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Phenolic Compounds of Sponge-associated Fungi (*Lecanicillium evansii*)

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This study was chiefly aimed at pursuing new biologically active secondary metabolites of microfungus species, *Lecanicillium evansii*, isolated from sponge *Callyspongia* sp collected from West Bali Sea, Indonesia. Sponges were collected by scuba diving. A tiny piece of sponge was inoculated on the surface of malt agar plates and incubated at 27 °C. In order to get a pure mono-culture of the fungus, repeated sub-culturing onto fresh malt agar plates were performed. The collected fungi were maintained on malt agar plates using the Wickerham medium. Mass cultivation of the fungus *L. evansii* (10 L) was carried out in 30 erlenmeyer flasks in Wickerham medium. After 10 days incubation, without shaking under constant room temperature (20 °C), fungal mycelium were separated from the culture broth. The mycelia were extracted with methanol and ethyl acetate was added to the media. Both methanol-added mycelia and ethyl acetate-added media were left overnight. Seven compounds were isolated from *L. evansii*. Those compounds comprised phenolic compounds (terphenylin, deoxyterphenylin, terprenin 2, terprenin epoxide), bipeptide (cyclo-tyrosylprolyl), and simple aromatic compounds (acetyl hydroxybenzamide, 4-hydroxybenzaldehyde). Detailed analysis by NMR and mass spectrometry enabled their identification to be new deoxyterphenylin, new terprenin 2, and new terprenin epoxide.

Key words: *Callyspongia*, deoxyterphenylin, *Lecanicillium evansii*, terprenin 2, terprenin epoxide

Penelitian bertujuan untuk mencari bahan bioaktif metabolit sekunder dari mikrofungi *Lecanicillium evansii* yang diisolasi dari sponge *Callyspongia* sp. yang dikoleksi dari perairan laut barat Bali, Indonesia. Sponge dikoleksi dengan penyelaman (scuba). Potongan kecil sponge diinokulasi pada media agar dan diinkubasi pada 27 °C. Untuk mendapatkan kultur tunggal dari mikrofungi, dilakukan beberapa kali sub kultur. Kultur missal mikrofungi *L.evanssi* (10 L) dilakukan pada 30 botol Erlenmeyer yang diisi medium Wickerham. Setelah 10 hari inkubasi tanpa goyangan pada suhu kamar 20 °C, miselium dan media dipisahkan. Miselium di diektrasksi dengan methanol, sedangkan pada media ditambahkan etil asetat. Keduanya didiamkan selama semalam. Tujuh senyawa berhasil diisolasi dari *L.evansii*. Senyawa-senyawa tersebut terdiri dari: fenolik (terphenylin, deoxyterphenylin, terprenin 2, terprenin epoxide), bipeptide (cyclo-tyrosylprolyl), dan senyawa aromatic sederhana (acetyl hydroxybenzamide, 4-hydroxybenzaldehyde). Analisis lanjut menggunakan NMR dan LCMS mengungkapkan bahwa deoxyterphenylin, terprenin 2, and terprenin epoxide merupakan senyawa baru yang belum pernah dilaporkan sebelumnya.

Kata kunci: *Callyspongia*, *Lecanicillium evansii*, deoxyterphenylin, terprenin 2, terprenin epoxide

Terrestrial fungi served an enormous resource for the discovery of novel natural product in the past 60 years, many of them being potential targets for biomedical developments. The discovery of penicillin in 1929 started the era of fungal antibiotic and was followed by other important fungal metabolites like cephalosphorins, cyclosporins, and griseofulvins. Until now, fungi have only been surpassed by actinomycetales as sources for biologically active metabolites. The terrestrial fungal biodiversity seems to be nearly exhausted. However, there are many fungal endophytes for example that have not been characterized. Thus, nowadays, researchers throughout the world pay increasing attention toward the potential of marine microorganism as an alternative source to isolate novel metabolites (Anke and Erkel 2002; Biabani and Laatsch 1998; Pietra 1997).

Natural products, such as secondary metabolites, isolated from plants, animals and microbes are important sources for bioactive molecules that, in many cases, have been developed into medications (Villa and Gerwick 2010). Some of the natural products isolated from marine invertebrates have been shown to be, or are suspected to be, of microbial origin and this is now thought to be the case for the majority of such molecules. Marine microorganisms, whose immense genetic and biochemical diversity is only beginning to be appreciated, seem likely to become a rich source of novel chemical entities for the discovery of more effective drugs (Haefner 2003). The marine environment has proven to be a very rich source of

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extremely potent compounds that have demonstrated significant activities as antitumors, antiinflammatories, analgesics, immunomodulators, antiallergies, and antivirals (Newman and Cragg 2004; Blunt *et al*. 2008; Simmons *et al*. 2005).

Although approximately three- to fourthousands known fungal secondary metabolites have been isolated, possibly not more than five to seven thousands taxonomic species have been studied in this respect. Genera such as: *Aspergillus*, *Penicillium*, *Fusarium*, and *Acremonium* are among fungi highly capable of producing high diversity of secondary metabolites (Dreyfuss and Chapela 1994).

The number of secondary metabolites isolated from marine-derived fungi has been increasing. This proves that they are rich sources of bioactive compounds with therapeutic potential. Almost one thousand marine natural product were discovered since 1990, with a pronounced increase between decades (Leal *et al*. 2012). The success story of cephalosporin isolation, which is now widely used in antibiotic therapy, from the fungus *Cephalosporium* sp. derived from the microbial flora of sea water collected from Cagliari, Italy, in the 1940's marked the onset of interest of pursuing natural product from marine fungi (Faulkner 2000). Cephalosporin C becomes the only compound from a fungus isolated from a marine source that up to now has been established as a medication or a source for partial synthetic derivatives (Biabani and Laatsch 1998).

Ziconotide (Prialt; Elan Pharmaceuticals), a peptide originally discovered in a tropical cone snail, was the first marine-derived compound to be approved in the United States in December 2004 for the treatment of pain. Then, in October 2007, trabectedin (Yondelis; PharmaMar) became the first marine anticancer drug to be approved in the European Union (Molinski *et al*. 2009).

This study was chiefly aimed at pursuing new biologically active secondary metabolites of new fungi species (*Lecanicillium evansii* strain 1), isolated from sponge *Callyspongia*.

MATERIALS AND METHODS

Isolation of the New Fungal Species. The strain of new fungal species of *Lecanicillium evansii* was isolated from the sponge *Callyspongia* sp. Microfungi was collected from West Bali Sea, Indonesia. Sponges were collected by scuba diving. A small piece of the inner part of the sponge was sliced under sterile conditions. This tiny piece was then inoculated on the surface of malt agar plates and incubated at 27 °C. In order to get a pure mono-culture of the fungi, purification through several sub-cultures onto fresh malt agar plates were repeatedly carried out. The collected fungi were maintained under malt agar plates using the Wickerham medium (Table 1). To eliminate bacterial contaminants, chloramphenicol (0.2 g L^{-1}), streptomycin sulphate (0.1 g L^{-1}), and penicillin G (0.1 g L^{-1}) were added to the medium.

Fungal species identification was conducted by the Centraalbureau voor Schimmelcultures, Netherlands, and stored at the Institute of Pharmaceutical Biology and Biotechnology, Duesseldorf University, Germany.

Table 1 Wickerham medium for marine fungi culture

Substances	Amounts
Yeast extract (Sigma)	3.0 g
Malt extract (Merck)	3.0 g
Pepton (Merck)	5.0 g
Glucose monohydrate (Caelo)	20.0 g
Agar (Merck)	16.0 g
Artificial sea water (Biomarine)	24.4 g
Distilled water	1000 ml
NaOH or HCl for pH adjustment (7.2 - 7.4)	Several drops

Mass cultivation of the fungus *L. evansii* (10 L) was carried out in 30 erlenmeyer flasks in Wickerham medium. After 10 days incubation, without shaking, under constant room temperature (20 °C), fungal mycelium were separated from the culture broth. The mycelia were extracted with methanol, and ethyl acetate was added to the media. Both methanol-added mycelia and ethyl acetate-added media were left overnight.

Extraction and Isolation of the Secondary Metabolites. Extraction and isolation of the secondary metabolites of *L. evansii* are presented in Fig 1. An

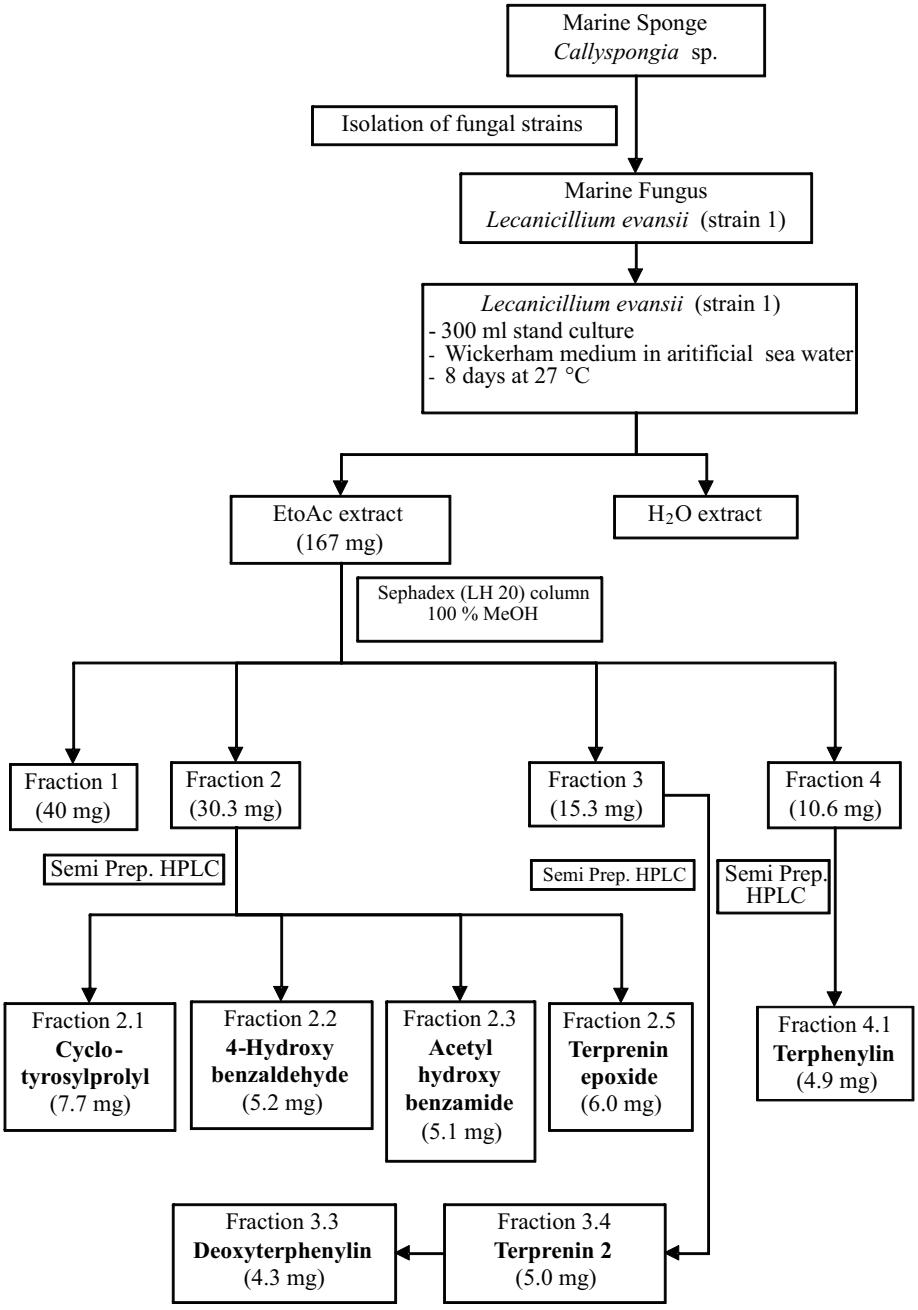

Fig 1 Isolation scheme of secondary metabolites from *Lecanicillium evansii*

unknown natural product sample often contains a mixture of many components. The components must be separated from one another so that each component can be analysed individually. Many organic liquids are immiscible with water. When such a liquid is added to water, two layers are formed. Whether the organic layer is in the upper or lower layer depends upon the relative density of organic liquid and water.

Analysis of the Secondary Metabolites. TLC was always conducted to each fraction prior to further chemical work, to get the overview of the identity of the fraction and the qualitative purity of theisolated compound. Band separation in TLC would also be very helpful in optimising the solvent system that would be applied later in column chromatography.

The use of analytical and semi preparative HPLC were intended to analyse the peaks distribution, either from raw extracts or fractions, as well as to evaluate the purity of isolated compounds. EI-MS (electron impact mass spectrometry), FAB-MS (fast atom bombardment mass spectrometry), and ESI-MS (electron spray ionisation mass spectrometry) were applied to identify molecular weight. Proton (1H) and carbon (13C) NMR spectra were recorded at 300 °K on Bruker DPX 300, ARX 400 or AVANCE DMX 600 NMR spectrometers (500 Mhz).

Table 2 NMR data of deoxyterphenylin

Position	δ^1H (ppm), multiplicity (*J* in Hz) (in MeOD)	δ^1H (ppm), multiplicity (*J* in Hz) (in DMSO)	COSY (H→H) (in MeOD)	ROESY (H→H) (in DMSO)
2	7.61 (d, 7.3)	7.61 (d, 8.5)	H3	H3, H4
3	7.42 (t, 7.3, 7.9)	7.46 (t, 7.4, 7.8)	H2, H4	H2, H4
4	7.34 (t, 7.3, 7.6)	7.37 (t, 7.3, 7.5)	H3, H5	H3, H5
5	7.42 (t, 7.3, 7 .9)	7.46 (t, 7.4, 7.8)	H4, H6	H4, H6
6	7.61 (d, 7.3)	7.61 (d, 8.5)	H5	H4, H5, H6'
5' OCH	3.68 (s)	3.65 (s)		
6'	6.46 (s)	6.45 (s)		H5' - OCH_3
2''	7.19 (d, 8.8)	7.11 (d, 8.5)	H3''	H3''
3''	6.80 (d, 8.8)	6.76 (d, 8.6)	H2''	H2''
4'' OCH	3.36 (s)	3.30 (s)		H3'', H5''
5''	6.80 (d, 8.8)	6.76 (d, 8.6)	H6''	H6''
6''	7.19 (d, 8.8)	7.11 (d, 8.5)	H5''	H5''

RESULTS

Seven compounds were successfully isolated from the fungus *L. evansii.* The compounds were deoxyterphenylin (new compound), terprenin 2 (new compound), terprenin epoxide (new compound), terphenylin, cyclo(tyrosylprolyl), acetyl hydroxybenzamide, and 4-hydroxybenzaldehyde. Only three new elucidated compounds would be discussed further in this paper.

Deoxyterphenylin. The ESI-MS spectrum of compound deoxyterphenylin presented intense ions at m/z 323.4 [M+H]+(positive) and 321.8 [M-H]-(negative) determining a molecular weight of 322 g $mole^{-1}$ and suggesting a molecular formula of $C_{20}H_{18}O_4$ which corresponded to deoxyterphenylin. The EI-MS high resolution spectrum displayed a molecular weight of 322.1195 g $mole^{-1}$. This was also confirmed by several ion peaks in the EI-MS spectrum at m/z 322 [M]+, 307 [M-CH3]+ (fragment 1) 292 [M-2(CH_3)]+ (fragment 2), and 276 [M-CH_3-CH_3O]+ (fragment 3) (Fig 2).

By analysing the chemical shifts, signal multiplicities, and coupling constants (7.25-8.82 Hz) of protons displayed in the 1H NMR spectrum, one mono-substituted benzene (ring A) δ 7.61 (H2, H6), 7.42 (H3, H5), 7.34 (H4), and one para-substituted phenyl (ring C) d 7.19 (H2", H6"), 6.80 (H3", H5") (Table 2, Fig 3) were identified.

The COSY correlations between H2"/6" and H3"/5" and H6" indicated an AA'BB' spin system and revealed the presence of a para-substituted phenyl in ring C. The second spin system of a mono-substituted

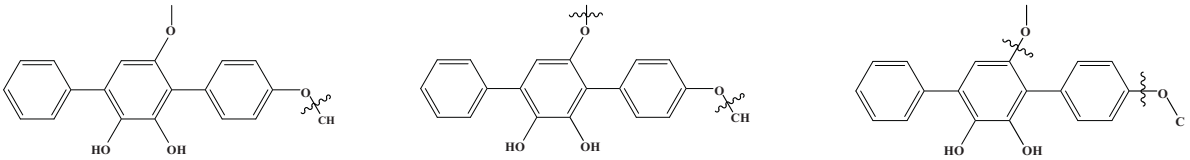

Fig 2 Hypothetical fragmentation of deoxyterphenylin in the EI-MS spectrum

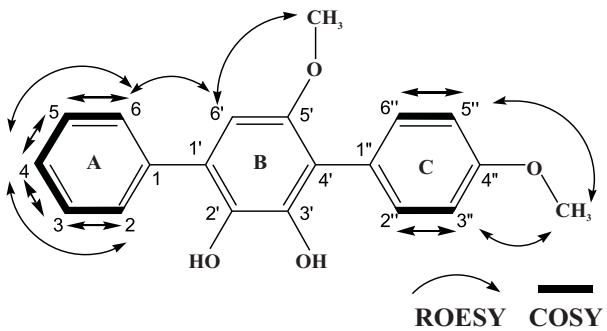

Fig 3 COSY and ROESY correlations of deoxyterphenylin

benzene in ring A was also distinctly observed in the COSY spectrum (Fig 3).

One penta-substituted phenyl (ring B) was clearly determined by one isolated singlet at δ 6.46 (H6'. In addition, two methoxy singlets (δ 3.68 and 3.36) were observed.

To determine the positions of the methoxy groups, NOE (Nuclear Overhauser Effect) experiments were performed. Irradiation of methoxy protons (δ 3.68, H5') resulted in the intensification of the singlet proton (H6'), assuring that the position of isolated singlet proton (H6') was adjacent to this methoxy group (H5').

Irradiation of the other methoxy group protons at

was also confirmed by the EI-MS spectrum with several intense ion peaks at m/z 406 [M]+, 340 [M-prenyl group]+ (fragment 1), 322 [M-prenyl group-H_2O]+ (fragment 2), 177 [M-prenyl group-ring A]+ (fragment 3), 69 [M of prenyl group]+ (fragment 4) (Fig 4). The ESI-MS high resolution spectrum displayed an ion peak at m/z 429.1673 [M+Na]+, supporting the assignments of molecular weight and molecular formula.

The placement of the two methoxy groups in ring B (positions 3' and 6') was identified by close inspection of the HMBC spectrum. In Terprenin 2, H5' displayed correlations to both oxygenated aromatic carbons (δ

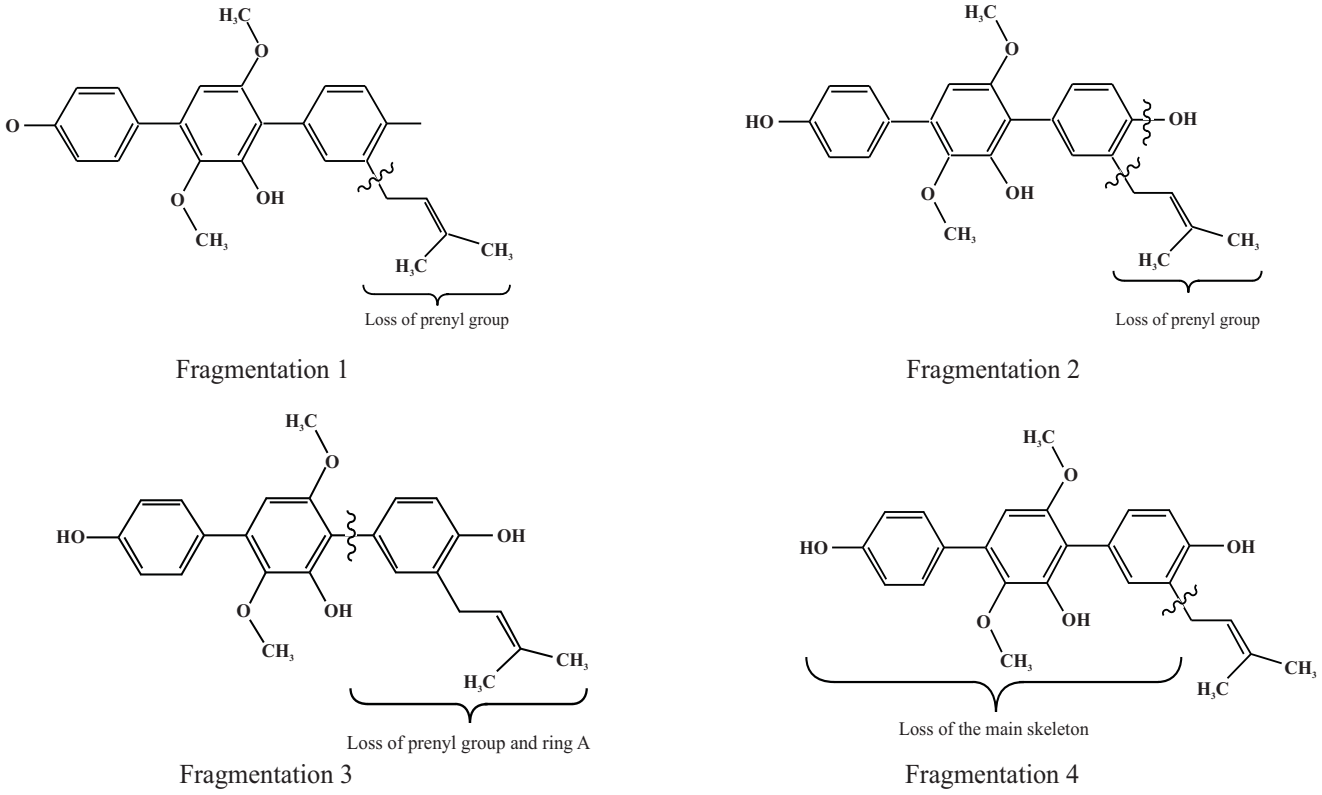

Fig 4 Hypothetical fragmentation of terprenin 2 in the EI-MS spectrum

the higher field (δ 3.36, H4") however did not cause any observable effects on other protons. Thus, a 2D-ROESY (Rotational Frame Nuclear Overhauser Effect Spectroscopy) experiment was conducted. Again a correlation between H6' and 5'-OCH_3 was clearly observed, while the correlation of 4"-OCH_3 and H3"/5" allowed for positioning of the second methoxy function. Thus, the structure of compound deoxyterphenylin was established as depicted in Fig 3.

Terprenin 2. The ESI-MS spectrum of compound Terprenin 2 showed the ion peak at m/z 407[M+H]+ suggesting a molecular weight of 406 g $mole^{-1}$ and a molecular formula of $C_{25}H_{26}O_5$. The molecular weight

138.50 and δ 153.72) that in turn were correlated to the methoxy signals.

Long range correlation in the HMBC spectrum confirmed the position of prenyl group through a number of prominent connections between H1'" and C3, C2, C4 (Fig 5). The correlation of H1'" and H2 in the ROESY spectrum also assured the location of the prenyl group (Fig 6). Likewise, the isolated proton of H5' displayed correlations with H2"/H6", and 6'-OCH_3, thus supporting the structure of terprenin 2 (Table 3).

Terprenin epoxide. The molecular formula of terprenin epoxide and molecular weight were

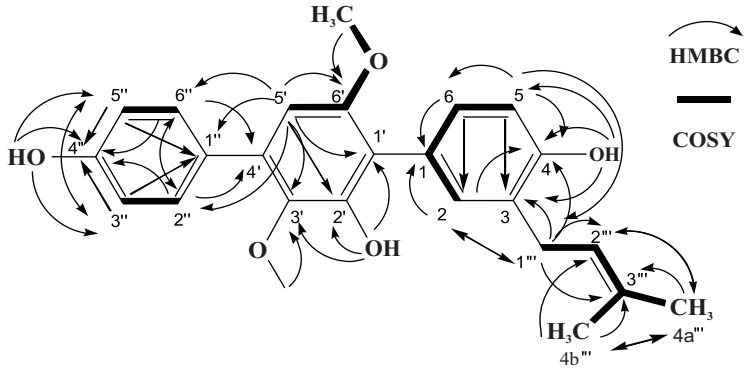

Fig 5 COSY and HMBC correlations of terprenin 2 (in $CDCl_3$)

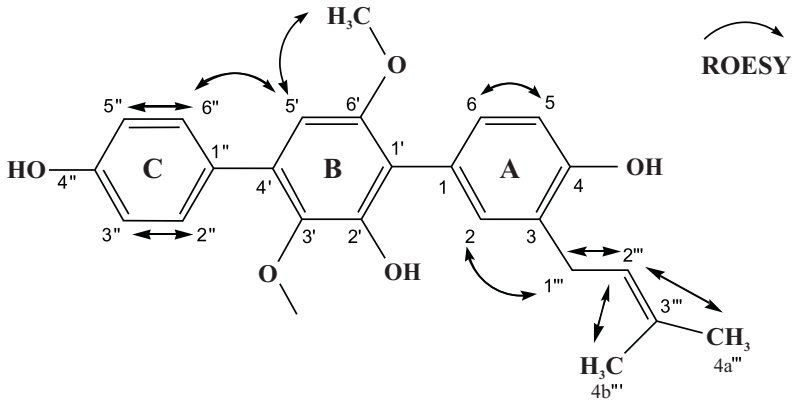

Fig 6 ROESY correlations of terprenin 2 (in Acetone)

established as $C_{25}H_{26}O_6$ and 422 g $mole^{-1}$, respectively, by the ESI-MS (m/z 423.3 [M+H]+, 421.6 [M-H]-). The high resolution of ESI-MS indicated the molecular weight of 422.1713 g $mole^{-1}$.

The 1H NMR data analysis indicated that terprenin epoxide had an epoxy prenyl side chain [δ 3.28 (H1'''A), 3.15 (H1'''B), 4.63 (H2'''), 1.19 (H4a'''), 1.14 (H4b''')], two methoxy groups [δ 3.37 (3'-OCH_3), 3.70 (6'-OCH_3)], three phenolic hydroxyl groups [δ 4.85 (4-OH), 5.90 (2'-OH), 4.85 (4''-OH)], and contained three phenyl rings with different substitution patterns as described above for terprenin epoxide (Table 4, Fig 7).

The presence of an epoxy group was assured by the molecular formula and by the HMBC correlations between H4a''' and H4b''' with C2''' and C3'''. The downfield shifts of the latter could only be explained by oxygen substitution, while the molecular formula only accounted for one additional oxygen atom in comparison to terprenin 2.

With the aid of proton multiplicity and coupling

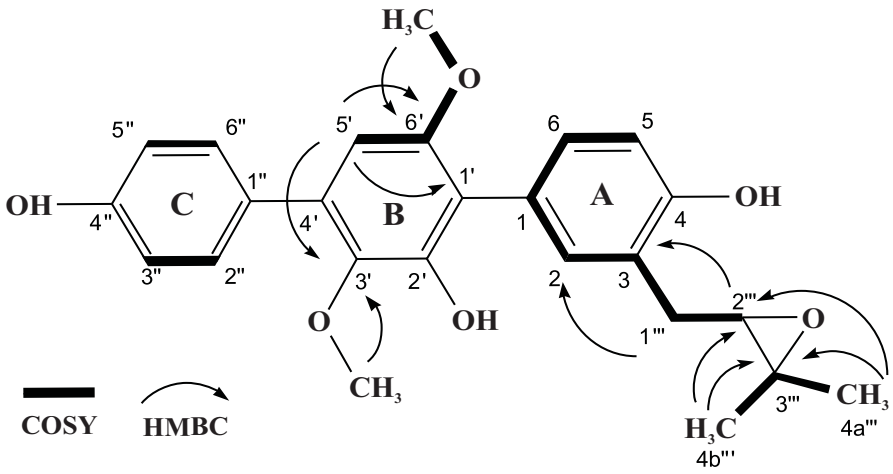

Fig 7 COSY and HMBC correlations of terprenin epoxide

Table 3 NMR data of terprenin 2

Position	δ^{13}C(ppm) (in $CDCl_3$)	δ^{1}H (ppm), multiplicity (*J* inHz) (in MeOD)	δ^{1}H (ppm), multiplicity (*J* in Hz) (in Acetone)	δ^{1}H (ppm), multiplicity (*J* in Hz) (in $CDCl_3$)	COSY (H H) (in $CDCl_3$)	HMBC (H C) (in $CDCl_3$)	ROESY (H H) (in Acetone)
1	129.94 (s)						
2	132.36 (d)	6.95 (d, 1.9)	7.13 (d, 2.5)	7.25 (d, 2.1)	H6	C1, C4, C1‘‘‘	H1‘‘‘
3	126.40 (s)						
4	153.72 (s)			5.15 (OH)		C3, C4, C5	
5	115.50 (s)	6.67 (d, 8.2)	6.83 (d, 8.2)	6.89 (d, 8.7)	H6	C3, C4, C6, C1‘‘‘	H6
6	125.23 (d)	6.91 (dd, 1.9; 8.2)	7.05 (dd, 1.9; 8.2)	7.22 (dd, 2.1; 8.5)	H2, H5	C1,C2	H5
1‘	115.64 (s)						
2‘	147.31 (s)			5.88 (OH)		C1’, C2’, C3’	
3‘	138.50 (s)						
3‘- OCH_3	56.04 (q)	3.35 (s)	3.38 (s)	3.46 (s)		C3’	
4‘	130.00 (s)						
5‘	103.83 (d)	6.34 (s)	6.46 (s)	6.45 (s)	H6’(OCH_3)	C6, C1’, C2’, C3’, C6’, C1“, C2”; 6’	H6’(OCH_3)
6‘	153.72 (s)						
6‘- OCH_3	60.73 (q)	3.66 (s)	3.69 (s)	3.74 (s)	H5’	C6’	H5’
1“	130.16 (s)						
2“	132.12 (d)	7.36 (d, 8.2)	7.50 (d, 6.3)	7.53 (d, 6.6)	H3”	C4’, C4”, C6”	H3”, H4”,H5”
3“	115.41 (d)	6.75 (d, 8.2)	6.92 (d, 6.3)	6.92 (d, 6.5)	H2”	C1”, C4”, C5”	
4“	155.13 (d)			4.86 (OH)		C3”, C4”, C5”	
5“	115.41 (d)	6.75 (d, 8.2)	6.92 (d, 6.3)	6.92 (d, 6.5)	H6”	C1”, C3”,C4”	
6“	132.28 (d)	7.36 (d, 8.2)	7.50 (d, 6.3)	7.53 (d, 6.6)	H5”	C4’, C2”, C4”	H5’, H3”, H4” H5”
1‘‘‘	30.02 (t)	3.35	3.36 (d, 6.9)	3.41 (d, 7.3)	H2’’’, H4a’’’, H4b’’’	C2, C3, C4, C2’’’, C3’’’	H2, H2’’’
2‘‘‘	121.90 (d)	5.35 (m)	5.39 (m)	5.40 (m)	H1’’’, H4a’’’, H4b’’’	C4a’’’	H1’’’
3‘‘‘	135.00 (s)						
4a‘‘‘	25.85 (q)	1.72 (s)	1.71 (s)	1.78 (s)	H1’’’, H2’’’, H4b’’’	C2’’’, C3’’’, C4b’’’	H2’’’
4b‘‘‘	17.93 (q)	1.71 (s)	1.70 (s)	1.77 (s)	H1’’’, H2’’’, H4a’’’	C2’’’, C3’’’, C4a’’’	H2’’’

constants observed in the 1H NMR spectrum, it was determined that the three phenyl rings possessan ABX spin system on ring A δ 7.17 (H2), 6.67 (H5), 7.08 (H6), a one proton system in ring B (δ 6.46 (H5'), an AA'BB' spin system on ring C [δ 6.92 (H3", H5"), 7.50 (H2", H6")]. An ABX spin system was also found in the side chain of epoxy prenyl group (Table 4, Fig 7). Proton signals of the 1H NMR spectrum recorded in $CDCl_3$ and methanol also supported the assignment of these three spin system types.

The epoxy group brought about a centre of asymmetric at position 2'". Consequently the methylene protons at position 1'" resonated as double doublet signals as clearly seen in the 1H NMR spectrum recorded in acetone.

An H/D exchange experiment using methanol as solvent to eliminate the hydroxyl protons that existed in the 1H NMR spectrum recorded in chloroform was conducted. Signals of the hydroxyl groups at positions 4, 2', and 4” disappeared.

COSY correlations also supported the assignment of the four spin systems in the molecule (Fig 7). Likewise, HMBC correlations assigned the position of the isolated proton H5' and the position of two methoxy groups in ring B. The side chain of epoxy prenyl group was bound to ring A through the position 3. This side connection was verified by the COSY correlations of H1'", H2'", and H2. The long range correlations between H1'" and C2 as well as between H2'" and C3 in the HMBC spectrum also clarified the side chain position (Fig 7).

DISCUSSION

Deoxyterphenylin has never been quoted in the literature before. Takahashi *et al.* (1976) isolated a related deoxyterphenylin from *Aspergillus candidus*. Both methoxy groups of this compound, however, are located at positions 2' and 5', whereas the methoxy groups of the new deoxyterphenylin at positions 5' and 4”.

Antimicrobial assay conducted using this newly elucidated deoxyterphenylin demonstrated no antimicrobial activity. Para-terphenyl metabolites showing a typical phenolic nature are found rarely as natural products. However, terphenylquinones were mostly isolated from *Basidiomycetes* (Thomson 1971). Takahashi *et al*. (1976) working with *Aspergillus*

Table 4 NMR data of terprenin epoxide

Position	δ^{13} C (ppm) (in Acetone)	δ^{1} H (ppm), multiplicity (*J* in Hz) (in Acetone)	δ^{1} H (ppm), multiplicity (*J* in Hz) (in CDCl 3)	COSY (H H) (in Acetone)	HMBC (H C) (in CDCl 3)
2	128.0	7.17 (br s)	7.25 (d)	H5, H6, H1‘‘‘	
3	136.0				
4			4.85 (OH)		
5		6.67 (d, 8.2)	6.87 (d, 8.2)	H6	
6		7.08 (d, 8.2)	7.20 (dd, 8.2, 2.0)	H2, H5	
1‘	131.0				
2‘			5.90 (OH)		
3‘	139.0				
3‘- OCH_3		3.37 (s)	3.45 (s)		C3‘
4‘					
5‘		6.46 (s)	6.45 (s)	6‘-OCH 3	C1‘, C3‘, C6‘
6‘	154.0				
6‘- OCH_3		3.70 (s)	3.74 (s)	H5‘	C6‘
2‘‘		7.50 (d, 8.8)	7.53 (d, 6.7)	H3‘‘	
3‘‘		6.92 (d, 8.8)	6.90 (d, 6.7)	H2‘‘	
4‘‘			4.85 (OH)		
5‘‘		6.92 (d, 8.8)	6.90 (d, 6.7)	H6‘‘	
6‘‘		7.50 (d, 8.8)	7.53 (d, 6.7)	H5‘‘	
1‘‘‘		H1‘‘‘A: 3.28 (dd, 15.7, 8.8) H1‘‘‘B: 3.15 (dd, 15.7, 9.5)	3.21 (dd, 19.6, 9.8)	H2‘‘‘	C2
2‘‘‘	72.0	4.63 (t, 9.5, 8.8)	4.65 (t, 9.3)	H1‘‘‘	C3
3‘‘‘	91.0				
4a‘‘‘	24.0	1.19 (s)	1.38 (s)	H4b‘‘‘	C2‘‘‘, C3‘‘‘, H4b‘‘‘
4b‘‘‘	26.0	1.14 (s)	1.25 (s)	H4a‘‘‘	C2‘‘‘, C3‘‘‘, H4a‘‘‘

candidus reported five p-terphenyl derivates. Terphenylin (3-10 g mL^{-1}) caused a specific toxicity on Hela cells. Certain quinones containing alkylating group have anti tumor activity. Cain (1961) performed several cytotoxicity tests on some quinone derivatives to find out the features of the quinone molecule responsible for antitumor activity.

Terprenin 2 has never been reported in the literature. A related compound with the prenyl group attached to the benzene ring at the position four through an oxygen bridge (terprenin) was reported by Kamigauchi *et al*. (1998) and Stead *et al*. (1999). There was no antimicrobial activity of this compound identified after carrying out a number of anti-microbiological assays.

Terprenin epoxide differs from the known terprenin derivatives in their side chains. The epoxy group on the side chain makes the terprenin epoxide a new natural product. This unusual characteristic of prenyl and epoxy group side chains in terprenin has never been mentioned in the literature before.

Terprenins possessed very strong proliferations against mouse spleen lymphocytes stimulated with Con A and LPS. The IC50 values of terprenin, 3-methoxyterprenin, and 4"-deoxyterprenin were calculated as 1.2, 2.0, and 5.6 ng mL^{-1} against Con A-induced proliferation and 4.5, 8.0, and 15.6 ng mL^{-1} against LPS-induced proliferation (Kamigauchi *et al*. 1998).

Stead *et al*. (1999) quoted that terprenin possesses a potent cytotoxicity against BALB/MK and other hyperproliferative cell lines. It is assumed that the existence of an oxygen-linked isoprene substituent brings about such an effect on cytotoxicity potency of this compound. This effect likely occurs through the inhibition of pyrimidine biosynthesis. However no antimicrobial activity against bacteria and fungi was reported. Antimicrobial assay conducted using terprenin epoxide as the test substance demonstrated no biological activity.

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