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Alliacane-type Secondary Metabolites from Submerged Cultures of the Basidiomycete *Clitocybe nebularis*

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ABSTRACT

Seven sesquiterpenoids, named nebucanes A – G (**1** – **7**), featuring a rare alliacane scaffold with unprecedented furan or pyrrole functions, were isolated from the fermentation broth of *Clitocybe nebularis*. Their structures were established on basis of 1D/2D NMR spectroscopic analyses, HR-(+)-ESIMS spectra, as well as comparison of measured and calculated CD spectra for determination of the absolute configuration. Assessing the biological activities, nebucane D (**4**) exhibited antifungal effects against *Rhodotorula glutinis,* while nebucane G (**7**) displayed significant cytotoxicity against MCF-7 and A431 cell lines.

Basidiomycetes are intriguing and creative organisms regarding their ecological behaviour and secondary metabolism. They are equipped with a powerful machinery to form unique and biological active secondary metabolites, holding their great potential as a source for novel bioactive compounds.[1]

The basidiomycete *Clitocybe nebularis* (Batsch) P. Kumm, (syn.: *Lepista nebularis* (Batsch) Harmaja; *Clitocybe robusta* Peck), commonly known as the 'clouded agaric' or 'cloud funnel', is an abundant medium-size mushroom (cap: diameter 40 – 150 [– 200] mm) belonging to the family Tricholomataceae.[2,3] Fruiting body formation of this saprotrophic species occurs during late summer and early autumn, usually in the form of characteristic fairy rings in both conifer-dominated and deciduous forests.[4] In 1946, the first study on chemical constituents of its basidiomata was conducted, leading to the investigation of nebularine, a purine riboside (9-*β*-D-ribofuranosyl-9*H*-purine), as the active principle of the extract with significant antibiotic effects against mycobacteria and cytotoxic properties.[5-8] Other known constituents from the basidiomata are 4-hydroxy-2,4-diphenyl-2-butenal and 2,4-diphenyl-2-butenal as well as several unsaturated and hydroxylated fatty acids, such as (8*E*)-10-hydroxy-8-decenoic acid and (8*E*)-10-oxo-8-decenoic acid.[9] Moreover, two nordrimane sesquiterpenoids, named nebularic acids A and B, as well as two drimane sesquiterpenoids, named nebularilactones A and B, have been isolated from cultures of this fungus.[10] Nebularic acid B exhibited significant activity against several fungi and bacteria due to its reactive *α*,*β*-epoxy, *α*-keto carboxylate moiety.

In the course of our study on the secondary metabolites of the genus *Laccaria*, we came across a culture from a public collection that had obviously been confused. We established that it does not belong to *Laccaria* but in fact was found to represent an isolate of *C. nebularis.* Since a preliminary check by HR-LC/DAD-MS on its secondary metabolites pointed toward the presence of unprecedented compounds, we decided to work on this strain. This paper describes the isolation and structure elucidation of seven unusual alliacane-type

sesquiterpenoids **1** – **7** including determination of their absolute configuration and their biological characterization.

RESULTS AND DISCUSSION

rDNA sequencing led to the reassignment of the strain CBS 378.89 as *C. nebularis*. A detailed HR-LC/DAD-MS analysis of submerged cultures of this strain in different media (YMG, Q6½, SYM) pointed to the presence of several unprecedented compounds with C_{15}-scaffolds and UV maxima between λ = 225 nm and 305 nm. For isolation of these interesting compounds, two large scale fermentations in Q6½ medium were conducted and extracted with ethyl acetate. The resulting crude extracts from mycelia and supernatants were purified in two separation steps with flash chromatography for the initial and preparative chromatography on an RP-C_{18} column for the final step.

Compound **1** was obtained as a white powder with UV absorption maxima at λ = 229 and 304 nm. The HR-(+)ESIMS spectrum of **1** exhibited an $[M+H]^+$ ion at *m*/*z* 246.1487, consistent with the molecular formula $C_{15}H_{19}NO_2$ and seven degrees of unsaturation. The ^{1}H NMR spectrum recorded at 298 K in acetone-d_6 showed 19 protons (Table 1). In accordance with the ^{1}H NMR and HSQC spectrum, compound **1** contained two exchangeable protons. Furthermore, correlations accounting for three methyl groups, two methylene groups, and four methine groups were identified in the HSQC spectrum. Consequently, the presence of 15 carbons in the ^{13}C NMR spectrum indicated that six carbons must be non-protonated. Apart from six olefinic carbon atoms (δ_C 127.4 ppm, C-3; δ_C 126.6 ppm, C-4; δ_C 131.3 ppm, C-5; δ_C 135.8 ppm, C-6; δ_C 122.9 ppm, C-11; δ_C 125.0 ppm, C-12) and one carbonyl group (δ_C 192.1 ppm, C-2), the remaining three degrees of unsaturation implied that the structure of **1** was tricyclic. The planar structure of **1** was deduced from 2D NMR data to be a 1,7,7-trimethyl hydrindane substructure (system AB). This substructure was assigned by ^{1}H,^{1}H COSY correlations indicating the linkage of C-8–C-9–C-1–C-10, while C-1 carried a terminal methyl group (CH_3-10).

Furthermore, key HMBC correlations from the diastereotopically split protons H-8α/β (δ_H 1.68 ppm/ 1.75 ppm) to C-7 (δ_C 46.3 ppm) and C-6 (δ_C 135.8 ppm), from H-6 (δ_H 5.90 ppm) to C-9 (δ_C 48.7 ppm), C-5 (δ_C 131.3 ppm) and C-4 (δ_C 126.6 ppm), from H-10 (δ_H 0.98 ppm) to the carbonyl carbon C-2 (δ_C 192.1 ppm) as well as from H-1 (δ_H 2.46 ppm) to C-3 (δ_C 127.4 ppm) and C-5 (δ_C 131.3 ppm) confirmed the connection of a five and six membered ring system (system AB) sharing C-5 and C-9 (Figure 1). On account of their chemical shift values, a double bond had to be located between C-5 (δ_C 131.1 ppm) and C-6 (δ_C 135.8 ppm). The presence of two geminal methyl groups (CH_3-14 and CH_3-15) attached to the quaternary carbon C-7 (δ_C 46.3 ppm) was revealed by HMBC correlations from H-14 (δ_H 1.12 ppm) and H-15 (δ_H 1.23 ppm) to C-6 (δ_C 135.8 ppm), C-7 (δ_C 46.3 ppm), and C-8 (δ_C 40.1 ppm) and correlations of these methyl protons to the other methyl carbon to each other. Moreover, key HMBC correlations from H-12 (δ_H 7.11 ppm) to the non-protonated carbons C-3 (δ_C 127.4ppm), C-4 (δ_C 126.6 ppm), and C-11 (δ_C 122.9 ppm) along with a characteristic chemical shift value for the olefinic methine proton H-12 (δ_H 7.11 ppm) indicated the presence of a pyrrole. Consequently, the hydrindane system (AB) is extended by the pyrrole substituent sharing carbons C-3 and C-4 (system C). The ^{1}H,^{1}H COSY spectrum exhibits the presence of an isolated methylene group (δ_C 56.9 ppm, CH_2-13) with a hydroxy group attached. According to HMBC correlations from H-13'/H-13" (δ_H 4.63 ppm and 4.57 ppm) to C-12 (δ_C 125.0 ppm), C-11 (δ_C 122.9 ppm), and C-4 (δ_C 126.6 ppm), the heterocycle carried the oxymethylene group at C-11 forming the tricyclic alliacane-type sesquiterpenoid, named nebucane A (**1**). The relative configuration of nebucane A (**1**) was assigned by analysis of NOESY data and coupling constants of the protons. NOE correlations between H-1/H-9 along with a coupling constant of 6 Hz (dihedral angle of $\alpha \approx 50°$) supported a synclinal arrangement of the C-1–C-9 bond (Figure 2). Furthermore, key NOE correlations were observed between H-10/H-8α, H-8α/H-15 and in contrast H-9/H-14 and H-8β/H-14 indicating that these protons are on the same side, respectively. To further assign the absolute configuration of **1**, ECD calculations were

performed. The calculated ECD spectrum was in good agreement with the experimental spectrum (Figure S13, *cf* SI). The absolute configuration of nebucane A (**1**) was thus be determined to be 1*S*,9*R*.

Compounds **2**, **3**, and **4** were isolated with comparable UV data to those of nebucane A (**1**). According to their HR-(+)ESIMS spectra, their molecular formulae were assigned as $C_{15}H_{19}NO_3$ for compound **2**, $C_{15}H_{19}NO_2$ for compound **3**, and $C_{15}H_{17}NO$ for compound **4**. Based on analysis of their molecular formulae and 1D/2D NMR data, compounds **2** – **4** were found to be structurally closely related to nebucane A (**1**), and **1** – **4** share the same tricyclic alliacane scaffold. In contrast to nebucane A (**1**), compound **2** is substituted by a hydroxy group at C-15 (δ_C 70.9 ppm). In addition to the characteristic downfield shifted chemical shift value of C-15, the HSQC spectrum exhibited a methylene group with diastereotopically split protons (δ_H 3.54 – 3.59 ppm/ δ_H 3.48 ppm) and a broad singlet at δ_H 3.71 ppm in the ^{1}H NMR spectrum, recorded at 298 K in acetone-d_6. Furthermore, ^{1}H,^{1}H COSY correlations between H-15'/H-15'' (δ_H 3.54 – 3.59 ppm/ δ_H 3.48 ppm) and OH-18 (δ_H 3.71 ppm) confirmed the presence of an oxymethylene group. The relative configuration of **2** was assigned by NOESY measurements. Key correlations between H-1/H-9, H-9/H-14, H-8α/H-10, H-8α/H-15 and H-8β/H-14 indicated the same relative configuration as nebucane A (**1**). Due to comparable ECD spectra between nebucane A (**1**) and **2** together with biosynthetic considerations, the absolute configuration for **2** was determined as 1*S*,9*R*,7*R* and the alliacane-type sesquiterpene was named nebucane B (**2**).

The ^{1}H NMR spectrum of compound **3** exhibited significant differences to that of compound **1** due to the presence of a singlet at δ_H 9.79 ppm and a doublet at δ_H 5.06 ppm. HMBC correlations from the proton H-13 (δ_H 9.79 ppm) to the carbons C-4 (δ_C 113.7 ppm), C-11 (δ_C 124.1 ppm), and C-12 (δ_C 131.3 ppm) indicated that an aldehyde group is attached to C-11, which was in agreement with its characteristic low field shifts (δ_C 184.5 ppm [C-13]; δ_H 9.79 ppm [H-13]). Furthermore, C-2 (δ_C 69.0 ppm) carries a hydroxy group instead of the carboxyl group found in **1** and **2**. This assumption was supported by HMBC correlations from H-2 (δ_H

5.06 ppm) to C-10 (δ_C 6.2 ppm), C-1 (δ_C 37.8 ppm), C-9 (δ_C 48.1 ppm), C-3 (δ_C 135.1 ppm), and C-4 (δ_C 113.7 ppm) along with a characteristic chemical shift value of δ_C 69.0 ppm for this carbon. The relative configuration was assigned by NOESY correlations of H-1/H-2, H-1/H-9, H-9/H-2, H-10/H-8α, H-9/H-14, H-14/H-8β, H-15/H-8α. This configuration was also supported by the coupling constants in the ^{1}H NMR (Table 1). The co-occurrence of compounds **1** – **3** in the same species suggested the same absolute configuration for **3** as for nebucanes A (**1**) and B (**2**) due to biogenetic considerations. Therefore, the absolute configuration of **3** is proposed to be 1*S*,2*S*,9*R*.

In contrast to nebucanes A – C (**1** – **3**), compound **4** was lacking the functionality at C-2 with simultaneous occurrence of an additional signal at δ_H 7.11 ppm in the aromatic region of the ^{1}H NMR spectrum. Furthermore, according to the HSQC spectrum, C-6 (δ_C 49.8 ppm) carried two protons at δ_H 3.30 ppm indicating that the double bond between C-5 and C-6 was missing. The HMBC spectrum exhibited key correlations from H-6 (δ_H 3.30 ppm) to C-5 (δ_C 135.3 ppm), C-9 (δ_C 136.6 ppm), C-1 (δ_C 130.3 ppm), C-4 (120.1 ppm) from H-8 (δ_H 2.72 ppm) to C-9, C-1, C-5, and C-4 and from H-2 (δ_H 7.11 ppm) to C-4 and C-9. The characteristic chemical shift values of these carbons allowed the construction of an aromatic six membered ring system (system B). Therefore, the molecular structure of **4** was determined to be an achiral aromatic sesquiterpenoid with alliacane scaffold, named nebucane D (**4**).

Nebucane C (**3**) was stored in dry state for four months as a solid at -20°C in the dark. HPLC-DAD/MS reanalysis of nebucane C (**3**) after this time pointed to the presence of an additional UV peak and corresponding $[M+H]^+$ ion at *m/z* 228 occurring at the same retention time as nebucane D (**4**) with concurrent loss of the previous UV peak of **3**. This observation indicated the conversion of nebucane C (**3**) into nebucane D (**4**).

According to its HR-(+)ESIMS spectrum, the molecular formula of compound **5** was assigned as $C_{15}H_{20}O_4$ with six degrees of unsaturation. Although **5** might possess a less

pronounced delocalized π-system compared to nebucanes **1** – **4** because of a shift in the UV maximum to shorter wavelengths ($\lambda \approx 270$ nm), the molecular formula suggested a sesquiterpenoid scaffold. The 1D NMR data of **5** (Table 2) demonstrated significant similarities to those of compound **1**, revealing the same alliacane core structure as nebucane A (**1**). Differences were determined in the shift values of the carbons C-3 (δ_C 146.6 ppm), C-4 (δ_C 138.7 ppm), C-12 (δ_C 145.3 ppm), and C-11 (δ_C 126.6 ppm) that were shifted to lower field compared to the pyrrole system in **1** (Table 2). According to the molecular formula, the structure of **5** lacked the nitrogen atom and possessed two additional oxygen atoms compared to nebucane A (**1**). Therefore, a furan system was replacing the pyrrole function in **1**, which was in agreement with the deviated shift values in **5** and the less pronounced aromaticity that was observed in the UV spectrum. As was found in nebucane D (**4**), the double bond between C-5 (δ_C 76.5 ppm) and C-6 (δ_C 55.10 ppm), that is present in **1** – **3**, is lacking. The ^{1}H NMR spectrum, measured at 298 K in DMSO-d_6, exhibited two additional signals at δ_H 5.43 ppm and δ_H 5.24 ppm. According to the HSQC spectrum, recorded at 298 K in DMSO-d_6, they were assigned as exchangeable protons of two hydroxy groups H-16 and H-17, respectively. Key correlations in the HMBC spectrum, measured at 298 K in DMSO-d_6, from H-16 (δ_H 5.43 ppm) to C-13 (δ_C 53.8 ppm) and C-11 (δ_C 126.9 ppm) as well as from H-17 (δ_H 5.24 ppm) to C-4 (δ_C 138.2 ppm) and C-6 (δ_C 53.7 ppm) confirmed the structure of **5**. For the assignment of the stereogenic centers in **5**, the relative configuration was determined by analysis of the ROESY data, measured at 298 K in DMSO-d_6. The key ROESY correlations between H-1/H-9, H-9/H-8β, H-8β/H-14, H-8α/H-15 were comparable to those in nebucanes A (**1**) and B (**3**). Further ROESY correlations between H-14/H-9, H-14/H-6β, H-17/H-6β, H-17/H-9, and H-17/H-1 on the one hand and H-15/H-6α on the other hand suggested that these protons were on the same side, respectively (Figure 2). Therefore, the data are in agreement with a *cis* ring junction at C-5/C-9 of the hydrindane system (AB). For the absolute configuration of **5**, ECD calculations were performed. The calculated ECD spectrum was in good agreement with the experimental

spectrum (Figure S61, *cf* SI). The absolute configuration of **5** was assigned as 1*S*,5*S*,9*R*, and the compound named nebucane E (**5**).

Compound **6** was isolated as a white powder with an absorption maximum at λ = 246 nm in the UV spectrum. The molecular formula $C_{15}H_{20}O_3$ was established based on the HR-(+)ESIMS analysis. Thus, compound **6** contains six degrees of unsaturation. Analysis of the 1D/2D NMR data suggested a structure which was comparable to the previously isolated nebucanes A – E (**1** – **5**). However, due to four olefinic carbon atoms (δ_C 131.7 ppm, C-4; δ_C 167.0 ppm, C-5; δ_C 137.0 ppm, C-11; δ_C 128.3 ppm, C-13) and two carbonyl groups (δ_C 195.8 ppm, C-3; δ_C 167.3 ppm, C-12), the remaining two degrees of unsaturation implied that the structure of **6** was bicyclic. HBMC correlations from the two protons H-13'/H-13" (δ_H 5.57 ppm/ δ_H 6.34 ppm) of an exo-methylene group (δ_C 128.3 ppm, C-13) to C-4 (δ_C 131.7 ppm), C-11 (δ_C 137.0 ppm, C-11), and C-12 (δ_C 167.3 ppm) revealed that a methacrylic acid functionality was attached to ring system B via C-4. Considering the carbon chemical shift values, a double bond could be placed between C-4 (δ_C 131.7 ppm) and C-5 (δ_C 167.0 ppm, C-5). Since there were ^{1}H,^{1}H COSY correlations between two diastereotopically split protons H-2*α* (δ_H 2.27 ppm) and H-2*β* (δ_H 2.59 ppm) and H-1 (δ_H 2.40 – 2.43 ppm) along with HMBC correlations from H-2*α*/*β* to C-3 (δ_C 195.8 ppm) and C-10 (δ_C 13.1 ppm) as well as from H-2*α* (δ_H 2.27 ppm) to C-4 (δ_C 131.7 ppm), C-2 was located between C-1 and C-3. According to the characteristic chemical shift value of δ_C 195.8 ppm, C-3 carried a ketone functionality, yielding nebucane F (**6**). To determine the relative configuration of **6**, assuming that H-1 and H-9 were *β* orientated, the NOESY data and coupling constants were analysed. NOESY correlations between H-1/H-9, H-8*β*/H-14, H-14/H-9, and H-14/H-6*β* indicated a comparable situation to **1** – **3** and **5**. Furthermore, correlations between H-2*β*/H-9 and H-2*α*/H-10 could be observed. The absolute configuration of **6** was established by ECD calculations. The calculated ECD spectrum of nebucane F (**6**) was in good agreement with the experimental spectrum (Figure S71, *cf* SI) and was found to be 1*R*,9*R*.

Compound **7** was purified as a white powder. The HR-(+)ESIMS data indicated a molecular formula of $C_{15}H_{20}O_5$ and six degrees of unsaturation. Since some of the proton signals were strongly broadened in the ^{1}H NMR spectrum, when recorded at 298 K in acetone-d_6, the ^{1}H NMR spectrum and the HMBC spectrum were measured at 273 K with much lower signal broadening (Table 2). According to the ^{13}C NMR spectrum, **7** possessed one ketone functionality (δ_C 207.3 ppm), one carbonyl group (δ_C 166.8 ppm), and two olefinic carbons atoms (δ_C 137.0 ppm, δ_C 128.6 ppm). On account of the molecular formula, **7** must be tricyclic. Furthermore, similarities in the 1D NMR spectra to nebucane F (**6**) indicated the same structural features, such as the 1,7,7-trimethyl hydrindane system, a ketone function at C-3 (δ_C 207.3 ppm), and a methacrylic acid functionality (δ_C 137.0 ppm, C-11; δ_C 166.8 ppm, C-12; δ_C 128.6 ppm, C-13). According to their downfield chemical shift values, carbons C-2 (δ_C 75.4 ppm), C-4 (δ_C 66.2 ppm), and C-5 (δ_C 81.8 ppm) were oxygenated. In contrast to **6**, a C-4/C-5 epoxide functionality and a hydroxy group at C-2 were required based on their ^{13}C NMR chemical shift values and the molecular formula. The key HMBC correlations confirmed this assumption (Figure 1), thus allowing **7** to be identified as nebucane G. The relative configuration was determined by analysis of the ROESY data, measured at 298 K in DMSO-d_6. Key ROESY correlations between H-2/H-1, H-2/H-9, H-8β/H-14 and H-6β/H-14 assigned the β-orientated protons. Weak ROESY correlations between H-10/H-6α, H-6α/H-8α, H-13"/H-6α, H-13"/H-6β suggested a *cis* ring junction at C-5/C-9 (Figure 2). The structure of nebucane G (**7**) is closely related to that of inonoalliacane I (**8**)[11], a member of the alliacane-type family, which was isolated from culture broth of the basidiomycete *Inonotus* sp. BCC22670. Due to analogous relative configurations in combination with similar optical rotation values of **7** and inonoalliacane I (**8**) (**7**: $[\alpha]^{20}_D$ +21.0 [*c* 0.2, MeOH]; **8**: $[\alpha]^{20}_D$ +26.0 [*c* 0.1, MeOH][11]), the absolute configuration of nebucane G (**7**) was supposed to be 1*S*,2*S*,4*S*,5*S*,9*R*.

Alliacane-type sesquiterpenoids are relatively rare in fungi and a few have been reported from Basidiomycota. The discovery of the alliacols A (alliacolide II) and B and alliacolides

from submerged cultures of the beech decaying *Mycetinis alliaceus* (Jacq.: Fr.) Earle (formerly named *Marasmius alliaceus*), featuring an epoxidized hydrindane structure with butyrolactone moiety, were the first reports of alliacane-type sesquiterpenoids.[12-14] Further examples for secondary metabolites possessing an alliacane skeleton are the group of clitocybulols, isolated from *Clitocybula oculus* (clitocybulols A – C)[15] and *Pleurotus cystidiosus* (clitocybulols D – O),[16,17] the purpuracolides A – C from *Gomphus purpuraceus*,[18,19] incarnetic acid from *Gloeostereum incarnatum*,[20] and the inonoalliacanes A – I from *Inonotus* sp.,[11] which are to some degree structurally related to nebucanes E – G (**5** – **7**). Notably, these fungi belong to various different families and orders in the Agaricomycetes and it is presently not possible to draw any conclusions on potential chemotaxonomic correlations.

Nebucanes A (**1**) and D – G (**4** – **7**) were tested against selected organisms and cell lines; **1**, **4**, and **7** showed antimicrobial and cytotoxic effects (Table 3). Nebucane D (**4**) showed significant antifungal activity against *Rhodotorula glutinis* [minimum inhibitory activity (MIC) of 4.2 µg/mL]. Nebucane G (**7**) exhibited cytotoxic effects against carcinoma cell lines A431 (human epidermoid carcinoma) and MCF-7 (human breast carcinoma), with a half maximal inhibitory concentration values (IC_{50}) between 7 and 8 µM, respectively.

To verify the taxonomic assignment of the producer strain CBS 378.89, fresh basidiomata of *C. nebularis* were collected, transferred into the corresponding culture (STMA 21071), and cultivated in Q6½ medium. HR-LC-(+)ESIMS analysis of the submerged cultivation of strain STMA 21071 exhibited the presence of nebucanes A (**1**), E (**5**), and G (**7**), according to retention times, MS spectra, and molecular formulae (Figure S88, *cf.* SI). Moreover, an acetone extract from the basidiomata of *C. nebularis* contained the purine riboside nebularine as the main compound, although the isolated alliacane-type sesquiterpenoids **1** – **7** could not be observed in the extract (Figure S89, *cf.* SI).

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded with a Bruker Avance III 700 spectrometer with a 5 mm TXI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz) and a Bruker AV III-600 (^{1}H 500 MHz, ^{13}C 126 MHz) spectrometer (Bruker BioSpin GmbH, Ettlingen, Germany). All the NMR spectra were referenced to the solvent used (acetone-d_6 [^{1}H: 2.05 ppm, ^{13}C: 29.32 ppm], dimethylsulfoxide (DMSO-d_6 [^{1}H: 2.50 ppm, ^{13}C: 39.51 ppm]). HPLC-DAD/MS analysis was performed using an amaZon speed ETD ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive and negative ionization modes for evaluating the extracts and the purity of isolated compounds. The HPLC system consisted of a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific Inc, Waltham, MA, USA) equipped with a C_{18} Acquity UPLC BEH (Waters, Milford, USA) column as stationary phase. Solvent A consisted of deionized H_2O + 0.1% formic acid (FA) (v/v) and solvent B was acetonitrile (ACN) + 0.1% FA (v/v). The applied gradient was 5% B for 0.5 min, increasing to 100% B over a period of 20 min and holding for 10 min at 100% B. The flow rate was 0.6 mL/min and UV/Vis detections were set to λ = 200–600 nm. HR-LC-(+)ESIMS data were recorded with Agilent 1200 series HPLC-UV system (Agilent Technologies, Berlin, Germany) equipped with a C_{18} column Acquity UPLC BEH (2.1×50 mm, 1.7 µm; Waters Limited, Wilmslow, UK), solvent A: deionized H_2O + 0.1% FA; solvent B: ACN + 0.1% FA, gradient: 5% B for 0.5 min increasing to 100% in 19.5 min and then maintaining 100% B for 5 min, flow rate 0.6 mL/min. UV detection was conducted at λ = 200 – 600 nm combined with ESI-TOF-MS (Maxis, Bruker Daltonics GmbH & Co. KG, Bremen, Germany), scan range 100–2500 *m*/*z*, capillary voltage 4500 V, dry temperature 200 °C. Optical rotation was recorded with an Anton Paar MCP 150 polarimeter. UV spectra were recorded with a J 815 (Jasco Deutaschland GmbH, Pfungstadt, Germany) UV/Vis spectrometer. CD spectra were obtained with a J 815 spectrometer. Analytical TLC was performed on silica gel 60 F_{254} aluminum foils (Merck KGaA, Darmstadt,

Germany). All chemicals and solvents (analytical and HPLC grade) were obtained from Avantor Performance Materials (Deventer, Netherlands), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), and Merck KGaA (Darmstadt, Germany).

Fungal Material. The fungal strain CBS 378.89 was ordered from Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands) in 2018. Reconsideration of its identification was achieved by rDNA sequence comparison [5.8S gene region, the internal transcribed spacer 1 and 2 (ITS) and the large subunit (LSU)] with others from Basidiomycota deposited in GenBank and identified as *C. nebularis* (Figure S91, *cf* SI). For verification of the identity of the producer strain CBS 378.89, a basidiome of *C. nebularis* was collected in Braunschweig, Germany on October 24, 2020 and identified based on its morphological characters (leg. and det. H. Andersson) and verified via rDNA sequence comparison [ITS4 and NL4]. Corresponding cultures were obtained from its basidiospores and are stored at the mycological strain collection at the Microbial Drugs Department of the Helmholtz Centre for Infection Research (Braunschweig, Germany) under accession number STMA 21071, as well as the Leibniz Institute DSMZ−German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany) under accession number DSM 113279, and the Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands) under accession number CBS 149282. Sequence data are deposited with GenBank, accession number SUB11837209 for NL4. A dried voucher specimen is deposited in the public herbarium of the Natural History Museum in Karlsruhe ([KR], Germany) under accession number KR-M-0047308.[21]

Sequencing. DNA extraction was performed using the EZ-10 Spin Column Genomic DNA Miniprep kit (Bio Basic Canada Inc., Markham, Ontario, Canada) following a previously published protocol.[22,23] A Precellys 24 homogenizer (Bertin Technologies, France) was used for cell disruption at a speed of 6000 rpm for 2 × 40 s. The DNA regions were amplified using standard primers. For ITS and LSU sequence processing, the PCR products were purified directly, using the Nucleo Spin® Gel and PCR Clean up kit (Macherey-Nagel GmbH & Co. KG,

Düren, Germany). PCR products were sequenced using Sanger Cycle Sequencing method at Microsynth Seqlab GmbH (Göttingen, Germany). Consensus sequences were obtained employing the Geneious® 7.1.8 program.[24] The sequences were then compared to reference data available by using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi/ [accessed 2020-04-20]).

Small-scale cultivation. CBS 387.89 was cultivated in Q6½ (10 g/L glycerol, 2.5 g/L D-glucose, 5 g/L cotton seed flour; pH 7.2) and SYM (30 g/L malt extract, 10 g/L sucrose, 5 g/L yeast extract; pH 6.3) media. A well-grown culture from YMG agar plate was cut into small pieces using a cork borer (7 mm), 5 pieces were inoculated into a 500 mL Erlenmeyer flask containing 200 mL media, and homogenized. The cultures were incubated at 24 °C on a rotary shaker (140 rpm). The growth of the fungus was monitored by checking the amount of free glucose with Medi-test Glucose (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The fermentation was terminated seven days after glucose depletion and analyzed via HPLC-DAD/MS and HR-LC-(+)ESIMS.

Scale-up of cultivation. The masses of the HR-LC-(+)ESIMS detected peaks and their molecular formulae obtained from HR-(+)ESIMS data were compared. HR-LC-(+)ESIMS results indicated that similar compounds were produced in all media but Q6½ medium produced the highest amounts of interesting secondary metabolites, it was used in the scale up process. All shake flasks cultivations were conducted in 500 ml Erlenmeyer flasks (200 ml working volume). A 200 ml liquid culture of the strain CBS 387.89 in Q6½ was used to inoculate a four L large scale cultivation in 20 Erlenmeyer flasks with Q6½ media for 17 days at 140 rpm and 23 °C. To obtain more material, a second 4 L large scale cultivation of the strain CBS 387.89 was performed in Q6½ medium as described before.

Preparation of the Extracts. Supernatant and biomass from small-scale fermentation were separated by filtration. The supernatant was extracted with equal amount of ethyl acetate and

filtered through anhydrous sodium sulphate. The resulting ethyl acetate extract was evaporated to dryness by rotary evaporator. The mycelia were extracted with 200 mL of acetone in ultrasonic bath for 30 min, filtered and the filtrate evaporated. The remaining water phase was suspended in equal amount of distilled water, extracted with equal amount of ethyl acetate and dried.

The mycelia and supernatant from large scale fermentation were separated via vacuum filtration. The mycelia were extracted with 2 × 500 mL acetone using an ultrasonic bath for 30 min and the solvent was evaporated. The remaining was solved in 100 mL distilled water, and extracted with 2 × 100 mL ethyl acetate. The resulting ethyl acetate layer was dried through anhydrous sodium sulfate and evaporated to complete dryness, leaving a brown solid (first large scale cultivation: 330 mg; second large scale cultivation: 90 mg). The supernatant was extracted by adding 2% (m/v) Amberlite XAD™-16N absorbent (Rohm & Haas Deutschland GmbH, Frankfurt am Main, Germany) and stirred for 2 h. The Amberlite resin was then harvested and eluted with 2 x 200 mL acetone. The resulting acetone extract was evaporated and the remaining water phase (100 mL) was extracted 2 times with equal amount of ethyl acetate. The organic phase was dried through anhydrous sodium sulfate and evaporated to complete dryness to yield a brown crude extract (first large scale cultivation: 380 mg, second large scale cultivation: 390 mg).

Isolation of Compounds 1 – 7. First large scale cultivation: The lipophilic compounds of the mycelium extract from large-scale liquid cultivation of CBS 387.89 were removed by MeOH/*n*-heptane (1:1) partition. The methanol layer of the partition was concentrated in *vacuo* at 40 °C to dryness (197 mg) and combined with the supernatant extract. Afterwards, the extract (577 mg) was prepurificated via normal phase flash chromatography (FC, GRACE Reveleris® X2 flash system; Büchi Labortechnik GmbH, Essen, Germany), using a Reveleris® 40 g silica cartridge (particle size: 40 µm). The column was eluted with a ternary gradient mobile phase

generated from three solvent mixtures [solvent A: dichlormethane (DCM); solvent B: DCM/acetone (8:2, v/v); solvent C: solvent B/methanol (7:3, v/v)] at a flow rate of 40 mL/min. The first gradient (AB system) was operating from 0% B to 20% B in 12.4 min, followed by a second gradient (BC system) from 20% C to 60% C in 25 min and from 60% C to 100% C in 13 min. UV detection was conducted at 220 nm, 254 nm, and 310 nm. Fractions were combined according to the major UV-absorbing peaks to yield 11 main fractions (I – XI).

Fractions with targeting molecules were further fractionated via RPLC utilizing a Gemini C_{18} column [250x50 mm, 10 µm 110 Å; Phenomenex, Aschaffenburg, Germany; solvent A: deionized water (Purelab®Flex, Veolia Water Technologies, Celle, Germany) + 0.1% formic acid [FA]; solvent B: acetonitrile + 0.1% FA, flow rate: 40 mL/min] at a Gilson PLC 2050 Purification System (Limburg, Germany) using individually established gradients for each fraction. UV detection was conducted at 210 nm, 250 nm, 280 nm, and 310 nm. The FC prefractions II – IV (t_R = 3 – 16 min) were combined (89 mg) and separated using a gradient operating with 5 min 22% B isocratic, then increasing to 30% B within 10 min, followed by increasing to 50% B within 20 min, and thereafter increasing to 100% B within 45 min, yielding to 3.4 mg of compound **4** (t_R = 53 – 54 min). Furthermore, prefraction V (t_R = 16 min, 154 mg), that was obtained via FC, was separated using a gradient of 6 min 20% B, then increasing to 45% B in 19 min, followed by 56 min isocratic conditions at 47% B, then increasing to 91% B over a period of 17 min, yielding to 3.5 mg of compound **5** (t_R = 64 min), 3.1 mg of compound **1** (t_R = 73 min), 10.8 mg of compound **7** (t_R = 75 min), and 6.7 mg of compound **3** (t_R = 77 min).
Second large scale cultivation: After discarding the mycelium extract due to lack of targeting molecules, the supernatant extract (390 mg) was prefractionated via normal phase FC as described previously. Fractions were combined according to the major UV-absorbing peaks to yield 7 main fractions (I – VII). Prefraction V (t_R = 13 min, 119 mg), obtained from FC, was purified via RPLC as described before, utilizing a Nucleodur 100-5 C_{18}ec column (250x21mm, 5 µm, 110 Å; Macherey-Nagel GmbH & Co. KG, Düren, Germany). The process was

performed with a flow rate of 20 mL/min using a gradient of 5 min isocratic conditions at 10% B, then increasing to 60% B in 40 min. The purification yielded 3.1 mg of compound **5** (t_R = 36 min) and 2.9 mg of compound **6** (t_R = 40 min). Prefractions VI and VII were combined (t_R = 14 – 16 min, 25 mg) and purified with a gradient of 5 min isocratic conditions at 10% B, then increasing to 50% B in 40 min, yielding 1.5 mg of compound **2** in combination with other impurities. To purify compound **2**, a TLC was performed using DCM/acetone + 0.1% FA (1:1) as mobile phase, yielding 0.9 mg of compound **2**.

Calculation of ECD Spectra. Geometry optimizations and calculations of electronic circular dichroism (ECD) spectra were performed using ORCA 5.0.0.[25,26] The structures were optimized at the B3LYP/cc-pVDZ[27,28] level of theory using the C-PCM[29,30] solvation model (MeOH, ε = 32.63). Conformational searches were performed for nebucanes A (**1**), E (**5**), and F (**6**) using Gabedit 2.5.0[31] with the Amber[32] model as implemented therein. For each possible configuration, 20 conformers were generated and then deduplicated. Each conformer was optimized and the vibrational frequencies were calculated. ECD spectra were calculated using TD-DFT as implemented in ORCA using the same level of theory and solvent model. ECD signals were converted into continuous spectra using SpecDis 1.71 [σ = 0.30 eV, UV shift is 10 nm for nebucane A (**1**), -5 nm for nebucane E (**5**), 0 nm for nebucane F (**6**)].[33,34] The spectra were weighted using a Boltzmann distribution based on each conformer's Gibbs free energy at 298.15 K as calculated by ORCA.

Verification of the producer strain. The identity of STMA 21071 was verified by comparison of its secondary metabolites with those of strain CBS 378.89 based on small-scale cultivation in liquid medium. A well-grown culture of *C. nebularis* (STMA 21071) on YMG agar plate was cut into small pieces using a cork borer (7 mm), 5 pieces were inoculated in a 500 mL Erlenmeyer flask containing 200 mL of Q6½ medium, and homogenized. The cultures were incubated at 24 °C on a rotary shaker (140 rpm). After day 17 and day 21, 5 mL of fungal

culture suspension was removed from the flask, extracted for 10 min with an equal amount of etyl acetate (5 mL) and centrifuged for 10 min (5000 rpm). The ethyl acetate layer was separated from the acqueous phase and dried under a nitrogen stream. The resulting residue was dissolved in acetone-acetonitrile (1:1, v/v, 100 µL), subjected to HR-LC-(+)ESIMS analysis (injection volume: 10 µL), and the chromatogram analyzed for the presence of nebucanes A – G (**1** – **7**).

Antimicrobial Assay. To evaluate the minimum inhibitory concentrations (MIC), compounds **1,** and **4** – **7** were tested against several bacterial and fungal strains using a 96-well serial dilution technique (Mueller-Hinton broth [MHB] media for bacteria and YMG media for filamentous fungi and yeasts) as previously described.[35] The selected organisms represent a broad spectrum of pathogens of clinical interest, as well as sensitive indicator strains, to discover new anti-infectives (bacteria: *Bacillus subtilis* [DSM 10], *Chromobacterium violaceum* [DSM 30191], *Escherichia coli* [DSM 1116], *Acinetobacter baumannii* [DSM 30008], *Mycolicibacterium smegmatis* [ATCC 700084], *Pseudomonas aeruginosa* [PA 14], *Staphylococcus aureus* [DSM 346]; fungi: *Mucor hiemalis* [DSM 2656], *Pichia anomala* [DSM 6766], *Rhodotorula glutinis* ([DSM 10134], *Candida albicans* [DSM 1665], and *Schizosaccharomyces pombe* [DSM 70572]). Cell density was adjusted to 6.7×10^5 cells/mL. The compounds were re-dissolved in MeOH (1 mg/mL), diluted to a final concentration range of 66.7 to 0.52 µg/mL, and incubated with the test organisms overnight (*B. subtilis*, *C. violaceum*, *E. coli*, *A. baumannii*, *M. smegmatis*, *P. aeruginosa*, *S. aureus*, *M. hiemalis*, *P. anomala*, *C. albicans*) or for 48 h (*S. pombe*, *R. glutinis*). 20 µL MeOH was used as negative control. Kanamycin (1.0 mg/mL; 2 µL [*M. smegmatis*]), gentamicin (1.0 mg/mL; 2 µL [*P. aeruginosa*]), ciprobay (2.54 mg/mL; 0.26 µL [*A. baumannii*]), nystatin (1.0 mg/mL; 20 µL [*S. pombe*, *P. anomala*, *M. hiemalis*, *C. albicans*, *R. glutinis*]), and oxytetracycline (1.0 mg/mL; 2 µL [*C. violaceum*, *B. subtilis*, *E. coli*, *S. aureus*] and 20 µL [*B. subtilis*]) were used as positive controls against tested organisms. Nebucanes **2** and **3** were not tested because of quantity available or lack of stability, respectively.

Cytotoxicity Assay. The evaluation of in vitro cytotoxicity (IC_{50}) of the isolated compounds was performed with mouse connective tissue fibroblast cell line [L929 (ACC 2)] and mammalian cervix carcinoma cells [KB3.1 (ACC 158)]. Compounds **1**, and **4** – **7** were tested as previously described.[35] Due to significant inhibition of viability of L929 and KB3.1 cells by compound **7**, further cell lines were evaluated: human ovary adenocarcinoma [SKOV-3 (ATCC HTB 77)], human prostate carcinoma [PC-3 (ACC 456)], human lung carcinoma [A549 (ACC 107)], human epidermoid carcinoma [A431 (ACC 91)], and human breast adenocarcinoma [MCF-7 (ACC 115)]. 6×10^3 cells/well were added to 96-well microtiter plates. The compounds were dissolved in MeOH (1 mg/mL) which was also used as negative control in this study; epothilone B (1 mg/mL) was used as positive control. The cell lines were incubated with a serial dilution of the test compounds (final range from 37 to 0.6 x 10^{-3} µg/mL) for five days. Afterwards, they were dyed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), which is only converted to its purple formazan derivative by living cells. To calculate the percentage of cell viability, absorption at 595 nm was measured using a microplate reader. Results were expressed as IC_{50}, the half maximal inhibitory concentration (µM). Nebucanes **2** and **3** and were not tested because of quantity available or lack of stability, respectively.

Nebucane A (1): white solid; $[\alpha]^{20}{}_D$ +11.1 (*c* 0.28, MeOH); UV/Vis (MeOH) λ_{max} (log ε) 229 (1.00), 304 (1.06); CD (MeOH): λ ($\Delta\varepsilon$) 235 (3.88), 298 (–3.25), 334 (3.83); HR-LC-(+)ESIMS: t_R = 7.8 min; HR-(+)ESIMS: *m/z* 513.2725 (20) $[2M+Na]^+$ (calcd for $C_{30}H_{38}N_2NaO_4$, 513.2724), 268.1307 (30) $[M+Na]^+$ (calcd for $C_{15}H_{19}NNaO_2$, 268.1308), 246.1487 (100) $[M+H]^+$ (calcd for $C_{15}H_{20}NO_2$, 246.1489), 228.1380 (12) $[M+H–H_2O]^+$.(calcd $C_{15}H_{18}NO$, 228.1383); ^{1}H NMR data see Table 1; ^{13}C NMR data see Table 2.

Nebucane B (2): white solid; $[\alpha]^{20}{}_D$ +75.3 (*c* 0.07, MeOH); UV/Vis (MeOH): λ_{max} (log ε) 231 (1.33), 304 (1.37); CD (MeOH): λ ($\Delta\varepsilon$) 238 (4.26), 297 (–3.13), 334 (6.03); HR-LC-

(+)ESIMS: t_R = 4.9 min; HR-(+)ESIMS: *m/z* 284.1257 (20) $[M+Na]^+$ (calcd for $C_{15}H_{19}NNaO_3$, 284.1257), 262.1442 (100) $[M+H]^+$ (calcd for $C_{15}H_{20}NO_3$, 262.1438); ^{1}H NMR data see Table 1; ^{13}C NMR data see Table 2.

Nebucane C (3): white solid; $[\alpha]^{20}{}_D$ –62.5 (*c* 0.2, MeOH); UV/Vis (MeOH): λ_{max} (log ε) 214 (1.76), 237 (1.62), 292 (1.07); CD (MeOH): λ ($\Delta\varepsilon$) 225 (–5.60), 291 (3.99), 329 (–2.69); HR-LC-(+)ESIMS: t_R = 8.4 min; HR-(+)ESIMS: *m/z* 513.2720 (25) $[2M+Na]^+$ (calcd for $C_{30}H_{38}N_2NaO_4$, 513.2724), 268.1304 (95) $[M+Na]^+$ (calcd for $C_{15}H_{19}NNaO_2$, 268.1308), 246.1492 (100) $[M+H]^+$ (calcd for $C_{15}H_{20}NO_2$, 246.1489), 228.1381 (99) $[M+H–H_2O]^+$.(calcd $C_{15}H_{18}NO$, 228.1383); ^{1}H NMR data see Table 1; ^{13}C NMR data see Table 2.

Nebucane D (4): white solid; UV/Vis (MeOH): λ_{max} (log ε) 213 (2.36), 252 (1.46), 305 (0.65); HR-LC-(+)ESIMS: t_R = 11.9 min; HR-(+)ESIMS: *m/z* 477.2515 (10) $[2M+Na]^+$ (calcd for $C_{30}H_{34}N_2NaO_2$, 477.2512), 250.1201 (20) $[M+Na]^+$ (calcd for $C_{15}H_{17}NNaO$, 250.1202), 228.1380 (100) $[M+H]^+$ (calcd for $C_{15}H_{18}NO$, 228.1382), 200.1426 (20) $[M+H–CO]^+$ (calcd $C_{14}H_{18}N$, 200.1434); ^{1}H NMR data see Table 1; ^{13}C NMR data see Table 2.

Nebucane E (5): white solid; $[\alpha]^{20}{}_D$ +24.0 (*c* 0.1 MeOH); UV/Vis (MeOH) λ_{max} (log ε) 225 (0.75), 272 (1.24); CD (MeOH): λ ($\Delta\varepsilon$) 269 (–0.28), 307 (0.89); HR-LC-(+)ESIMS: t_R = 6.7 min; HR-(+)ESIMS *m/z* 551.2624 (70) $[2M+Na]^+$ (calcd for $C_{30}H_{40}NaO_8$, 551.2615), 287.1257 (70) $[M+Na]^+$ (calcd for $C_{15}H_{20}NaO_4$, 287.1254), 265.1443 (100) $[M+H]^+$ (calcd for $C_{15}H_{21}O_4$, 265.1434), 247.1330 (30) $[M+H–H_2O]^+$.(calcd $C_{15}H_{19}O_3$, 247.1329), 229.1223 (40) $[M+H–2H_2O]^+$ (calcd $C_{15}H_{17}O_2$, 229.1223); ^{1}H NMR (500 MHz, acetone-d_6, 298 K) data see Table 2; ^{1}H NMR (500 MHz, DMSO-d_6, 298 K): δ_H [ppm, Hz] = 7.80 (1H, dd, ^4J = 1.1; 1.1, H-12), 5.43 (OH, s, H-17), 5.24 (OH, t br, ^2J = 4.3, H-16), 4.49 (2H, d, ^2J = 4.3, H-13), 3.07 (1H, qd, ^3J = 7.0; 4.7, H-1), 2.60 (1H, ddd, ^3J = 12.7; 8.1; 4.7, H-9), 2.19 (1H, d, ^2J = 13.4, H-6), 1.90 (1H, d, ^2J = 13.4, H-6), 1.70 (1H, dd, ^2J = 12.7, ^3J = 8.1, H-8β), 1.08 (3H, s, H-14), 1.04 (3H, d, ^3J = 7.0, H-10), 0.99 (1H, dd br, ^2J = 12.7; ^3J = 12.7, H-8α), 0.72 (3H, s, H-15); ^{13}C NMR (500

MHz, acetone-d_6, 298 K) data see Table 2, ^{13}C NMR (500 MHz, DMSO-d_6, 298 K): δ_C [ppm] = 187.6 (C-2), 145.3 (C-12), 145.1 (C-3), 138.2 (C-4), 126.9 (C-11), 75.4 (C-5), 53.8 (C-13), 53.7 (C-6), 53.2 (C-9), 41.6 (C-8), 39.9 (C-1), 33.7 (C-7), 31.6 (C-14), 30.3 (C-15) [chemical shift values were assigned using HSQC/HMBC].

Nebucane F (6): white solid; $[\alpha]^{20}{}_D$ +16.0 (*c* 0.1, MeOH); UV/Vis (MeOH) λ_{max} (log ε) 246 (1.19); CD (MeOH): λ ($\Delta\varepsilon$) 269 (–0.28), 319 (–0.44); HR-LC-(+)ESIMS: t_R = 8.6 min; HR-(+)ESIMS *m/z* 519.2715 (95) $[2M+Na]^+$ (calcd for $C_{30}H_{40}NaO_6$, 519.2717), 271.1306 (100) $[M+Na]^+$ (calcd for $C_{15}H_{20}NaO_3$, 271.1305), 249.1488 (100) $[M+H]^+$ (calcd for $C_{15}H_{21}O_3$, 249.1485), 231.1398 (100) $[M+H–H_2O]^+$.(calcd $C_{15}H_{19}O_2$, 231.1380); ^{1}H NMR data see Table 1; ^{13}C NMR data see Table 2.

Nebucane G (7): white solid; $[\alpha]^{20}{}_D$ +21.0 (*c* 0.2, MeOH); UV/Vis (MeOH) λ_{max} (log ε) 297 (–0.18); CD (MeOH): λ ($\Delta\varepsilon$) 220 (3.63), 294 (–1.16); HR-LC-(+)ESIMS: t_R = 7.7 min; HR-(+)ESIMS *m/z* 583.2519 (80) $[2M+Na]^+$ (calcd for $C_{30}H_{40}NaO_{10}$, 583.2514), 561.2700 (10) $[2M+H]^+$ (calcd for $C_{30}H_{41}O_{10}$, 561.2694), 303.1205 (100) $[M+Na]^+$ (calcd for $C_{15}H_{20}NaO_5$, 303.1203), 281.1385 (60) $[M+H]^+$ (calcd for $C_{15}H_{21}O_5$, 281.1384), 263.1279 (50) $[M+H–H_2O]^+$ (calcd $C_{15}H_{19}O_4$, 263.1278), 245.1172 (40) $[M+H–2H_2O]^+$ (calcd $C_{15}H_{17}O_3$, 2455.1172); ^{1}H NMR data (500 MHz, acetone-d_6, 273 K) see Table 2; ^{1}H NMR (700 MHz, DMSO-d_6) δ_H [ppm, Hz] = 6.21 (1H, br s, H-13'), 5.68 (1H, br s, H-13''), 5.07 (OH, br s,), 4.48 (1H, br s, H-2), 2.91 (1H, br s, H-9), 2.19 (1H, br s, H-1), 1.66 (1 H, d, ^3J = 14.6, H-6α), 1.58 (1H, dd, J = 9.7; 8.0, H-8β), 1.47 (1H, dd, J = 14.2; 12.3, H-8α), 1.18 – 1.23 (1H, m, H-6β), 1.12 (3H, s, H-14), 1.04 (3H, s, H-15), 0.87 (3H, d, ^3J = 6.9, H-10), ^{13}C NMR data (126 MHz, acetone-d_6, 298 K) see Table 2; ^{13}C NMR (700 MHz, DMSO-d_6, 298 K): δ_C [ppm] = 205.4 (C-3), 165.6 (C-12), 135.7 (C-11), 79.5 (C-5), 73.2 (C-2), 64.5 (C-4), 44.1 (C-6), 40.7 (C-8), 42.3 (C-9), 39.3 (C-1), 37.8 (C-7), 28.7 (C-15), 26.5 (C-14), 8.2 (C-10) [chemical shift values were assigned using HSQC/HMBC].

ASSOCIATED CONTENT

Supporting Information.

The supporting Information is available free of charge on the ACS Publication website at DOI: xxxx

UV spectra, HR-LC/DAD, HR-(+)ESIMS data, HR-(+)ESIMS/MS data, 1D and 2D NMR data, ECD spectra (experimental/calculated), 5.8S/ITS DNA sequence of the fungal organism.

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Notes

The authors declare no competing financial interests.

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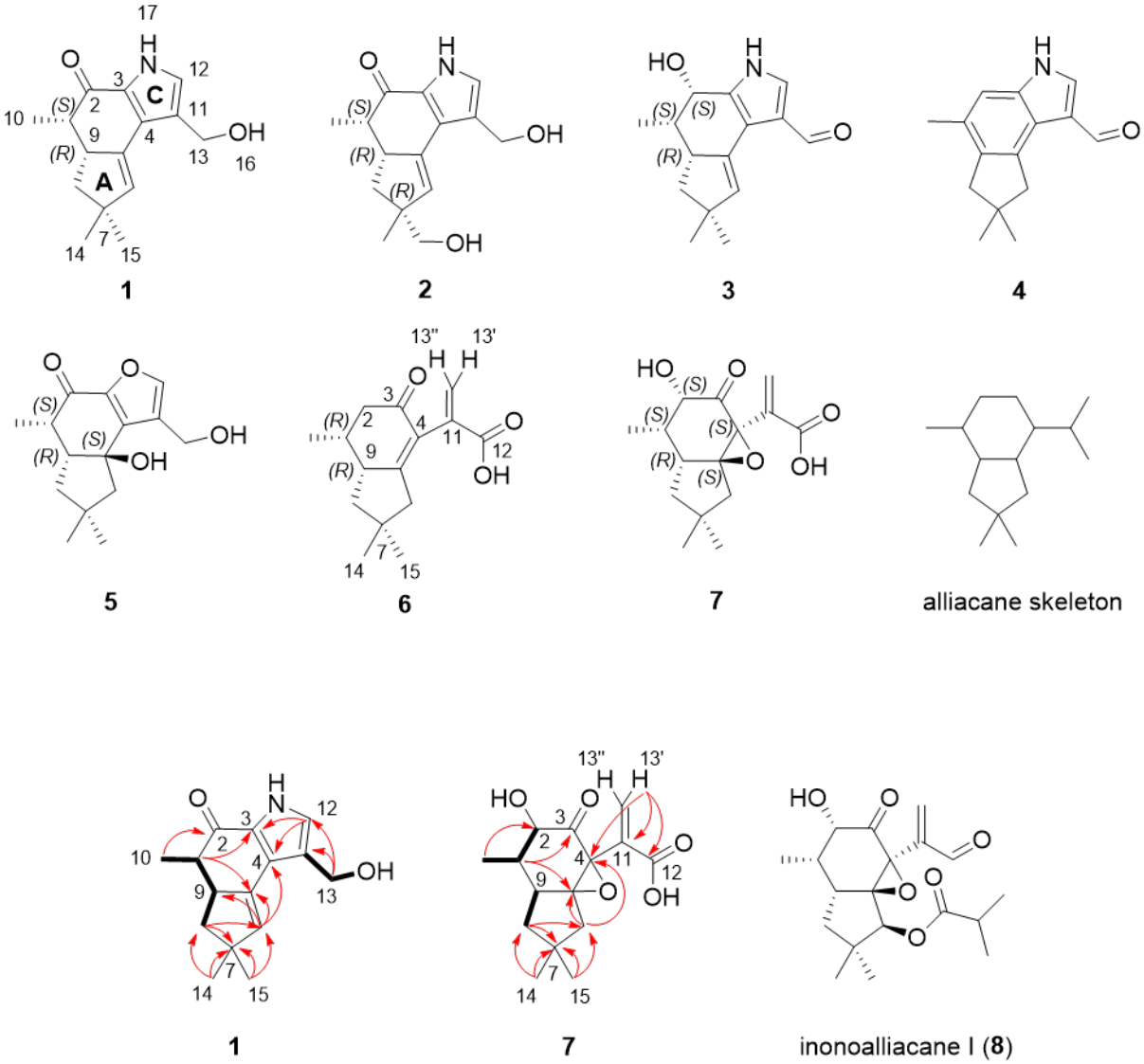

Figure 1. Key (—) ^{1}H,^{1}H COSY and ( ) HMBC correlations of nebucanes A (**1**) and G (**7**) and the chemical structure of the known inonoalliacane I (**8**).

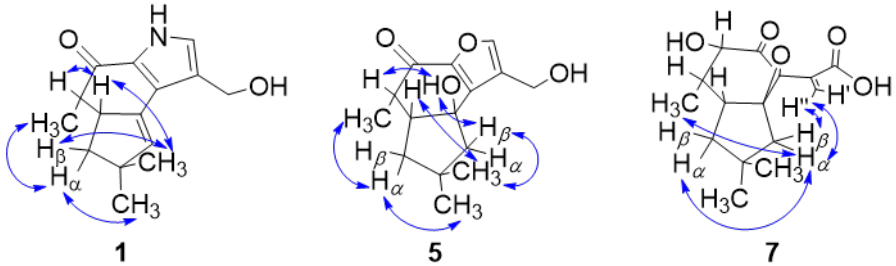

Figure 2. Key ( ) NOESY/ROESY correlations of nebucanes A (**1**), E (**5**), and G (**7**).

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Compounds 1 – 4 in acetone-d_6, J in Hz

	1[a]		**2**[bc]		**3**[a]		**4**[a]	
no.	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	45.1, CH	2.46 qd (7.2, 6.0)	45.3, CH	2.49 qd (7.3, 5.2)	37.8, CH	2.13–2.20 m	130.3, C	-
2	192.1, C	-	192.5, C	-	69.0, CH	5.06 d (5.3)	110.3, CH	7.11 dd (1.7, 0.8)
3	127.4, C	-	127.4, C	-	135.1, C	-	138.1, C	-
4	126.6, C	-	126.7, C	-	113.7, C	-	120.1, C	-
5	131.3, C	-	132.9, C	-	131.8, C	-	135.3, C	-
6	135.8, CH	5.90 d (2.1)	132.4, CH	5.95 d (2.2)	134.2, CH	6.34 d (2.2)	49.8, CH_2	3.30 d (0.5)
7	46.3, C	-	53.1, C	-	45.1, C	-	39.3, C	-
8α	40.1, CH_2	1.68 dd (12.4, 9.8)	34.9, CH_2	1.91 dd (12.5, 9.6)	41.6, CH_2	1.60 dd (10.0, 12.3)	46.8, CH_2	2.72 s
8β	-	1.75 dd (12.4, 8.0)	-	1.59 dd (12.5, 8.2)	-	1.69 dd (7.9, 12.3)	-	-
9	48.7, CH	3.56 dddd (9.8, 8.0, 6.0, 2.1)	48.3, CH	3.54–3.59 m*	48.1, CH	3.20 ddddd (10.0, 7.9, 3.5, 2.2, 0.5)	136.6, C	-
10	12.9, CH_3	0.98 d (7.2)	12.8, CH_3	0.99 d (7.3)	6.2, CH_3	0.72 d (6.7)	19.3, CH_3	2.29 s
11	122.9, C	-	123.3, C	-	124.1, C	-	120.2, C	-
12	125.0, CH	7.11 d (2.9)	125.0, CH	7.12 d (2.8)	131.3, CH	7.54 s	137.0, CH	8.02 d (3.2)
13'	56.9, CH_2	4.63 d (12.4)	56.8, CH_2	4.64 dd (13.1, 3.2)	184.5, CH	9.79 s	183.7, CH	9.90 s
13"		4.57 d (12.4)		4.59 dd (13.1, 5.0)				
14	28.0, CH_3	1.12 (s)	23.1, CH_3	1.12 s	27.7, CH_3	1.07 s	29.2, CH_3	1.20 s
15'	29.2,[c] CH_3	1.23 (s)	70.9, CH_3	3.54–3.59 m*	29.8,[c] CH_3	1.17 s	29.2, CH_3	1.20 s
15"				3.48 dd (10.3, 6.0)				
16-OH	-	3.88 s (br)	-	3.85 t br (5.0)	-	-	-	-
17-NH	-	10.61 s br	-	10.64 s br	-	10.71 s br	-	10.98 s br
18-OH	-	-	-	3.71 t br (6.0)	-	-	-	-

[a] Measured in 500 [126] MHz at 298 K; [b] measured in 700 MHz at 298 K; [c] chemical shift values assigned using HSQC/HMBC; * overlapping multiplet.

Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data of Compounds 5 – 7 in acetone-d_6, *J* in Hz

	5[a]		**6**[ac]		**7**[ab]	
no.	δ_C	δ_H	∂_C	δ_H	δ_C	δ_H
1	41.3, CH	3.06 qd (7.0, 4.8)	31.8, CH	2.40–2.43 m*	41.2, CH	2.33 qdd (7.2, 5.3, 4.5)
2*α*	187.9, C	-	46.5, CH_2	2.27 dd (16.3, 2.4)	75.4, CH	4.57 d (4.5)
2*β*	-	-		2.59 dd (16.3, 4.9)	-	-
3	146.6, C	-	195.8, C	-	207.3, C	-
4	138.7, C	-	131.7, C	-	66.2, C	-
5	76.5, C	-	167.0, C	-	81.9, C	-
6*α*	55.1,[c] CH_2	2.34 d (13.7)	47.3, CH_2	2.23 dd (19.1, 2.8)	46.1, CH_2	1.77 d (14.5)
6*β*		2.05–2.08 m[c]		2.40–2.45 m*		1.28 dd (14.5, 2.1)
7	35.0, C	-	38.2, C	-	39.7, C	-
8*α*	42.4, CH_2	1.14 dd, (≈ 13.0, 13.0)[c]	43.0, CH_2	1.53 dd (12.3, 12.1)	42.8, CH_2	1.58 dd (12.6, 12.6)
8*β*		1.80 dd (13.0, 8.0)		1.67 ddd (12.1, 8.0, 2.0)		1.62 – 1.67 m
9	53.7, CH	2.77 ddd (13.0, 8.0, 4.8)	46.0, CH	3.36 ddddd (12.3, 8.0, 4.7, 3.1, 1.1)	44.5, CH	2.99 ddd (12.6, 7.4, 5.3)
10	12.1, CH_3	1.12 d (7.0)	13.1, CH_3	0.95 d (7.2)	9.3, CH_3	0.93 d (7.2)
11	126.6, C	-	137.0, C	-	137.0, C	-
12	145.3, CH	7.72 s	167.3, C	-	166.8, C	-
13'	55.1,[c] CH_2	4.71 d (14.0)	128.3, CH_2	6.34 d (1.8)	128.6, CH_2	6.35 d (1.2)
13"		4.68 d (14.0)		5.57 d (1.8)		5.82 d (1.2)
14	31.6, CH_3	1.14 s	28.3, CH_3	1.09 s	27.8, CH_3	1.16 s
15	30.9, CH_3	0.80 s	29.5, CH_3	1.13 s	29.9,[c] CH_3	1.08 s

[a] Measured in 500 [126] MHz, 298 K; [b] measured in 500 [126] MHz, 273 K; [c] chemical shift values/coupling constant assigned using HSQC/HMBC; * overlapping multiplet.

Table 3. Antimicrobial and Cytotoxic Activities of Compounds 1 and 4 – 7

	MIC (µg/mL)					
Test organisms	**1**	**4**	**5**	**6**	**7**	**positive control**
Bacillus subtilis DSM10	-	66.6	-	-	-	9.2[a]
Candida albicans DSM1665	-	66.6	-	-	-	9.2[b]
Mucor hiemalis DSM2656	-	16.6	-	-	-	9.2[b]
Pichia anomala DSM6766	-	33.3	-	-	-	4.6[b]
Rhodotorula glutinis DSM 10134	66.6	4.2	-	-	-	1.0[b]
Schizosaccharomyces pombe DSM 70572	-	33.3	-	-	-	4.2[b]
	Cytotoxicity (IC_{50} µM)					
Cell lines						**positive control[c]**
L929 (ACC 2)	68	41	-	-	22	1.0×10^{-3}
KB3.1 (ACC 158)	47	21	-	-	18	2.8×10^{-5}
MCF-7 (ACC 115)	nt	nt	nt	nt	8	2.7×10^{-5}
SKOV-3 (ATCC HTB 77)	nt	nt	nt	nt	20	7.5×10^{-5}
PC-3 (ACC 465)	nt	nt	nt	nt	36	3.0×10^{-5}
A431 (ACC 91)	nt	nt	nt	nt	7	3.1×10^{-5}
A549 (ACC 107)	nt	nt	nt	nt	53	3.4×10^{-5}

[a] oxytetracyclin; [b] nystatin; [c] epothilone B; -: no activity; nt: not tested.

Graphical abstract