) were identified as (12*S*)-11*α*,14*α*-epoxy-13*α*,14*β*,15-trihydroxycyath-3-ene (**7**),¹⁸ (12*R*)-11*α*,14*α*-epoxy-13*α*,14*β*,15-trihydroxycyath-3-ene (**8**),²¹ cyathin E (**9**),²² erinacol (**10**),³⁰ cyathatriol (**11**),³¹ cyathin B₂ (**12**),²⁸ cyathin Q (**13**),²⁹ 14-*oxo*-cyatha-3,12-diene (**14**),³² neoallocyathin A₄ (**15**),³³ respectively, by comparing their NMR and MS data with those reported in literatures.

The neurite-promoting activities of all isolated compounds were evaluated using PC-12 cell line. Compounds **9** and **13** displayed significant cytotoxicity at the test concentration (10 μ M) and the other compounds showed neurite-promoting activities. Notably, compounds **1–3**, **5**, **7**, **8** and **11** exhibited conspicuous neurite outgrowth-promoting effects compared to control cells and compound **3** appeared to be the most potent in promoting neurite outgrowth (Figure 7). The PC-12 cells treated with different concentrations of **3** were further investigated and the number of neurite bearing cells was counted. The percentage of neurite-bearing cells reached $28.1 \pm 1.1\%$ and $32.0 \pm 0.5\%$ for 5 and 10 μ M compound **3**, respectively (Figure 8). These values were significantly higher than those obtained from cells treated with 20 ng/mL NGF ($15.7 \pm 0.1\%$). The cells treated with compounds **1–6** (10 μ M) in combination with NGF (20 ng/mL) were stained with methylene blue and photographed under microscope to observe the morphological differences (Figure 9).

Nitric oxide (NO) is an important messenger in a variety of physiological systems

and the overproduction of the NO is involved in many pathogenic diseases. The inhibition against LPS-induced NO production in BV-2 microglial cells was conducted to evaluate the anti-inflammatory activities of compounds **1–15**. As shown in [Table 3](#), most of the isolated compounds, except for compounds **4**, **7**, **8** and **15** ($IC_{50} > 50 \mu M$), exhibited inhibitory effect with IC_{50} values ranging from 6.9 to 36.8 μM , and compound **9** exerting the most potent inhibitory effect with an IC_{50} value of 6.9 μM . From comparing the NO generation inhibition of the compounds tested, the following observations can be made: (i) among **5**, and **9–11**, the presence of a free aldehyde group at C-12 seems crucial for enhanced activity; (ii) regarding **6**, **12**, and **13**, incorporation of a hydroxy group at C-14 improved activity (IC_{50} 19.7 μM of **6** vs 9.1 μM of **13**), and a hydrogen at C-13 may help heighten activity (**6** vs **12**); (iii) with respect to **1**, **7** and **8**, and **2**, **3** and **4**, introduction of a low polar acetal unit at C-12 highly improved activity (IC_{50} of **1** and **2** far less than that of **7** and **8**, and **3** and **4**). Overall, the α , β -unsaturated aldehyde moiety in the molecule as with **9**, **12** and **13**, may play role in improved inhibition of NO production.

Molecular docking studies were performed for compounds **2**, **4**, **9**, **12**, and **13** in order to rationalize potency and selectivity against iNOS as described previously.³⁴ The binding mode predicted for compounds **2**, **9**, **12**, and **13**, in the docking studies is shown in [Figure 10](#). All four compounds exhibited a very similar binding mode in the selectivity pocket of iNOS with the binding energy ranging from -6.63 kcal/mol to -6.31 kcal/mol, while compound **4** applied as a negative control with binding affinity of 6.06 kcal/mol. The hydroxy group at the C-14 position in the seven-member ring in compounds **2**, **9** and **13** was favorably placed in the pocket facilitating hydrogen-bonding interaction with Tyr347. Furthermore, the ketone group at C-14 in compound **12** also displayed the hydrogen-bonding interaction with Ile-265, Leu-264

and Ala-351. These observations may help rationalize the inhibitory potency against iNOS of **2**, **9**, **12**, and **13**.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in MeOH using an Autopol III automatic polarimeter (Rudolph Research Analytical). IR spectra were obtained by a Bruker Tensor 27 FT-IR spectrometer with KBr pellets. UV spectra were recorded using a Thermo Scientific Evolution-300 UV-visible spectrophotometer. A Bruker AM-400, AVANCE III-500 or AV-600 spectrometers with TMS as internal standard, was used to acquire 1D and 2D NMR spectra. Mass spectra were measured on an LTQ Fleet instrument (Thermo Fisher Scientific Inc., USA). High-resolution electrospray ionization mass spectrometry (HRESIMS) of new compounds was performed on an AB SCIEX Triple TOF 5600+ spectrometer (AB SCIEX, Boston, MA, USA). Single crystal X-ray diffraction measurements were carried out on a Bruker D8 Venture diffractometer outfitted with a PHOTON-100 CMOS detector. The high-speed counter-current chromatography (HSCCC) system was composed of OptiChrome-300 PLUS apparatus (OptiChrome, Jiangyin, China), a UV3000D metering pump, a UV3000 spectrometer, a BSZ-100 fraction collector (QiTe, Shanghai, China) and CXTH-3000 ChemStation. Analytic HPLC were performed on a Waters 1525 instrument (Waters Corp., USA), equipping with a 4.6 mm $\times$ 250 mm, 5 μ m, Hypersil BDS C₁₈ column. Semi-preparative HPLC was achieved on a Shimadzu LC-20AP system, using a 10 mm $\times$ 250 mm, 5 μ m, YMC C₁₈ column. Column chromatography (CC) was performed on silica gel (200-300 mesh, Qingdao Marine Chemical Co. Ltd., Qingdao, China), Sephadex LH-20 (40–70 μ m; Amersham Pharmacia Biotech AB, Sweden), and reversed-phase (RP)-18 gel (20–45 μ m, Fuji Silysia Chemical Ltd., Japan). Fractions were monitored by thin-layer

chromatography (TLC) equipped with silica GF254 (10–20 μ m, Qingdao Marine Chemical Ltd., China).

Biological Material. The fungus *C. hookeri* Berk was purchased from China General Microbiological Culture Collection Center (CGMCC) with the accession number CGMCC 5.1116. This strain was collected at Da Long Gou (Gansu Province, China) in August 2005, and identified by Dr. Zhou Tonghu. A voucher specimen (No. CA20150822) is deposited at the College of Chemistry & Pharmacy, Northwest A&F University, Shaanxi, China.

Fermentation, Extraction, and Isolation. The culture medium consisted of yeast extract (2%), and sucrose (15%). Azacitidine (0.1 mmol/L), as an inhibitor of DNA methylation, was added to enrich the diversity of secondary metabolites of *C. hookeri*. The fermentation was carried out in one hundred 500 mL Fernbach flasks each containing 250 mL of YES medium, which were incubated at 28 °C for 14 days. After fermentation, the culture broth (63 L) filtered to afford the supernatant and the mycelia. The mycelia were extracted ultrasonically with MeOH–acetone (3/1, v/v) three times to break down the cell wall and release the secondary metabolites. The supernatant was evaporated under vacuum to yield an aqueous residue (5 L), which was extracted three times with 5 L petroleum ether and subsequently five times with 5 L EtOAc, respectively. The petroleum ether extract was discarded and the EtOAc layer was concentrated under reduced pressure to afford a crude extract (23.6 g), which was applied to a RP-18 column eluted with a gradient of MeOH–H₂O (10%–100%) to obtain fractions A (70% MeOH, 899 mg), B (90% MeOH, 4.9 g) and C (90% MeOH, 817.8 mg).

Fr. A (899 mg) was fractionated using Sephadex LH-20 column in MeOH to give three fractions, A1–A3. Fr. A2 (258.1 mg) was separated on HSCCC with a two-phase

solvent system composed of *n*-Hexane-EtOAc-MeOH-H₂O (HEMWat, 9:1:9:1, v/v), affording thirteen fractions, A2.1–A2.13. Fr. A2.3 was purified by RP-C₁₈ HPLC (65%, MeOH–H₂O, 2 mL/min) to obtain **15** (*t*_R, 27.6 min, 4.6 mg).

Fr. B (4.9 g) was fractionated by silica gel column chromatography eluting with a gradient of CHCl₃–MeOH (200:1–1:1), to yield fourteen fractions (B1–B14). Fr. B5 was isolated by RP-C₁₈ HPLC (75%, MeOH–H₂O, 2 mL/min) to obtain **3** (*t*_R, 36.3 min, 5.6 mg). Fr. B7 was subjected to RP-C₁₈ HPLC (75%, MeOH–H₂O, 2 mL/min) to give **7** (*t*_R, 34.2 min, 29.8 mg). Fr. B10 was further purified by semipreparative HPLC (72%, MeOH–H₂O, 2 mL/min) to obtain **8** (*t*_R, 37.4 min, 20.4 mg), **11** (*t*_R, 39.7 min, 28.6 mg) and **4** (*t*_R, 46.1 min, 28.6 mg).

Fr. C (817.8 mg) was subjected to Sephadex LH-20 column in MeOH to remove pigments, giving Fr. C1–C2. Fr. C2 (604.6 mg) was subjected to HSCCC separation using *n*-Hexane-EtAc-MeOH-H₂O (9:1:9:1, v/v) solvent system, yielding Fr. C2.1–C2.10. Among them, Fr. C2.7 and Fr. C2.10 corresponded to **9** (77.9 mg), and **14** (55.8 mg), respectively. Fr. C2.3 was purified by semipreparative HPLC on a RP-C₁₈ column (85%, MeOH–H₂O, 2 mL/min) to afford **13** (*t*_R, 28.7 min, 2.9 mg), **2** (*t*_R, 31.4 min, 8.2 mg) and **1** (*t*_R, 35.6 min, 27.8 mg). Fr. C2.4 was purified with RP-C₁₈ column HPLC (80%, MeOH–H₂O, 2 mL/min) to give **12** (*t*_R, 34.4 min, 56.8 mg). **6** (*t*_R, 30.2 min, 3.0 mg) was obtained from Fr. C2.6 with RP-C₁₈ HPLC (85%, MeOH–H₂O, 2 mL/min). **5** (*t*_R, 37.5 min, 5.0 mg) was obtained from Fr. C2.8 with RP C₁₈ HPLC (85%, MeOH–H₂O, 2 mL/min). Fr. C2.9 was further purified by semipreparative HPLC on a reversed-phase (RP) C₁₈ column (95%, MeOH–H₂O, 2 mL/min) to yield **10** (*t*_R, 19.1 min, 3.3 mg).

Cyahookerin A (1): White crystal (MeOH); mp 147.1 – 149.3 °C; [α]_D²⁰ = – 25 (*c* 0.25, MeOH); IR (KBr) ν_{max} 3451, 2941, 2865, 2312, 1717, 1454, 1370, 1217, 1145,

1104, 1035 cm^{-1} ; ^1H and ^{13}C NMR data, see [Table 1](#); negative ion HRESIMS m/z 405.2628 $[\text{M}-\text{H}]^-$ (calcd. for $\text{C}_{24}\text{H}_{37}\text{O}_5$, 405.2636).

Cyahookerin B (2): White powder; $[\alpha]_{\text{D}}^{20} = -73$ (c 0.25, MeOH); IR (KBr) ν_{max} 3397, 2939, 2869, 2312, 1716, 1454, 1370, 1230, 1090, 1047 cm^{-1} ; ^1H and ^{13}C NMR data, see [Table 1](#); positive ion HRESIMS m/z 389.2668 $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_{24}\text{H}_{37}\text{O}_4$, 389.2686).

Cyahookerin C (3): White powder; $[\alpha]_{\text{D}}^{20} = -96$ (c 0.25, MeOH); IR (KBr) ν_{max} 3397, 2938, 2864, 2313, 1736, 1454, 1369, 1228, 1130, 1048 cm^{-1} ; ^1H and ^{13}C NMR data, see [Table 1](#); positive ion HRESIMS m/z 408.2744 $[\text{M}+\text{NH}_4]^+$ (calcd. for $\text{C}_{23}\text{H}_{34}\text{O}_5\text{Na}$, 408.2744).

Cyahookerin D (4): White crystalline (CH_2Cl_2); mp 95.5 – 97.2 $^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{20} = -84$ (c 0.25, MeOH); IR (KBr) ν_{max} 3453, 3000, 2956, 2870, 1732, 1439, 1367, 1219, 1048 cm^{-1} ; ^1H and ^{13}C NMR data, see [Table 2](#); positive ion HRESIMS m/z 350.2313 $[\text{M}+\text{NH}_4]^+$ (calcd. for $\text{C}_{20}\text{H}_{32}\text{NO}_4$, 350.2326).

Cyahookerin E (5): White powder; $[\alpha]_{\text{D}}^{20} = -16$ (c 0.25, MeOH); IR (KBr) ν_{max} 3409, 2933, 2312, 1723, 1451, 1367, 1217, 1076 cm^{-1} ; ^1H and ^{13}C NMR data, see [Table 2](#); HRESIMS m/z 401.2670 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_{23}\text{H}_{38}\text{O}_4\text{Na}$, 401.2662).

Cyahookerin F (6): White powder; $[\alpha]_{\text{D}}^{20} = -16$ (c 0.25, MeOH); IR (KBr) ν_{max} 2935, 2867, 2312, 1704, 1454, 1368, 1226 cm^{-1} ; ^1H and ^{13}C NMR data, see [Table 2](#); positive ion HRESIMS m/z 353.2078 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_{21}\text{H}_{30}\text{O}_3\text{Na}$, 353.2087).

Acid Hydrolysis of Cyahookerin E (5). Compound **5** (0.5 mg) was dissolved in acetone (1.0 ml) and water (0.1 ml), followed with the addition of $p\text{-TsOH}\cdot\text{H}_2\text{O}$ (1.0 mg) at room temperature. After stirring for 1 h, the reaction was quenched with saturated aqueous NaHCO_3 (1 ml) and then extracted twice with AcOEt .²⁷ The combined organic layer was concentrated under reduced pressure. The product **5a** was

submitted to HRESIMS analysis using a LTQ Fleet mass spectrometer. The HRESIMS data gave m/z 355.2240 (calcd. for $C_{21}H_{32}O_3Na$, 355.2244).

Single-Crystal X-ray Structure Determination of Cyahookerin A (1). A single crystal of **1** was obtained and the X-ray diffraction data collection was carried out with an I μ S Cu K α ($\lambda = 1.54178$ Å) source which was operated at 50 kV and 40 mA at 153 K. Single crystals were selected and mounted on a nylon loop in Paratone-N cryoprotectant. Unit cell determination was performed in the Bruker SMART APEX III software suite. The data sets were reduced and a multi-scan spherical absorption correction was implemented in the SCALE interface. Crystal data: $C_{24}H_{37}O_5$, 405.26, space group monoclinic, P 21; unit cell dimensions were determined to be $a = 9.684$ (3) Å, $b = 7.892$ (2) Å, $c = 15.352$ (4) Å, $\alpha = 90.00^\circ$, $\beta = 95.198$ (10) $^\circ$, $\gamma = 90.00^\circ$, $V = 1168.5$ (6) Å³, $Z = 23$, $d_x = 1.117$ Mg/m³, $F(000) = 1200$, $\mu(\text{Cu K}\alpha) = 0.753$ mm⁻¹. 2388 unique reflections were collected to $\theta_{\text{max}} = 68.44^\circ$, where 1168 reflections were observed [$F^2 > 4\sigma(F^2)$]. The structures were solved using direct methods (SHELXTL, 2008) and refined through full-matrix least squares on F^2 . The final $R = 0.1707$, $R_w = 0.3812$, $S = 1.794$. The summary crystallographic data of **1** were deposited to The Cambridge Crystallographic Data Centre as CCDC 1877507.

Single-Crystal X-ray Structure Determination of Cyahookerin D (4). Single crystal X-ray diffraction measurements were carried out using a monochromatic I μ S Cu K α ($\lambda = 1.54184$ Å) source that was operated at 50 kV and 40 mA at 153 K. Single crystals were selected and mounted on a nylon loop in Paratone-N cryoprotectant. Unit cell determination was performed using the Bruker SMART APEX III software suite. The data sets were reduced and a multi-scan spherical absorption correction was implemented in the SCALE interface. Crystal data: $C_{20}H_{32}NO_4$, 350.23, space group monoclinic, P 21; unit cell dimensions were determined to be $a = 14.1618$ (13) Å, $b =$

15.0341 (2) Å, $c = 20.4035$ (4) Å, $\alpha = 90.00^\circ$, $\beta = 96.330$ (3)°, $\gamma = 90.00^\circ$, $V = 4317.6$ (7) Å³, $Z = 2$, $d_x = 3.176$ Mg/m³, $F(000) = 1200$, μ (Cu K α) = 0.753 mm⁻¹. 9232 unique reflections were collected to $\theta_{\max} = 68.45^\circ$, where 4317 reflections were observed [$F^2 > 4\sigma(F^2)$]. The structures were solved using direct methods (SHELXTL, 2008) and refined through full-matrix least squares on F^2 . The final $R = 0.0993$, $R_w = 0.2893$, $S = 1.480$ and Flack = 0.18 (4). The summary crystallographic data of **4** was deposited to The Cambridge Crystallographic Data Centre as CCDC 1877508.

Neurotrophic Activity. The neurotrophic activities of the isolated compounds were examined according to an assay using PC-12 cells as reported.¹⁷ Rat pheochromocytoma PC-12 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences). Briefly, the PC-12 cells were cultured in DMEM supplemented with 10% heat-inactivated HS (Gibco, Grand Island, NY, USA), 5% FBS (Hyclone, Logan, UT, USA), and 100 U/ml penicillin and 100 µg/ml streptomycin (Shanghai BasalMedia Technologies, Shanghai, China) in a humidified incubator with 5% carbon dioxide at 37 °C. During logarithmic growth phase, PC-12 cells (1×10^4 cells/well) were placed into a 24-well plate coated with poly-L-lysine (Sigma-Aldrich, MO, USA) in normal serum medium for 24 h, followed with low serum (2% HS and 1% FBS) medium treatment for 14 h. The cells were treated with compounds at various concentrations (0.1–10 µM, DMSO as solvent) in the absence or presence of NGF (20 ng/mL). Cells treated with 20 ng/mL of NGF served as a positive control. The same concentration experiment was repeated in three wells. After an additional 48 h of incubation, neurite outgrowth of PC-12 cells was observed under an inverted microscope using phase-contrast objectives and photographed with a digital camera. For each well, ten images were randomly captured under a microscope. In each selected field, at least one hundred cells were

scored. The cells with neurites which were greater than or equal to the length of cell body served as positive hit, and then expressed as a percentage of the total cell number in ten fields.

NO Inhibition in BV-2 cells. BV-2 murine microglial cells were purchased from Cell Resource Centre of Peking Union Medical College (Beijing, China). Cells were incubated in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 U/mL). BV-2 cells were seeded in 96-well plates at the concentration of 2×10^4 /well. After 24 h incubation at 37 °C, the cells were treated with 1 µg/mL of LPS (*Escherichia coli* 0111:B4, Sigma, MO, USA) and various concentrations of test compounds (DMSO as solvent) for 24 h. An equal amount of DMSO and LPS served as the controls, quercetin (J&K Scientific, Beijing, China), was taken as the positive control. The NO production in microglial culture was measured indirectly from the supernatant using a NO assay kit (Beyotime Institute of Biotechnology Company, Jiangsu, China) based on the Griess reaction.³³ Briefly, 50 µL of the culture supernatants were put into a new 96-well plate. Subsequently, 50 µL Griess reagents I and II was added to each well. The absorbance at 540 nm was measured on a microplate reader (Mode 680, Bio-Rad, Tokyo, Japan). The concentration of nitrite was measured by using a standard curve via the standard sodium nitrite solutions. The IC₅₀ was calculated as the concentrations that reduced NO production on 50%.

Molecular–Docking Studies. Autodock 4.2 Vina software and AutoDock Tools (ADT 1.5.6) were used for the molecular–docking studies as reported previously.³³ The three-dimensional (3D) crystal structure of iNOS (PDB code, 3E7G) was obtained from the RCSB Protein Data Bank. The standard 3D structures (PDB format) of **2**, **9**, **12**, and **13** were constructed using the “SKETCH” option function in

SYBYL-X, and a cubic grid box of $50 \times 60 \times 50 \text{ \AA}$ (x, y, z) with a spacing of $0.375 \text{ \AA}$ and grid maps were generated. The docking parameters were used the default settings.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website

AUTHOR INFORMATION

Corresponding Author

*Tel: 86-29-87092335. E-mail: jinminggao@nwsuaf.edu.cn

*Tel: 86-29-87038381. E-mail: leonie@nwsuaf.edu.cn

ORCID

Jin-Ming Gao: [0000-0003-4801-6514](https://orcid.org/0000-0003-4801-6514)

Marc Stadler: [0000-0002-7284-8671](https://orcid.org/0000-0002-7284-8671)

Ling-Li Liu: [0000-0003-2081-4493](https://orcid.org/0000-0003-2081-4493)

Notes

The authors declare no competing financial interest.

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Table 1. ^1H and ^{13}C NMR Spectroscopic Data for Compounds **1–3**^a.

Position	1		2		3	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	41.0	1.50, m 1.74, m	41.1	1.47, m 1.70, m	41.1	1.46, m 1.70, m
2	29.5	2.33, m	29.5	2.28, m	29.5	2.24, m
3	141.6		140.2		143.5	
4	136.9		138.4		138.1	
5	39.0	2.24, m	40.7	2.62, m	40.8	2.54, m
6	44.1		43.3		43.3	
7	30.7	1.60, m 2.30, m	28.9	1.68, m 2.22, m	28.0	1.62, m 2.24, m
8	38.1	1.62, m	38.2	1.57, m	38.3	1.56, m
9	49.1		49.3		49.3	
10	32.5	1.56, m 2.20, td, (3.5, 12.0)	31.5	1.40, m 1.56, m	31.5	1.38, m 1.55, m
11	75.0	4.41, m	79.5	4.83, m	80.6	4.73, m
12	52.9	2.28, m	145.5		140.4	
13	69.7	4.48, d, (7.5)	132.6	6.31, s	131.0	6.17, s
14	107.7		111.3		111.3	
15	102.9	5.08, d, (6.5)	99.5	5.55, s	61.3	4.80, d, (14.0) 4.94, d, (14.0)
16	12.9	1.08, s	12.1	1.04, s	12.0	1.04, s
17	24.1	1.09, s	24.3	1.00, s	24.4	1.00, s
18	27.4	3.00, sept, (6.8)	27.6	3.02, sept, (7.0)	27.6	3.03, sept, (7.0)
19	21.6	0.98, d, (6.8)	21.8	0.96, d, (7.0)	21.6	0.96, d, (7.0)
20	22.6	1.07, d, (6.8)	22.6	1.09, d, (7.0)	22.7	1.06, d, (7.0)
21	76.0	4.23, m	76.2	4.24, m	175.8	
22	15.7	1.20 ^b , d, (6.5)	15.6	1.17 ^c , d, (6.2)	67.9	4.32, q, (6.9)
23	75.4	4.18, m	76.1	4.21, m	20.7	1.42, d, (6.9)
24	15.9	1.22 ^b , d, (6.5)	15.7	1.16 ^c , d, (6.2)		

^a Recorded in CD₃OD at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR.^{b, c} Signals are interchangeable.

Table 2. ^1H and ^{13}C NMR Spectroscopic Data for Compounds **4–6**^a.

Position	4		5		6	
	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)
1	41.1	1.44, m 1.68, m	38.7	1.56, m 1.62, m	38.9	1.69, m
2	29.6	2.26, m	29.5	2.36, m	29.5	2.41, m
3	140.8		141.2		141.7	
4	137.6		140.2		138.9	
5	40.7	2.40, m	36.5	3.26, m	38.3	4.20, m
6	42.9		44.9		55.7	
7	27.8	1.65, m 2.25, m	35.0	1.02, m 2.42, m	34.1	1.28, m 2.51, td, (4.5, 13)
8	38.2	1.35, m 1.55, m	37.6	1.45, m 1.60, m	37.3	1.63, m
9	49.2		50.7		50.7	
10	31.6	1.35, m 1.60, m	32.3	2.11, m 2.31, m	34.1	3.09, m
11	79.6	4.90, m	76.2	4.04, m	162.0	7.23, m
12	140.4		142.8		137.1	
13	143.6	6.98, s	136.0	6.30, d, (7.5)	83.3	4.79, s
14	111.6		77.4	3.54, d, (8.0)	212.4	
15	166.2		108.6	4.57, s	194.7	9.50, s
16	11.8	1.02, s	16.9	0.81, s	14.7	1.09, s
17	24.2	0.96, s	24.6	1.15, s	24.7	1.20, s
18	27.6	2.99, sept, (7.0)	28.3	3.07, sept, (7.0)	28.3	3.12, m
19	21.6	0.92, d, (7.0)	22.0	1.03, d, (7.0)	21.9	1.10 ^b , d, (6.8)
20	22.7	1.03, d, (7.0)	22.4	1.04, d, (7.0)	22.2	1.10 ^b , d, (6.8)
21			57.2	3.42, s	58.7	3.47, s
22			55.0	3.41, s		
23			54.6	3.39, s		

^a Recorded in CD₃OD at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR.^b Signals are interchangeable.

Table 3. Inhibitory Effects of Compounds on LPS-Activated NO Production in BV-2 Microglial Cells.

Compounds	IC ₅₀ (μM) ^a	Compounds	IC ₅₀ (μM) ^a
1	15.5	9	6.9
2	12.0	10	32.8
3	27.3	11	22.1
4	> 50	12	10.9
5	36.8	13	9.1
6	19.7	14	27.8
7	> 50	15	> 50
8	> 50	Quercetin ^b	2.4

^a Half-Inhibitory Concentrations (IC₅₀) in μM.

^b Quercetin (99.9% purity) was used for comparison.

Figure legends

Figure 1. Key two-dimensional correlations of compounds **1–6**.

Figure 2. NOE correlations of compounds **1–6**. (conformation of **1–6** was assumed with minimized energy)

Figure 3. ORTEP drawing of Cyahookerin A (**1**).

Figure 4. ORTEP drawing of Cyahookerin D (**4**).

Figure 5. Hydrolysis reaction of compound **5**.

Figure 6. Experimental and theoretical ECD spectra of **1–6**.

Figure 7. Percentage of neurite-bearing PC-12 cells. Cell with one or more neurites whose lengths were at least twice the diameter of the cell body were counted as neurite-bearing cells. In all panels, error bars indicates $\pm$ SD (n=3). * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control.

Figure 8. Dose-effect relationship of **3**. In all panels, error bars indicates $\pm$ SD (n=3). (* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control,)

Figure 9. Effects of compounds **1–6** on neurite outgrowth in PC-12 cells after 48h treatment. Scale bar: 100 μ m.

Figure 10. Molecular-docking simulations of compounds **2** (A), **9** (B), **12** (C) and **13** (D) into iNOS binding site obtained in the lowest-energy conformation.

R

R

R₂

R₁

R

6 OCH₃

12 H

13

14

15

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 10.

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