An unprecedented spiro [furan-2,1'-indene]-3-one derivative and other nematicidal and antimicrobial metabolites from *Sanghuangporus* sp. (Hymenochaetaceae, Basidiomycota) collected in Kenya

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Abstract

Bioassay guided fractionation of extracts derived from submerged cultures of a *Sanghuangporus* sp. (i.e., the genus that was until recently referred to as the "*Inonotus linteus* complex" of medicinal mushrooms) originating from Kenya led to the isolation of a new spiro [furan-2,1'-indine]-3-one derivative, for which we propose the trivial name phelligridin L (**1**) together with the known compounds 3,14'-bihispidinyl (**2**), hispidin (**3**), ionylideneacetic acid (**4**), 1*S*-(2*E*)-5-[(1*R*)-2,2-dimethyl-6-methylidenecyclohexyl]-3-methylpent-2-enoic acid (**5**), phellidine E (**6**) and phellidine D (**7**). Compounds **1** -**3**, showed moderate nematicidal activity against *Caenorhabditis elegans* with LD_{50} of 12.5 µg/m. The nematicidal activity of 3, 14'-bihispidinyl and hispidin (**1**, **2**) has not been reported before. Furthermore, compounds **1**-**5** demonstrated moderate antimicrobial activity against various test organisms.

Key words: Hymenochaetaceae, secondary metabolites; Basidiomycota ; medicinal mushroom; structure elucidation

1.1 Introduction

Fungi have been recognized as source of structurally unique and bioactive metabolites (Karwehl and Stadler, 2016). The fungal diversity in the tropical rainforest ecosystems has been underexplored, since the majority of the fungal species found in these habitats have not been described nor has their chemistry been studied. In an effort to document this diversity and its rich chemistry, we embarked on extensive study of the secondary metabolites production of several tropical basidiomycetes collected from Kenya's tropical rain forest Kakamega. Novel structurally diverse and bioactive metabolites like laetiporins, calocerins, 9-oxostrobilurins, laxitextines, microporenic acids and aethiopinolones are some of the metabolites that we have reported recently (Chepkirui et al., 2018a, 2018b, 2017, 2016; Mudalungu et al., 2015) from our ongoing study.

The present paper deals with a species belonging to the genus *Sanghuangporus* (Hymenochaetaceae), whose extracts from mycelial cultures had shown prominent antimicrobial effects during the course of the aforementioned study. The specimen was collected from the Kakamega Nature Reserve, a spot of rain forest at medium elevation in Kenya. The genus *Sanghuangporus* was erected for the "*Inonotus linteus* complex", and several additional species from the Paleotropics previously included in *Phellinus* (Zhou et al., 2015). It originally included *S. alpinus, S. baumii, S. lonicericola, S. lonicerinus*, *S. microcystideus*, *S. sanghuang, S. vaninii*, *S. weigelae, S. weirianus* and *S. zonatus* (Zhou et al., 2015). Three more species have been added later, *viz. S. ligneus, S. pilatii* and *S. quercicola* (Ghobad-Nejhad, 2015; Tomsovsky, 2017; Zhu et al. 2015). The Asian species have been referred to in the literature and some of them are regarded as medicinal mushrooms. Only one species in the genus, *S. microcystideus*, has been reported from Eastern Africa (Zhou et al., 2015). In this study we report the isolation, structure elucidation and biological activities of secondary metabolites from another African *Sanghuangporus* sp., which probably represents an undescribed species.

2.1 Experimental section

2.1.1 General Experimental Procedure: Optical rotations were determined with a Perkin-Elmer (Überlingen, Germany) 241 spectrometer; UV spectra were recorded with a Shimadzu (Duisburg, Germany) UV-vis spectrophotometer UV-2450. NMR spectra were recorded with a Bruker (Bremen, Germany) Ascend 700 spectrometer with 5mm TXI cryoprobe (^{1}H 700MHz, ^{13}C 175 MHz) and Bruker AV II-600 (^{1}H 500 MHz, ^{13}C 150 MHz) spectrometers. HR-ESI-MS mass spectra were recorded with Bruker (Bremen, Germany) Agilent 1200 series

HPLC-UV system (column 2.1 x 50 mm, 1.7 µm, C18 Acquity UPLC BEH (waters), solvent A: H2O + 0.1% formic acid; solvent B: AcCN + 0.1% formic acid, gradient: 5% B for 0.5 minutes increasing to 100% B in 19.5 minutes and then maintaining 100% B for 5 minutes, flow rate 0.6 ml/min-1, uv/vis detection 200-600 nm combined with ESI-TOF-MS (Maxis, Bruker) [scan range 100-2500 m/z, capillary voltage 4500V, dry temperature 200 ^{0}C]. Chemicals and solvents were obtained from AppliChem GmbH, Avantor Performance Materials, Carl Roth GmbH & Co. KG and Merck KGaA in analytical and HPLC grade.

2.1.2 Fungal material: The specimen MUCL 55592 was collected from Kakamega equatorial rainforest, located in the western part of Kenya (0°17′3.19" N 34°45′8.24" E) by C. Decock on Feb. 17, 2015. The dried herbarium specimen and culture are deposited at MUCL, Louvain-la-Neuve, Belgium (designation no. MUCL 55592). The fungus was identified as a species of the genus *Sanghuangporus* by sequencing of the rDNA (5.8S gene region, the internal transcribed spacer (ITS) and part of the nuclear ribosomal large subunit (nLSU).

DNA extraction was performed as reported previously (Wendt et al., 2018) with the EZ-10 Spin Column Genomic DNA Miniprep kit (Bio Basic Canada Inc., Markham, Ontario, Canada). A Precellys 24 homogenizer (Bertin Technologies, France) was used for cell disruption at a speed of 6000 rpm for 2 × 40 s. The gene regions were amplified with primers ITS 1f and NL4 for sequencing of the rDNA (5.8S gene region, the internal transcribed spacer ITS1 and ITS2). Genomic DNA Miniprep kit (Bio Basic Canada Inc., Markham, Ontario, Canada). The gene regions were amplified with primers ITS 1f and ITS4 for ITS and LROR and LR7 for nLSU.

2.1.3 Fermentation: *Sanghuangporus sp.* was cultivated in 500 mL Erlenmeyer flask containing 200 mL of the three different liquid media YMG, Q6 ½ and ZM ½ (for details on the composition of these media see Supplementary information) . These three media were selected because previous studies had revealed that they were optimal for attaining complementary secondary metabolites profiles in filamentous fungi (Bitzer et al., 2008). A well grown culture grown on an YMG agar plate was cut into small pieces using a cork borer (7 mm) and five pieces inoculated in each flask. The cultures were incubated at 23 °C on a rotary shaker (140 rpm). The growth of the fungus was monitored by constantly checking the amount of free glucose (using Bayer Diastix Harnzuckerstreifen). The fermentation was terminated five days after glucose depletion.

2.1.4 Extraction of crude extracts from small scale fermentation: The supernatant and the mycelia from the 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 means of 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 and subjected to same procedure as the supernatant. The mycelia and supernatant crude extracts from the three media HRMS were measured. Analysis of the MS spectra by comparing the masses of the detected peaks and their molecular formula obtained from HRMS with those in the data base (Dictionary of natural products) led to the identification of the new compound on the ZM ½ supernatant crude extract (Dictionary of Natural Products on DVD, 2017).

2.1.5 Scale-up fermentation: A well-grown seven days old YMG agar plate of the mycelial culture was cut into small pieces using a 7 mm cork borer and five pieces inoculated in 500 mL Erlenmeyer flask containing 200 mL (30 flasks) of ZM ½ medium. The culture was incubated at 23 °C on a rotary shaker (140 rpm). The growth of the fungus was monitored by constantly checking the amount of free glucose (using Bayer Diastix Harnzuckerstreifen). The fermentation was terminated five days after glucose depletion.

*2.1.6 Isolation of compounds **1**-**7***: The 500 mL supernatant culture crude extracts (700 mg) were fractionated using preparative reverse phase liquid chromatography (PLC 2020, Gilson, Middleton, USA). VP Nucleodur 100-5 C 18 ec column (250 ×40 mm, 7 µm: Macherey-Nagel) used. Deionized water (Milli-Q, Millipore, Schwalbach, Germany) (solvent A) and acetonitrile (solvent B) were used as the mobile phase. The elution gradient used was 5-100% solvent B in 52 min and thereafter isocratic condition at 100% solvent B for 10 min. UV detection was carried out at 210, 254 and 350 nm and flow rate 35mL/min. Five fractions (F1-F5) were collected according to the observed peaks.

Fraction F1 and F2 were further purified by reversed phase LC (solvent A/solvent B), elution gradient 20-30% solvent B for 30 minutes followed by gradient shift from 35-100 % in 3 minutes and finally isocratic condition at 100% solvent B for 5 minutes with a preparative Nucleodur Phenyl hexyl column (Macherey-Nagel, Düren, Germany; 250 x 21 mm, 5 µm) as stationary phase and a flow rate of 15 mL/min., to afford compound **1** (3 mg) and compound **3** (50 mg). Using the same column and a modified elution gradient (25-45% solvent B for 30 minutes fraction) F3 was purified to afford 45 mg of **2**. Fractions F4 and F5 were purified by

reversed phase HPLC (solvent A/solvent B), elution gradient 78-100% solvent B for 25 minutes followed by isocratic condition at 100% solvent B for 5 minutes with a preparative (Kromasil, MZ Analysentechnik, Mainz, Germany) 250 x 20 mm 7 µm C-18 column as stationary phase to give compound **4** (125 mg) and **5** (10 mg). The same separation and purification conditions were applied to the mycelial culture and the 1000 mL fermentation products. Compound **6** (5 mg) and **7** (2mg) were purified from F4 by reverse phase LC (solvent A/solvent B), elution gradient 65-85% solvent B for 20 minutes followed by gradient shift from 85-100 % in 3 minutes and finally isocratic condition at 100% solvent B for 5 minutes with a preparative HPLC column (Kromasil, 250 x 20 mm, 7 µm C-18) as stationary phase.

2.1.7 Antimicrobial Assay: Minimum Inhibition Concentrations (MIC) against different test organisms were determined in serial dilution assay as described previously by Teponno et al., 2017, against *Candida tenuis* MUCL 29982, *Mucor plumbeus* MUCL 49355, *Escherichia coli* DSM498 and *Bacillus subtilis* DSM10 and *Micrococcus luteus* DSM 1790. The assays were carried out in 96-well microtiter plates in YMG medium for filamentous fungi and yeasts and MH for bacteria. The stock solution concentration was 300 µg/mL.

2.1.8 Nematicidal Assay: Compounds **1-5** were assessed for nematicidal activity against *Caenorhabditis elegans* according to Rupcic et al., 2018 and Kuephadungphan et al., 2017 with slight modification. *Caenorhabditis elegans* were inoculated monoxenically on nematode agar at room temperature for 4-5 days. Thereafter, nematodes were washed down from the plates with M9 buffer. The final nematodes concentration was adjusted to 500 nematodes/mL of M9 buffer. Assay was performed in 24-well microtiter plate at four different concentration (100, 50, 25 and 12, 5 µ/mL) of each compound. Ivermectin was used as the positive control and methanol as a negative control. The plates were incubated at 20 °C in the shaker in the dark and nematicidal activity was recorded after 18 h of incubation and expressed as a LD_{50}.

3.1 Results and Discussion

The fungus strain *Sanghuangporus* sp. (MUCL 55592 was identified to generic level by sequencing parts of the rDNA (5.8S gene region, including the internal transcribed spacers (ITS1 and ITS2) and part of the large subunit (LSU) as described in the Experimental. A BLAST search in GenBank confirmed the identity affinities of this strain with members of *Sanghuangporus* since the most homologous sequences were derived from this genus. This

was in agreement with the morphological features of the basidiomata. Currently, further taxonomic studies are ongoing to assess whether MUCL 55592 constitutes a new species; still a bulk of *Inonotus* and *Phellinus* species need to be re-evaluated in a modern taxonomic scheme.

Sanghuangporus sp. MUCL 55592 was investigated for active secondary metabolites as its crude extract exhibited antimicrobial activity against *Bacillus subtilis*. Analysis of the HPLC-MS data and subsequent search in public databases such as the Dictionary of Natural Products (Dictionary of Natural Products, 2017), revealed the presence of a potentially new metabolite. The chemical profile of the crude extracts is shown in (Fig. 1). Bioassay guided fractionation led to the isolation of one new compound of the phelligridrin family named phelligridin L (**1**) together with known compounds: 3, 14'-bihispidinyl (**2**), hispidin (**3**), ionylideneacetic acid (**4**), 1S-(2E)-5-[(1R)-2,2-dimethyl-6-methylidenecyclohexyl]-3-methylpent-2-enoic acid (**5**), phellidine E (**6**) and phellidine D (**7**) (Fig. 2) (Klaar et al., 1977; Edwards et al., 1961; Kobayashi et al., 2010). The known compounds were identified by comparing their NMR and HR-MS data with those reported in the literature.

Phelligridin L was isolated as a brown powder. The molecular formula $C_{25}H_{16}O_9$ with 18 degrees of unsaturation was deduced from the HRMS data. Furthermore, the ion peaks $[M+H]^+$ at *m/z* 461.0871, $[M+Na]^+$ at *m/z* 483.0690 and $[2M+H]^+$ at *m/z* 921.1660 were identified in the HR mass spectrum. The ^1H NMR spectrum revealed 6 singlets, 4 doublets and one doublet of doublets attributed to aromatic and/or olefinic protons. From the ^{13}C and DEPT/HSQC NMR spectra 11 methines and 14 quaternary carbons were identified (Table 1).

The HMBC correlations (Fig. 3) of H-3 to C-2, C-5 and H-5 (δ_H 5.72) to C-3, C-5, C-6, C-2' established the 4,6-disubstituted pyrone moiety in the molecule. Although C-3 (δ_C 90.4) and C-5 (δ_C 100.9) were missing in the ^{13}C spectra their existence was confirmed from the HSQC and HMBC correlations of H-5 (Fig. 4). The oxygenated substituents were attached to C-2 (δ_C163.0), C-4 (δ_C 174.8) and C-6 (δ_C156.3) based on their chemical shifts. Further HMBC correlations of H-3' (δ_H 7.63) to C-6, C-7'a, C-1', C-3'a, H-4' (δ_H 6.93) to C-7'a, C-3', C-6' and H-7' (δ_H 6.62) to C-3'a, C-5', C-1' unambiguously established the disubstituted-5',6'-dihydroxyindene moiety. The chemical shifts of the quaternary carbons C-5' (148.4) and C-6' (δ_C148.1) indicated that these carbons were oxygenated. The long range HMBC correlations of H-5 to C-2' and H-3' to C-6 confirmed the linkage of the pyrone moiety to the indene moiety part of the molecule through C-2' and C-5 bond.

The HMBC correlations of a doublet occurring at δ_H7.16 (H-4''') with a coupling constant 1.94 Hz to C-2''', C-5''', C-6''', C-8''', a doublet H- 7''' (δ_H 6.80, *J*=8.17 Hz) to C-3''', C-5''', C-6''' and a doublet of doublets H-8''' (δ_H 7.07, *J*=1.94, 8.17 Hz) to C-4''', C-2''', C-6''' observed pointed to a 1,2,4-trisubstituted benzene ring. Based on their chemical shifts δ_C 147.2 and δ_C 150.5 for C-5''' and C-6''' respectively, hydroxyl groups were attached to these carbons. Further HMBC correlations of H-1''' (δ_H7.01) to C-3''', C-5'', 4''' and H-2''' to C-4''', C-8''', C-5'' were observed. Cross peaks between H-1''' and H-2''' were also observed in the COSY spectrum with the olefinic bond between them being *trans* because of the large coupling constant of 15.92 Hz recorded. From these HMBC and COSY correlations a trans-5''', 6'''-dihydroxystyryl moiety was elucidated.

The singlet at δ_H 6.08 (H-4'') long range HMBC correlations to C-1', C-3'', C-5'', C-1''' were recorded thus establishing the connection of C-1' to C-5'' through C-3'' and C-4''. The HMBC correlations of H-4'' to C-5'', C-1'''and H-4'' to C-1' established the connection between this part of the molecule to the trans-5''', 6'''-dihydroxystyryl moiety and the disubstituted-5', 6'-dihydroxyindene moiety respectively. The molecular formula of compound **1** had been established from the HRMS data as $C_{26}H_{16}O_9$ and the 18 degrees of unsaturation, to these far 26 carbons, 16 hydrogens, 8 oxygens and 17 degrees of unsaturation have been accounted for. Therefore based on the chemical shifts of C-5'' (δ_C 188.0) and C-1' (δ_C 96.9) and the molecular formula, C-1' and C-5'' were connected via an oxygen atom establishing the spiroindene moiety in the molecule. Therefore the structure of compound **1** was established elucidated as 5',6'-dihydroxy-2'-(4-hydroxy-2-oxo-2H-pyran-6-yl)-5-[(E)-2-(4-hydroxy-phenyl)ethenyl]-3H-spiro[furan-2,1'-inden]-3-one.

Phelligridin L (**1**) is closely related to other naturally occurring antioxidant and cytotoxic agents such as phelligridin G, phelligridin E and inoscavin A (Wang et al., 2005; mo et al., 2004; Kim et al., 1999). Like the above mentioned compounds phelligridin L was optically inactive. The biogenetic formation of the chiral centre at C-1' of the spiroindene moiety has been reported before to be nonstereo-selective (Wang et al., 2005).

The nematicidal effects of compounds **1-7** on *Caenorhabditis elegans* are shown in Table 2. Compounds **1** and **3** showed moderate nematicidal activity with LD_{50} of 12.5 µg/mL. Compound **2** exhibited a weak effect with LD_{50} of 50 µg/mL. The other compounds **4-7** did not show any significant nematicidal effects on *C. elegans*. Although the biological activities of hispidin (**3**), and 3, 14'-Bihispidinyl (**2**) have already been studied for decades, to the best of our knowledge their nematicidal activities have not been reported. Furthermore **1-7** were

tested for antimicrobial activities against various test organisms (Table 2). Compound **1** and **3** were found to be active against *Micrococcus luteus* with MIC of 25 µg/mL and 100 µg/mL respectively. Further **4-5** demonstrated non selective antibacterial and antifungal activities. Antimicrobial activities of these two compounds have been reported before (Kobayashi et al., 2010). However, when studied by us, phellidines D and E exhibited no significant antimicrobial activities at concentration up to 100 µg/mL. This observation may relate to the different test organism and protocols used by us in comparison to the aforementioned reference.

4.1 Conclusion

Sanghuangporus sp. belong to the *Inonotus linteus* complex of medicinal mushrooms. A new highly oxygenated spiro [furan-2,1'-indine]-3-one derivative (**1**) together with six known compounds were isolated from *Sanghuangporus* sp. Compounds **1-5** showed moderate antimicrobial activity. Furthermore, compound **1-3** showed moderate nematicidal activities against *C. elegans*. Even though 3,14'-bihispidinyl and hispidin have been studied for several decades their nematicidal activity have not been reported before. Some of the biological activities of hispidin and other hispidin derivatives include antimicrobial, antioxidants, cytotoxic, anti-inflammatory and β-secretase inhibition activities (Chen et al., 2016; De Silva et al., 2013; Hsieh et al., 2013; Lee et al., 2006; Lee et al., 2011)

5.1 Chemical data

Phelligridin L (**1**): Brown powder; UV (MeOH, c= 0.25) λ_{max} (log ε) 202 (3.663), 256 (3.3950), 385 (3.646); ^{1}H NMR (700 MHz, Methanol-d_4 see Table 1, ^{13}C NMR (175 MHz, Methanol-d4) see Table 1; HRESIMS *m/z* 461.0871 $[M+H]^+$ (calcd for $C_{25}H_{17}O_9$, 461.0872).

Acknowledgements

We are grateful to Christel Kakoschke and Cäcilia Schwager for their technical support. Financial support by the "ASAFEM" Project (Grant no. IC-070) under the ERAfrica Programme to J.C.M. and M.S. and a personal PhD stipend by the German Academic Exchange Service (DAAD) and the Kenya National Council for Science and Technology (NACOSTI) to C.C. is gratefully acknowledged (programme-ID: 57139945).

Supplementary information

UV, HRMS data and ^{1}H, ^{13}C, COSY, HSQC, HMBC NMR spectra of metabolites **1** are available as Supporting Information. The ITS and LSU sequences of the producing organism are also included.

The authors declare no competing financial interests.

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Table 1

^{1}H and ^{13}C data of phelligridin L (**1**) recorded in methanol-d_4 (^{1}H 700 MHz, ^{13}C 150 MHz)

No	δ_C	DEPT	δ_H
2	163.0	C	
3	90.4	CH	5.34
4	174.8	C	
5	100.9	CH	5.72 (s)
6	156.3	C	
1'/2''	96.9	C	
2'	134.7	C	
3'	141.4	CH	7.63 (s)
3a'	134.4	C	
4'	112.1	CH	6.93 (s)
5'	148.4	C	
6'	148.1	C	
7'	110.5	CH	6.62 (s)
7a'	136.1	C	
3''	201.6	C	
4''	104.5	CH	6.08 (s)
5''	188.0	C	
1'''	113.4	CH	7.01 (d), *J*=15.92 Hz
2'''	143.9	CH	7.60 (d), *J*=15.92 Hz
3'''	128.3	C	
4'''	115.7	CH	7.16 (d), *J*=1.94 Hz
5'''	141.4	C	
6'''	150.5	C	
7'''	116.8	CH	6.80 (d), *J*=8.17 Hz
8'''	124.0	CH	7.07 (dd), *J*=1.94, 8.17

Table 2: Antimicrobial and nematicidal activities of compounds **1-7**

Test strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Reference**
Antimicrobial activities				**MIC (µg/mL)**				
Bacillus subtilis DSM 10	/	/	/	6.25	/	/	/	2.3[a]
Micrococcus luteus DSM 1790	25	25	100	25	≤100	/	/	8.3[a]
Escherichia coli DSM 498	/	/	/	/	/	/	/	2.3[a]
Candida tenuis MUCL 29982	/	/	/	50	/	/	/	2.3[b]
Mucor plumbeus MUCL 49355	100	/	/	12.5	100	/	/	9.4[b]
Nematicidal activities				**LD_{50} (µg/mL)**				
Caenorhabditis elegans	12.5	12.5	25	/	/	/	/	≤3.1[c]

[a]ciprofloxacin; [b]nystatin; [c]ivermectin; / no activity; stock solution 100 µg/mL

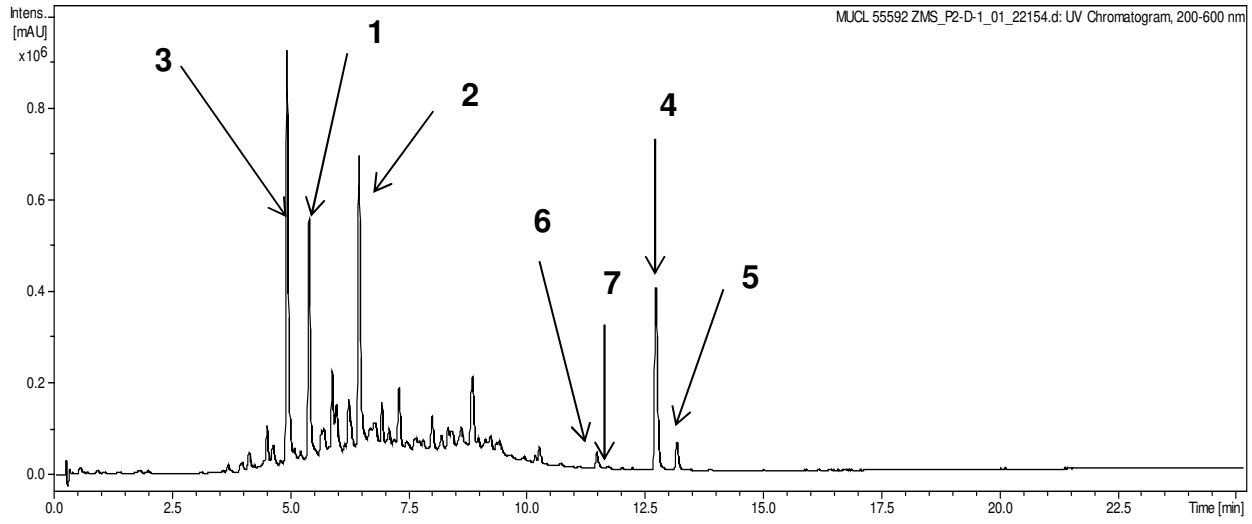

Fig. 1 Chemical profile of *Sanghuangporus sp* supernatant crude extract

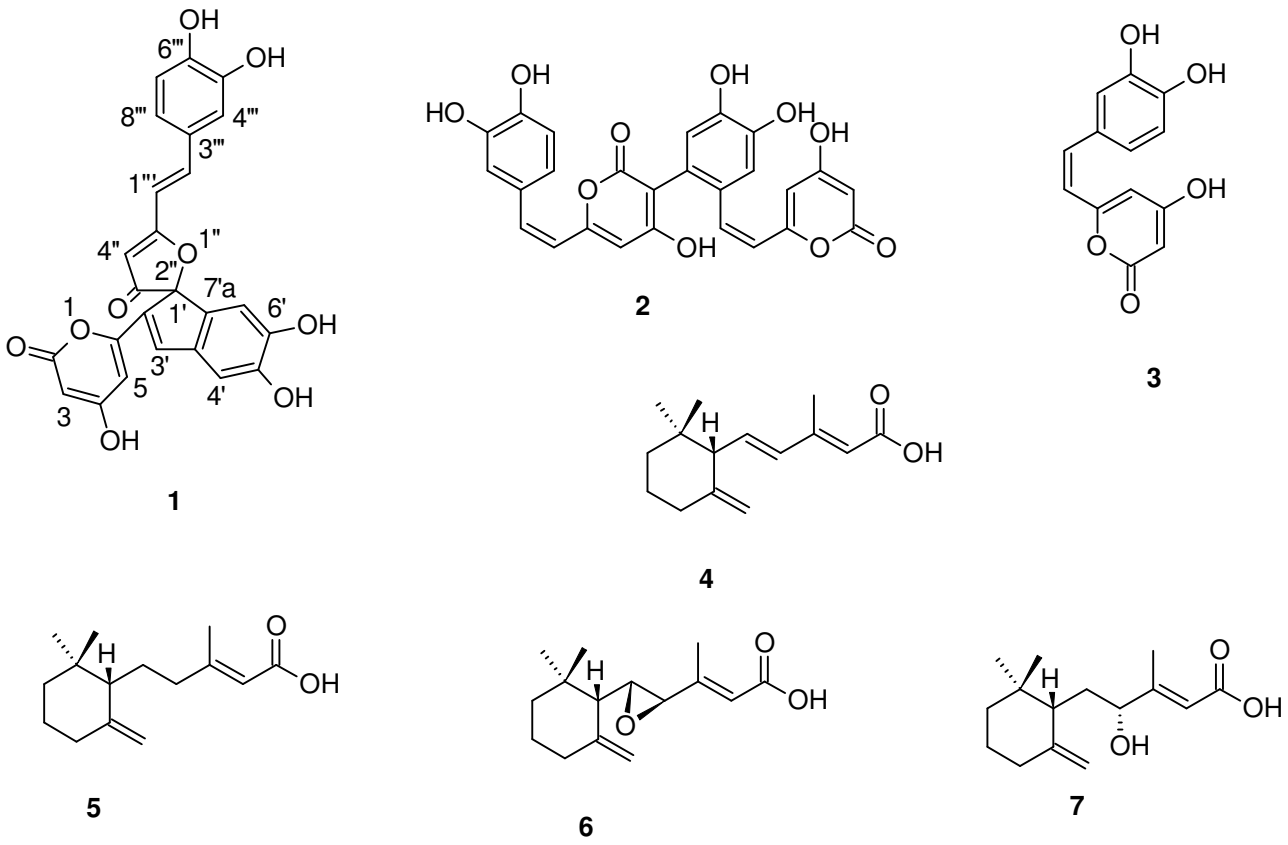

Fig. 2. Chemical structures of **1-5**

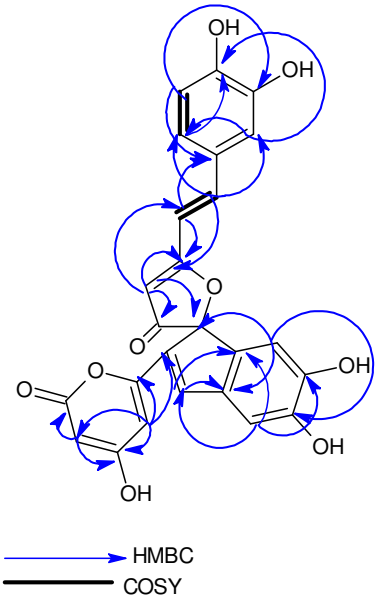

Fig. 3. HMBC and COSY correlations of 1

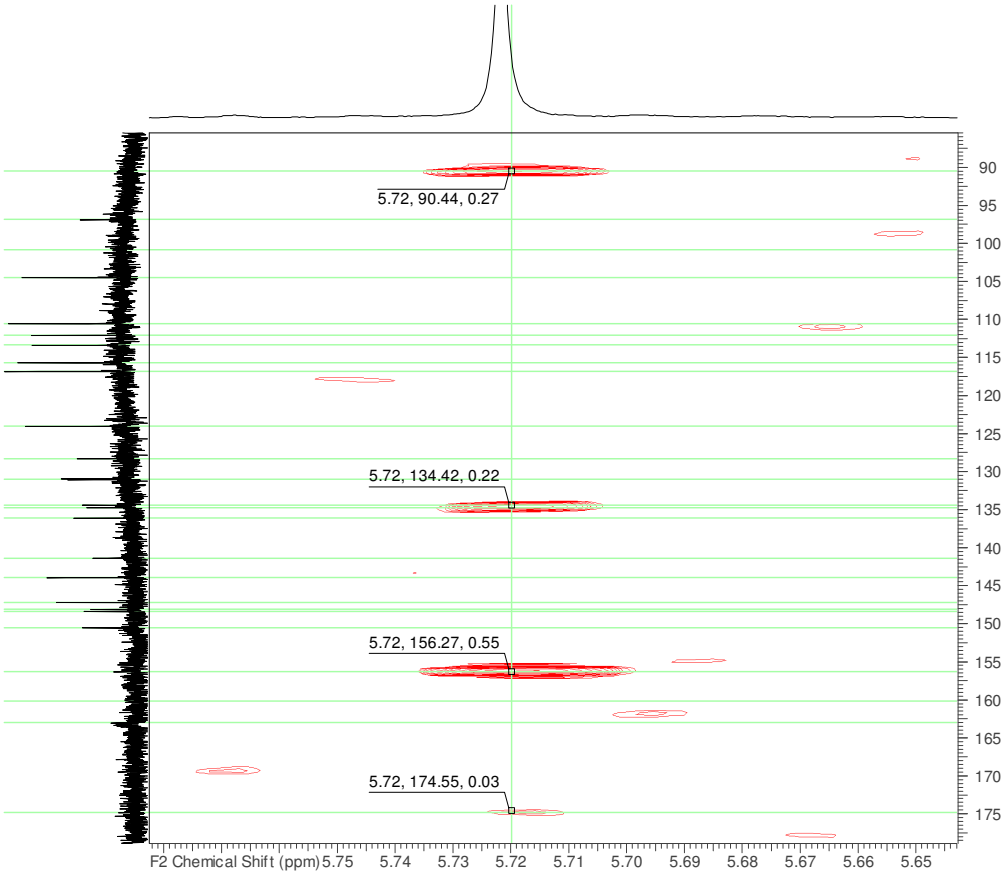

Fig. 4. Extracted part of HMBC spectra of phelligridin L (**1**) showing H-5 HMBC correlations