Bukti korespondensi

Steroids from Toona sureni-derived Endophytic Fungus Stemphylium sp. MAFF 241962 and Their Heme Polymerization Inhibition Activity

1. **Pengiriman artikel dan balasan dari jurnal**
2. **Permintaan Revisi sesuai arahan reviewer**
3. **Pernyataan Accepted dari jurnal**
4. **Persetujuan penulisan akhir**
5. **Artikel publish**

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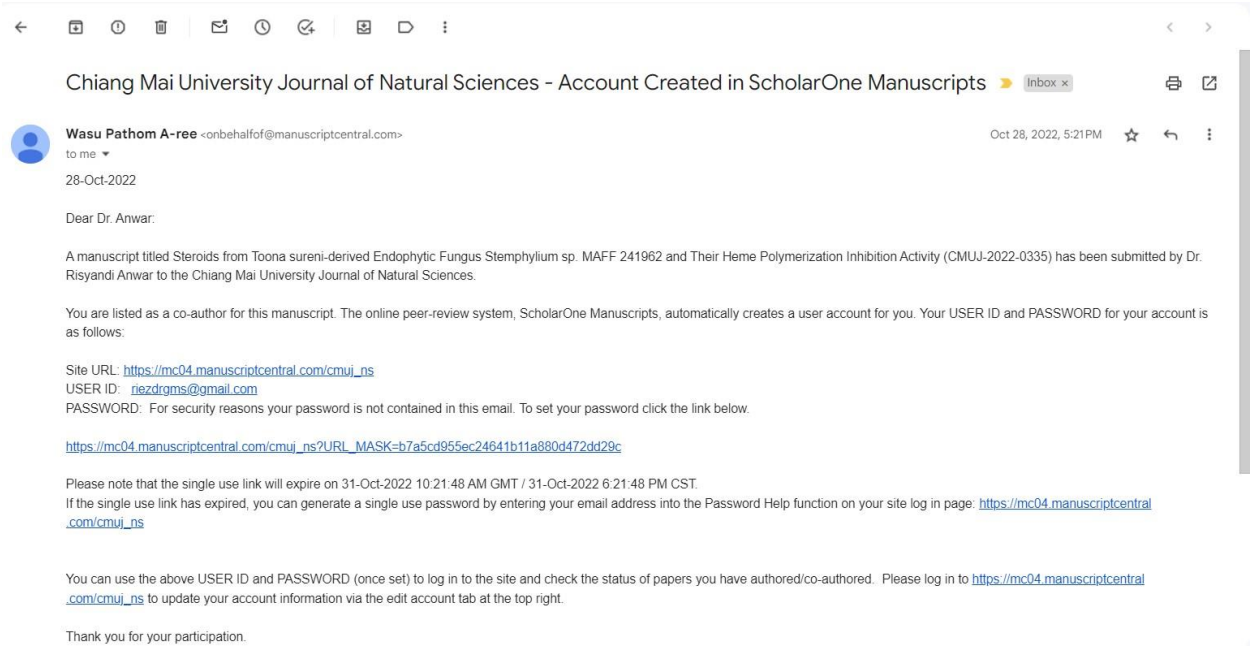

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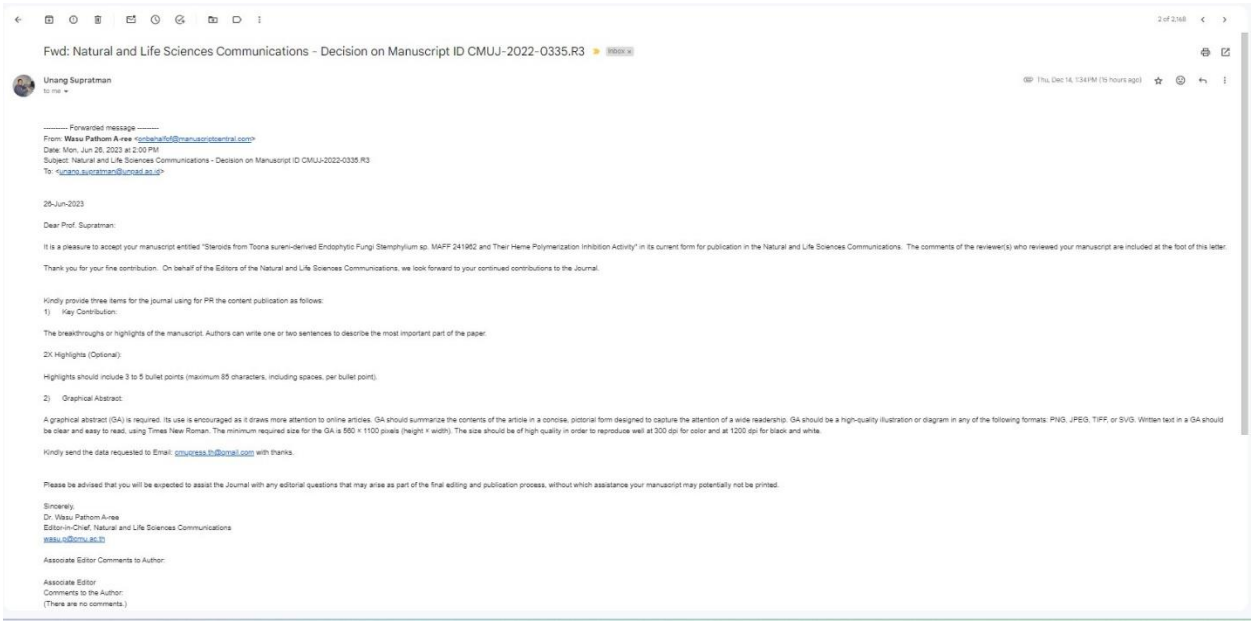

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26-Jun-2023

Dear Prof. Supratman:

It is a pleasure to accept your manuscript entitled "Steroids from Toona sureni-derived Endophytic Fungi Stemphylium sp. MAFF 241962 and Their Heme Polymerization Inhibition Activity" in its current form for publication in the Natural and Life Sciences Communications. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

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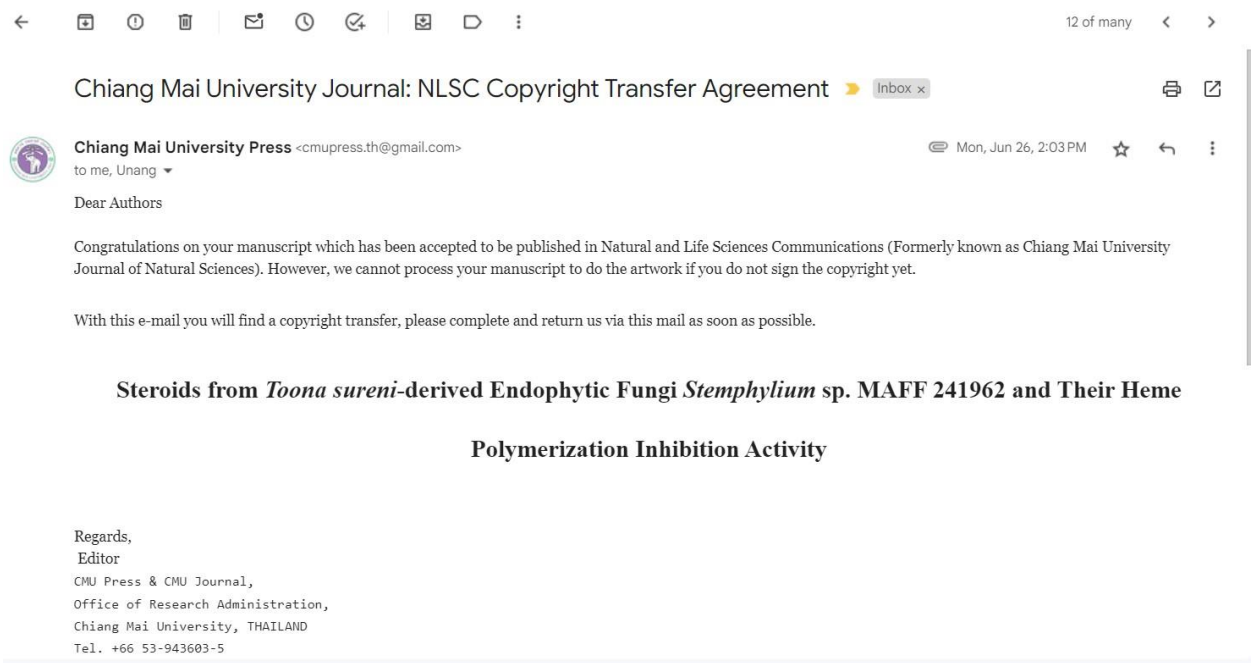

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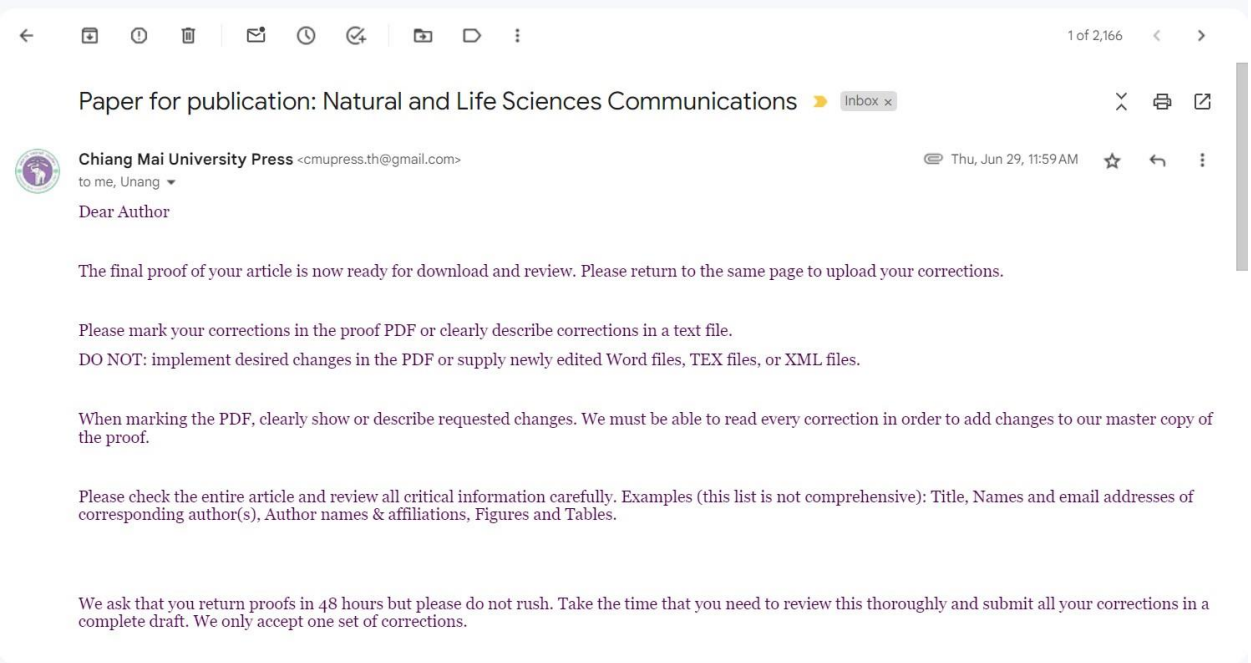

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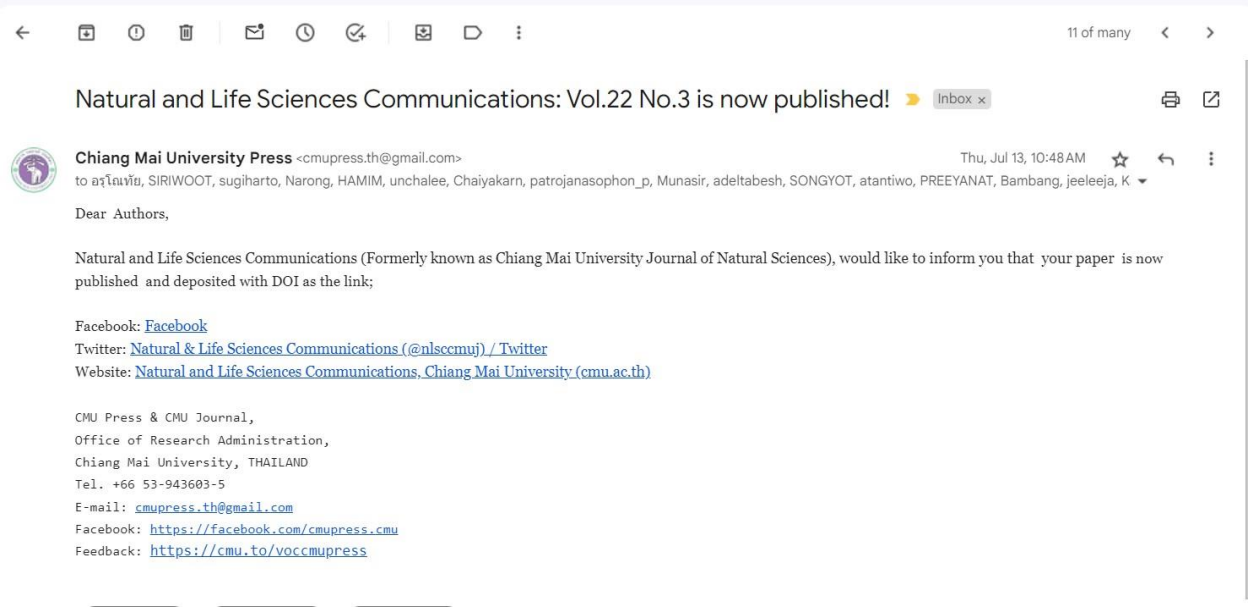

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12-May-2023

Dear Prof. Supratman:

Manuscript ID CMUJ-2022-0335 entitled "Steroids from Toona sureni-derived Endophytic Fungus Stemphylium sp. MAFF 241962 and Their Heme Polymerization Inhibition Activity" which you submitted to the Natural and Life Sciences Communications, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

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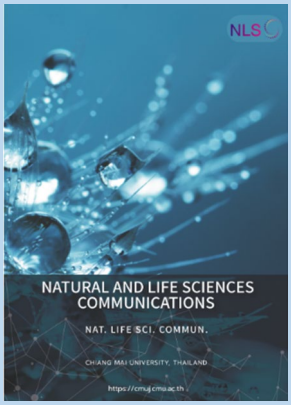

Editor:
Nisit Kittipongpatana,
Chiang Mai University, Thailand

Article history:
Received: October 18, 2022;
Revised: June 16, 2023;
Accepted: June 26, 2023;
Published online: ...., 2023

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Steroids from *Toona sureni*-derived Endophytic *Fungi Stemphylium* sp. MAFF 241962 and Their Heme Polymerization Inhibition Activity

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ABSTRACT

Antimalarial drug resistance is a major cause of the increasing incidence of malaria worldwide, necessitating an urgent demand for the development of new antimalarial drugs. However, the availability of bioactive natural compounds derived from plants is often limited. To address this issue, continuous exploration of bioactive secondary metabolites from endophytic fungi derived from medicinal plants has been recognized as a viable alternative. Therefore, this research aimed to isolate and characterize three ergosteroids, namely isocyathisterol (1), ergosterol-5, 8-peroxide (3), cerevisterol (4), and a phytosterol, β-sitosterol (2), from the rice cultures of endophytic fungus *Stemphylium* sp. MAFF 241962, derived from *Toona sureni*. Endophytic fungi species were determined using molecular analysis of the internal transcribed region (ITS) of the ribosomal DNA. After comparing the sequence data to the NCBI database using BLAST, endophytic fungi were identified as *Stemphylium* sp. 241962 with 100% similarity. The chemical structures were elucidated using spectroscopic methods, including 1D and 2D NMR. Antimalarial activities of compounds 1-4 were evaluated using heme polymerization inhibition activity (HPIA) method. The results showed moderate inhibition activities with IC_{50} values of 7.70 ± 0.11, 9.48 ± 0.09, 7.88 ± 0.10, and 8.36 ± 0.56 mg/mL, respectively, compared to positive control chloroquine diphosphate with IC_{50} values of 1.59 ± 0.03 mg/mL.

Keywords: Steroid, *Stemphylium* sp., *Toona sureni,* Antimalarial activity, Heme polymerization inhibition

Funding: The authors are grateful to the Universitas Muhammadiyah, Semarang, Indonesia for the Research Grant for International Collaboration Grant, 2022, No. 0001/UNIMUS.L/PT/PJ.INT/2022 by Risyandi Anwar

Citation: Anwar, R., Pratama, G. B., Unang Supratman, U., Harneti, D., Azhari, A., Fajriah, S., Azmi, M. N., and Shiono, Y. 2023. Steroids from *Toona sureni*-derived Endophytic Fungi *Stemphylium* sp. MAFF 241962 and Their Heme Polymerization Inhibition Activity. Natural and Life Sciences Communications. 22(3): e2023055.

INTRODUCTION

Malaria is still the most serious public health problem and lethal parasite infection globally due to the emergence and spread of its drug resistance (Ouattara et al., 2014; Fitriastuti et al., 2017). In 2020, the number of malaria cases worldwide increased to 241 million, with an estimated 627.000 deaths, of which 77% were children under the age of five (World Health Organization, 2021). Therefore, there is an urgent need to investigate new sources of antimalarial drugs.

Endophytic fungi are renowned as valuable sources of bioactive natural products with a wide spectrum of biological and pharmacological properties (Zhou et al., 2014; Deshmukh et al., 2018; Supratman et al., 2021a). These fungi live symbiotically inside the plant tissues without harming the plant and provide bioactive substances that may give the host plant advantages for survival and protection (Shiono et al., 2013). The host plants receive antimicrobials, growth regulators, insecticides, antivirals, and tolerance to abiotic stresses from endophytes (Ibrahim et al., 2018; Rana et al., 2020). Based on previous research, endophytic fungi that live inside medicinal plants have acquired the capacity to generate biologically active metabolites similar to those produced by their host plants (Supratman et al., 2021b; Suzuki et al., 2019). Some notable examples include taxol from Taxomyces andreanae and the host plant *Taxus brevifolia* (Uzma et al., 2018), which is also produced from *Stemphylium sedicola* SBU-16 and the host plant *Taxus baccata* (Mirjalili et al., 2012). These also include the antimalarial compounds artemisinin from *Pseudonocardia* sp. and the host plant *Artemisia annua* (Li et al., 2012), and quinine from *Diaporthe* sp. and *Cinchona ledgeriana* (Maehara et al., 2010). Therefore, endophytic fungi can be an alternative, dependable, and efficient way to produce bioactive secondary metabolites originating from plants (Uzma et al., 2018). Endophytic fungi, mostly found inside medicinal plants, have been recognized for its capability to produce a variety of bioactive compounds (Aly et al., 2010; Moussa et al., 2016; Li et al., 2020; Stricker et al., 2021).

Toona sureni is a medicinal plant from the *Meliaceae* family that contains a variety of secondary metabolites with a wide range of bioactivities and is popularly used for the conventional treatment of several illnesses, including malaria (Chen et al., 2009; Ekaprasada et al., 2015; Chini et al., 2016). This plant also produces some antimalarial compounds with potential activity (Cuong et al., 2007). A previous report described the isolation of a new steroid, (22E)-3α,6α,9a-ergosta-7,22-diene-3,6,9-triol from *Periconia pseudobyssoides* K5, an endophytic fungus derived from *Toona sureni* (Azhari et al., 2023). In further exploration for antimalarial substances, the chemical investigation of other endophytic fungi derived from *Toona sureni, Stemphylium* sp. MAFF 241962, was conducted. In this research, the isolation and structure elucidation of three ergosteroid, isocyathisterol (1), ergosterol-5,8-peroxide (3), and cerevisterol (4), as well as phytosterol, β-sitosterol (2), along with antimalarial activity using in vitro assay, the heme polymerization inhibition activity (HPIA) method, are described.

MATERIALS AND METHODS

General experimental procedures

IR spectra were recorded in a KBr plate using PerkinElmer Spectrum 100 FT-IR spectrometer (PerkinElmer, Shelton, USA). High resolution of mass spectra (HR-TOFMS) was determined on a Water Xevo Q-TOF direct probe/MS system, using ESI mode and microchannel plates MCPs detector (Milford, MA,

USA). NMR spectra were recorded on JEOL JNM-ECX500R/S1 spectrometer (JEOL, Tokyo, Japan) and Bruker Topspin spectrometer (Karlsruhe, Germany) using 500 MHz for ^{1}H and 125 MHz for ^{13}C with TMS as an internal standard. Subsequently, column chromatography (CC) was conducted on silica gel 60 (70-230 and 230-400 mesh, Merck, Darmstadt, Germany) and octa desylsilane (Chromatorex® C18 DM1020 M, 200-400 mesh, Fuji Sylisia, Tokyo, Japan). Thin-layer chromatography (TLC) plates were precoated with silica gel GF_{254} (0.25 mm, Merck, Darmstadt, Germany), and spot detection was obtained by spraying with 10% H_2SO_4 in EtOH, followed by heating.

Materials

The stem of *Toona sureni* was collected from a residential garden in Kuningan, West Java, Indonesia, in September 2019. Plant determination was conducted at Herbarium Jatinangor, Plant Taxonomy/Biosystematics Laboratory, Department of Biology, Universitas Padjadjaran, Indonesia. Endophytic fungi was isolated from the inner tissues of the *Toona sureni* stem using a surface sterilization method based on a previously reported method (Shiono et al., 2014). Subsequently, the fresh mycelia from the single endophytic fungi strain were cultivated on 5.5 kg of unpolished red rice (25g/flask x 220) at room temperature for 4 weeks.

Identification of endophytic fungi species

Fungi species were identified by Genetika Science Indonesia using molecular analysis of the internal transcribed region (ITS) of the ribosomal DNA with several steps, namely (1) Genomic DNA extraction with Quick-DNA Fungi/Bacterial Miniprep Kit (Zymo Research, D6005), (2) PCR amplification with MyTaq HS Red Mix (Bioline, BIO-25048), and (3) Bi-directional sequencing. The resulting sequences were compared for similarity to the NCBI database known as Basic Local Alignment Search Tool (BLAST). Based on the comparison of the sequence data with the NCBI database using BLAST, endophytic fungus K2 was identified as Stemphylium sp. 241962 with 100% similarity.

Extraction and isolation

The cultured rice was macerated using ethyl acetate (EtOAc), and the extract was concentrated under a vacuum to yield EtOAc extract (258 g). The concentrated extract was mixed with 500 mL of distilled water and partitioned using n-hexane, followed by EtOAc. The n-hexane extract (101 g) was subjected to vacuum liquid chromatography (VLC) using a gradient elution of *n*-hexane, EtOAc, and MeOH, which was concentrated separately to yield 10 fractions (A – J) based on TLC profiles. Fraction D (4.87 g) was separated with silica gel column chromatography using *n*-hexane:EtOAc 10% stepwise to yield 9 sub-fractions (D1 – D9) that were combined according to TLC profiles. Sub-fraction D5 (659 mg) was separated using silica gel column chromatography with n-hexane:EtOAc 5% stepwise (100:0 to 1:1) to yield 12 sub-fractions (D5A – D5L). Subsequently, sub-fraction D5F (101 mg) was separated using silica gel column chromatography with *n*-hexane:EtOAc (10:1) to yield D5F1 as 2 (8.3 mg) and D5F7 (17 mg), which was further separated using silica gel column chromatography with *n*-hexane:EtOAc (7:3) to give 1 (4.4 mg). D5I (113 mg) was subjected to separation using column chromatography with *n*-hexane:EtOAc (9:1) to give 3 (23.2 mg). Fraction G (710 mg) was also subjected to separation using column chromatography with *n*-hexane: CH_2Cl_2 10% stepwise to yield 10 subfractions (G1 – G10). This was followed by the separation of subfraction G7 (93 mg) using column chromatography on ODS (200 – 400 mesh) eluted with MeOH:water (7:3) to yield G7E as 4 (5.7 mg).

Heme polymerization inhibitory activity assay

The heme polymerization inhibitory activity test was conducted following the previously reported method (Tjitraresmi et al., 2020). 100 µL of sample was prepared with a series of concentrations of 5.0, 2.5, 1.25, 0.625, and 0.3125 mg/mL diluted in DMSO 10% solution. 50 µL of each sample was added to an Eppendorf tube containing 100 µL of 1 mM hematin dissolved in 0.2 M NaOH. Chloroquine diphosphate was prepared as the positive control and DMSO 10% as the negative control. All samples and controls were added to 50 µL of glacial acetic acid (pH 2.6) to start the polymerization reaction and incubated at 37°C for 24 hours. Subsequently, all samples were centrifugated at 8000 rpm for 10 minutes, and the precipitate was separated from the supernatant. The precipitate from each sample was washed using DMSO and centrifugated at 8000 rpm for 10 minutes with four repetitions. The washed precipitate was diluted with 200 µL of 0.1 M NaOH. Each 100 µL of the solution obtained was added to a 96-well microplate, and the absorbance was recorded by a microplate reader at λ 405 nm. Values for heme polymerization inhibitory activity were presented as IC_{50}, or a concentration that can reduce heme polymerization by 50% compared to the negative control. A standard curve of hematin was constructed by making a series of concentrations, namely 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, and 3.9063 mM. 100 µL of each concentration was added to a 96-well microplate, and the absorbance was recorded by a microplate reader at λ 405 nm (Figure 1). The percentage of inhibition was calculated using Equation (1) and the IC_{50} was determined using the linear equation from each sample. The standard curve of hematin is shown in Figure 1.

$$\% \text{ Inhibition} = \frac{(\text{Control Absorbance} - \text{Sample Absorbance})}{\text{Control Absorbance}} \text{ x } 100\% \qquad (1)$$

RESULTS

Isocyathisterol (1). White powder; UV ($CHCl_3$) λmax 275 nm; IR (KBr) vmax 3414, 2920 & 2850, 1651, and 1463 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz): δH 6.13 (^{1}H, d, *J* = 9.5 Hz, H-7), 6.06 (^{1}H, d, *J* = 9.5 Hz, H-6), 5.70 (^{1}H, s, H-4), 5.16 (^{1}H, dd, *J* = 8.0, 16 Hz, H-23), 5.12 (^{1}H, dd, *J* = 8.0, 16 Hz, H-22), 1.31 (^{3}H, s, Me-19), 1.00 (^{3}H, s, Me-18), 0.97 (^{3}H, d, *J* = 7.0 Hz, Me-21), 0.89 (^{3}H, d, *J* = 7.0 Hz, Me-28), 0.81 (^{3}H, d, *J* = 7.0 Hz, Me-26), 0.80 (^{3}H, d, *J* = 7.0 Hz, Me-27), ^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 1; HR-TOFMS m/z found 411.3263 $[M+H]^+$, (calculated for $C_{28}H_{43}O_2$+, *m/z* 411.3263).

β-sitosterol (2). White crystalline powder; IR (KBr) vmax 3421, 2937 & 2867, and 1462 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz): δH 5.35 (^{1}H, m, H-6), 3.51 (^{1}H, m, H-3), 0.99 (^{3}H, s, Me-18), 0.90 (^{3}H, d, *J* = 6.0 Hz, Me-21), 0.83 (3H, d, *J* = 6.0 Hz, Me-26), 0.81 (^{3}H, d, *J* = 6.0 Hz, Me-27), 0.80 (^{3}H, t, *J* = 7.2 Hz, Me-29), 0.66 (^{3}H, s, Me-19), ^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 1; HR-TOFMS *m/z* found 397.4008 $[M+H]^+$, (calculated for $C_{29}H_{49}$-, *m/z* 397.4046).

egosterol-5,8-peroxide (3). White crystalline powder; IR (KBr) vmax 3471, 2955 & 2869, 1458, dan 967 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz): δH 6.48 (^{1}H, d, *J* = 8.5 Hz, H-7), 6.22 (^{1}H, d, *J* = 8.5 Hz, H-6), 5.20 (^{1}H, dd, *J* = 8.0, 15.2 Hz, H-23), 5.11 (^{1}H, dd, *J* = 8.0, 15.2 Hz, H-22), 3.95 (^{1}H, tt, *J* = 5.0, 10.5 Hz, H-3), 0.97 (^{3}H, d, *J* = 6.5 Hz, Me-21), 0.89 (^{3}H, d, *J* = 6.8 Hz, Me-28), 0.86 (^{3}H, s, Me-19), 0.81 (^{3}H, d, *J* = 6.8 Hz, Me-27), 0.79 (^{3}H, d, *J* = 6.7 Hz, Me-26), 0.79 (3H, s, Me-18), ^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 1; HR-TOFMS *m/z* found 451,3184 $[M+Na]^+$, (calculated for $C_{28}H_{44}O_3Na$, *m/z* 451.3188).

cerevisterol (4). White powder; IR (KBr) vmax 3295, 2955 & 2870, dan 1458 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz): δH 5.25 (^{1}H, m, H-7), 5.21 (^{1}H, m, H-23), 5.16 (^{1}H, m, H-22), 4.84 (^{1}H, d, *J* = 6.5 Hz, H-6), 4.07 (^{1}H, m, H-3), 1.05 (3H, s, Me-19), 1.03 (3H, d, *J* = 6.6 Hz, Me-21), 0.91 (^{3}H, d, *J* = 6.8 Hz, Me-28), 0.83 (3H, dd, *J* = 6.8, 9.3 Hz, Me-27), 0.81 (3H, dd, *J* = 6.8, 9.3 Hz, Me-26), 0.58 (^{3}H, s, Me-18), ^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 1; HR-TOFMS *m/z* found 429.3346 $[M+Na]^+$, (calculated for C28H45O3- *m/z* 429.3346).

Table 1. ^{13}C-NMR data of Compounds 1-4 (125 MHz, in $CDCl_3$).

Position Carbon	Compounds			
	1 δc	**2** Δc	**3** δc	**4** δc
1	35.1	37.3	34.7	32.5
2	33.7	31.7	30.1	30.7
3	199.9	71.8	66.5	67.5
4	125.2	42.3	36.9	39.3
5	163.4	140.8	82.2	75.4
6	128.0	121.7	135.5	73.5
7	139.3	31.7	130.8	114.2
8	71.8	31.9	79.4	145.8
9	53.1	50.2	51.1	43.5
10	36.2	36.5	37.0	37.4
11	17.9	21.1	23.4	22.1
12	40.8	39.8	39.4	39.2
13	44.1	42.3	44.6	43.9
14	57.0	24.3	51.7	55.0
15	22.7	28.3	20.7	22.9
16	28.2	56.1	28.7	28.0
17	56.4	11.9	56.2	56.1
18	14.5	19.8	12.9	12.4
19	18.9	36.2	18.2	18.3
20	39.8	18.8	39.7	40.5
21	20.6	34.0	20.9	19.7
22	135.3	26.1	135.2	135.5
23	132.2	45.9	132.3	132.3
24	42.8	29.2	42.8	42.9
25	33.1	19.1	33.1	33.2
26	20.0	19.4	19.7	20.1
27	19.7	23.1	20.0	21.2
28	17.7	12.0	17.6	17.6
29	-	24.3	-	22.9

Table 2. The IC_{50} values of compound 1 – 4, n-hexane extract, and positive control chloroquine diphosphate according to HPIA assay.

Samples	IC_{50} (mg/mL)
Isocyathisterol (1)	7.70 ± 0.11
β-sitosterol (2)	9.48 ± 0.09
Ergosterol-5,8-peroxide (3)	7.88 ± 0.10
Cerevisterol (4)	8.36 ± 0.56
n-hexane extract	2.46 ± 0.04
*Chloroquine diphosphate	1.59 ± 0.03

Note: * Positive control

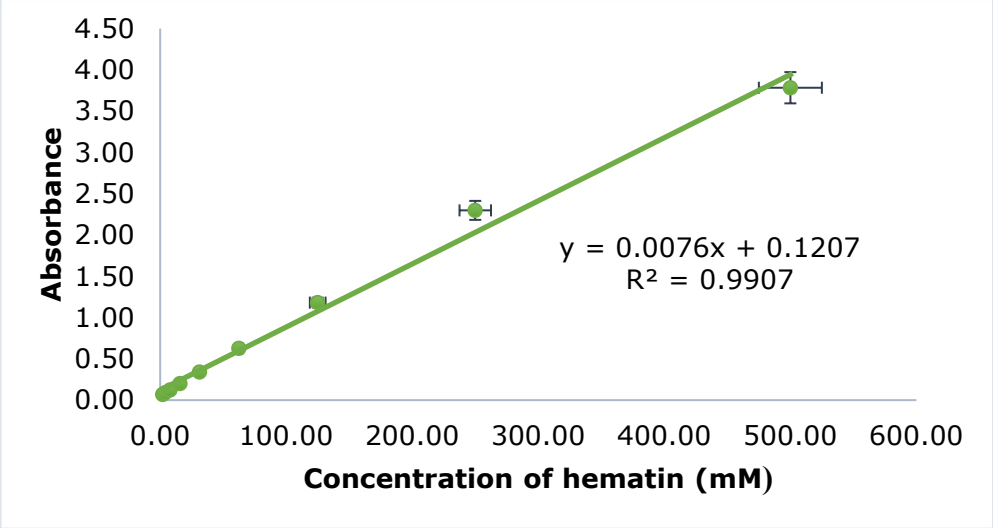

Figure 1. Hematin standard curve.

DISCUSSION

Compound 1 was obtained as a white powder. The molecular formula was identified by HR-ESI-TOFMS measurement using positive-ion-high-resolution as $C_{28}H_{42}O_2$ with the presence of $[M+H]^+$ molecular ion peak at *m/z* 411.3263 (calculated for $C_{28}H_{43}O_2^+$, *m/z* 411.3263) indicated as eight degrees of unsaturation. The IR absorption bands implied the presence of hydroxyl (3414 cm^{-1}), aliphatic (2920 cm^{-1} and 2850 cm^{-1}), conjugated carbonyl (1651 cm^{-1}), and alkene (1463 cm^{-1}) groups. The ^{1}H-NMR spectrum exhibited proton resonances related to six methyl signals, including two methyl singlets at δ_H 1.00 (3H, s) and 1.31 (3H, s) and four methyl doublets at δ_H 0.80 (3H, d, 7 Hz), 0.81 (^{3}H, d, 7 Hz), 0.89 (3H, d, 7 Hz) and 0.97 (^{3}H, d, 7 Hz). Furthermore, methines and methylene signals accumulated in δ_H 1.10 – 2.50 ppm, which were indicated as characteristics of steroid groups. There were also five olefinic proton signals assigned as four typical ergosterol olefinic proton with two doublets at δ_H 6.07 (^{1}H, d, 9.5 Hz) and 6.14 (^{1}H, d, 9.5 Hz), two double doublets at δ_H 5.10 (^{1}H, dd, 8 Hz, 16 Hz) and 5.20 (^{1}H, dd, 8 Hz, 16 Hz), and one additional singlet olefinic signal at δ_H 5.70 (^{1}H, s). The ^{13}C NMR and DEPT-135 spectrum analysis revealed 28 signals defined as six methyl, six methylenes, 11 methines, and five quaternary carbons, with one oxygenated quaternary carbon and one typical carbonyl signal. Based on HMQC experiments, compound 1 was confirmed to have an ergostane-type steroid skeleton. Therefore, the eight degrees of unsaturation can be attributed to the presence of three pairs of C sp^2, one C carbonyl group, and the remaining tetracyclic ergostane-type steroid.

The carbonyl group position was determined by the HMBC correlation of δ_H 2.06 (H-1) to 2.41 ppm (H-2) and δ_H 5.70 ppm (H-4) to δ_C 199.9 ppm (C-3). The HMBC correlation of δ_H 6.13 ppm (H-7) with δ_C 71.8 ppm (C-8) revealed the hydroxyl group was attached to C-8. Additionally, the HMBC and 1H-1H COSY correlations also revealed sp2 carbon positions forming conjugated double bonds from C-4 to C-7 (δ_C 125.2, 163.4, 128.0, and 139.3 ppm, respectively), as well as the double bond between C-22 (δ_C 135.3 ppm) and C-23 (δ_C 132.2 ppm). The ^{1}H-^{1}H COSY and HMBC correlation analysis, as shown in Figure 2, confirmed the identity of compound 1 as 8-hydroxyergosta-4,6,22-trien-3-one. Based on the literature comparison, compound 1 had the same planar structure as cyathisterol and isocyathisterol, the previously reported

ergosteroid from *Calvatia cyathiformis* and *Aspergillus ustus*, respectively (Kawahara et al., 1994; Liu et al., 2014).

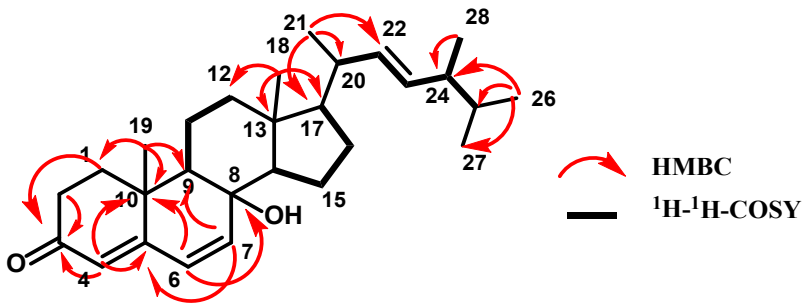

Figure 2. HMBC and ^{1}H-^{1}H COSY correlations of compounds 1.

Based on the difference in NMR chemical shifts around C-8, the isocyathisterol, and cyathisterol were expected to be pairs of isomers in C-9 and/or C-14. The specific optical rotation of isocyathisterol was ⟦[α]⟧ _D^20+61.3, while that ofthe cyathisterol was ⟦[α]⟧ _D^25+133 (Liu et al., 2014). Compound 1 was expected to be isocyathisterol according to the similarity between the C-8 chemical shift andwith previously reported NMR data (δ_{C-8} 71.8 ppm for isocyathisterol and δ_{C-8} 82.0 ppm for cyathisterol) (Kawahara et al., 1994; Liu et al., 2014). The structure of isocyathisterol in Figure 3 was isolated from *Stemphylium* sp. MAFF 241962 for the first time.

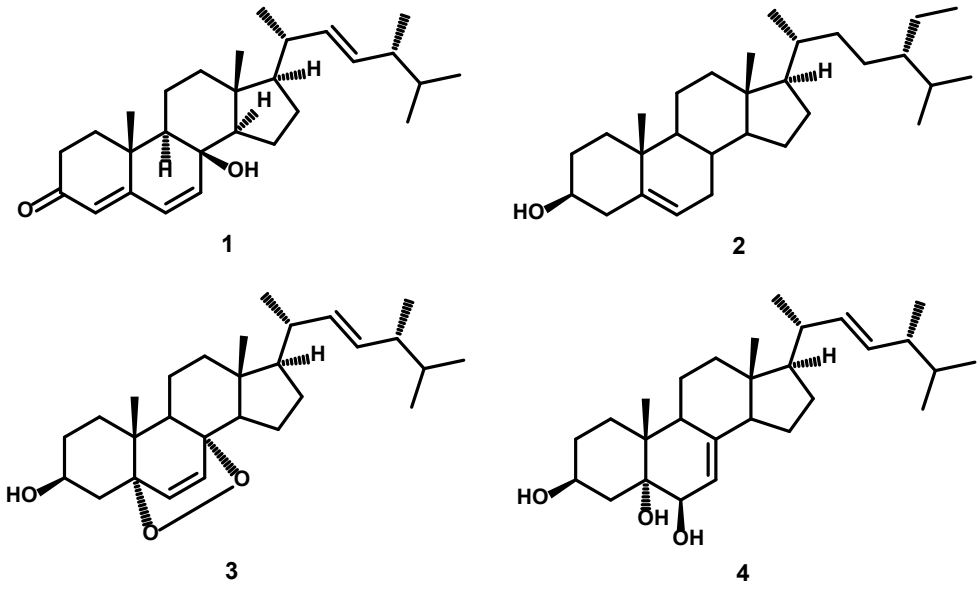

Figure 3. Structures of compounds 1-4

Compound 2 was obtained as a white crystalline powder, and the molecular formula of $C_{29}H_{50}O$ was determined using HR-ESI-TOFMS analysis. The results showed a $[M\text{-}OH]^+$ molecular ion peak at *m/z* 397.4008 (calculated for $C_{29}H_{49}$- *m/z* 397.4046), indicating five degrees of unsaturation. The IR spectrum analysis showed similar absorption as 1, but with the absence of carbonyl absorption, indicating the presence of hydroxyl (3421 cm^{-1}), aliphatic (2937 and 2867 cm^{-1}), and alkene groups (1462 cm^{-1}). The ^{1}H NMR spectrum

revealed similar methine and methylene signals as 1 accumulated in δ_H 1.00 – 2.30 ppm and six methyl resonances, indicating the characteristics of steroid group (Jaeger & Aspers, 2012). However, the difference was identified with the presence of one methyl triplet at δ_H 0.80 (3H, t, 7.2 Hz), which showed the possibility of stigmastane steroid structure. Furthermore, the presence of one multiplet signal from an olefinic proton (δ_H 3,51, ^{1}H, m) and one signal from oxymethine (δ_H 5,35, ^{1}H, m) supported the presence of double bond and hydroxyl groups in the structure, respectively. The ^{13}C NMR and DEPT-135 spectra revealed 29 carbon signals ascribed as six methyls, 11 methylenes, nine methines, including one oxymethine and one unsaturated methine, and three quaternary carbons with one unsaturated quaternary carbon. The presence of one pair of unsaturated carbons in ^{13}C NMR supported by IR absorption indicated one double bond group corresponding to one degree of unsaturation, leaving the remaining four degrees of unsaturation related to tetracyclic structure as a typical steroid group characteristic. Compound 2 showed spectral data that was similar to the known phytosterol, β-sitosterol, isolated from Rubus suavissimus S. Lee by Chaturvedula & Prakash (2012). The phytosterols were originally biosynthesized from plants, although their occurrence in fungi was found to be relatively unusual, especially in endophytic fungi (Carvalho et al., 2016; Jiang et al., 2020). This occurred due to the ability of plant-associated endophytic fungi to produce the same class of compounds known as horizontal gene transfer (HGT), which played an important role in the evolution and adaptation of microorganisms (Tiwari & Bae, 2020). Based on these results, further investigation is recommended to understand HGT signatures and their potential effects on related organisms due to the limited research on gene transfer between plants and endophytes. Therefore, this is the first report of β-sitosterol isolated from Stemphylium sp. MAFF 241962.

Compound 3 was isolated as a white crystalline powder with a molecular formula of $C_{28}H_{44}O_3$ corresponding to HR-ESI-TOFMS analysis with a [M+Na]+ molecular ion peak at m/z 451,3184 (calculated for $C_{28}H_{44}O_3Na$ m/z 451.3188), indicating seven degrees of unsaturation. The IR spectra exhibited similar functional groups as 2, with an additional absorption of C-O-O stretching at 967 cm^{-1} as a typical peroxide group. The 1H NMR spectral data showed similarities to 1 with six methyl signals and four typical olefinic signals for ergosterol derivatives, but with the absence of one additional olefinic proton observed in 1. Although the same total of 28 carbons as in 1 was observed in ^{13}C NMR and DEPT-135 spectral data, the differences were also identified with the absence of carbonyl from 1 and the appearance of two oxygenated quaternary carbons, indicating the presence of a peroxide functional group, and one oxymethine suggested to be the attached hydroxyl group. Based on the analysis, the presence of two pairs of C ^{sp2} was responsible for two degrees of unsaturation. The peroxide group identified in the spectral data was also suggested to form one cyclic with one degree of unsaturation, resulting in a total of four degrees of unsaturation corresponding to tetracyclic skeletal ergosterol derivatives. A comparison of compound 3 spectral data with those of isolated compounds from Eunicella cavolini and Tridemnum inarmatum by Ioannou et al. (2009) revealed a similar structure of compound 3 as ergosterol-5,8-peroxide in Figure 3, which was isolated for the first time from Stemphylium sp. MAFF 241962.

Compound 4 was obtained as a white powder with a molecular formula of $C_{28}H_{46}O_3$ according to HR-ESI-TOFMS experiments of molecular ion peak at *m/z* 429.3346 $[M-H]^+$ (calculated $C_{28}H_{45}O_{3-}$ *m/z* 429.3346), which indicated six degrees of unsaturation. The IR spectrum of 4 revealed a similar absorption as that of 2, originating from the presence of hydroxyl, aliphatic, and alkene groups. The NMR spectrum of 4 showed the typical ergosterol derivatives with a typical proton and a total of 28 carbon signals, as shown in 1 and 3 NMR data. However, the differences were observed as the presence of two

oxygenated methine signals at δ_H 4.07 (1H, m) and 4.84 (^{1}H, d, 6.5 Hz) as well as one oxygenated quaternary carbon from 13C NMR and DEPT-135. This was suggested to be attached to hydroxyl groups as described in IR absorption and the molecular formula. Therefore, the six degrees of unsaturation were defined as two pairs of C sp^2 responsible for two degrees of unsaturation and a tetracyclic skeletal ergosterol structure responsible for the remaining four degrees of unsaturation. The chemical shifts of 4 were further compared with the isolated compound from Cladosporium sp. CYC38 by Jiang et al. (2018). Based on the results, compound 4 was identified as ergosta-7,22-diene-3,5,6-triol or cerevisterol (Figure 3), which was first isolated in Stemphylium sp. MAFF 241962.

Compounds 1 – 4 and the n-hexane extract were tested for their antimalarial activity using a simple colorimetric in vitro assay, namely HPIA. Based on their chemical structures, the carbonyl, hydroxyl, and methyl groups were suggested to exhibit good antimalarial activity in the HPIA assay. This heme polymerization inhibition assay mimics the degradation of hemoglobin into globin and free heme, which occurs in the food vacuole of plasmodium when it infects human erythrocytes. During this process, the globin component will be degraded into amino acids, which are valuable for protein synthesis in plasmodium. Meanwhile, free heme is a toxic substance because it can form oxygen radical species that have the potential to kill plasmodium. For protection, plasmodium detoxifies the free heme through the polymerization reaction to convert the free heme into hemozoin, an insoluble substance that can be excreted through the secretion system (Fitriastuti et al., 2017). This method inhibits this reaction using a polymer analog to hemozoin, namely β-hematin.

Polymerization was conducted in incubation at 37°C for 24 hours, which was the optimum condition for hematin to crystallize. Hematin was used as a substrate in the polymerization reaction, with the isoelectric point at pH 5, similar to the pH in the plasmodium food vacuole. To start polymerization, glacial acetic acid was used as an acidity regulator in the reaction (Fitriastuti et al., 2017). After the incubation, all samples were centrifuged to separate the precipitate from the supernatant. The precipitate was washed four times using DMSO 10% to remove the unreacted hematin entrapped in the β-hematin precipitate. Subsequently, the amount of β-hematin polymer formed was measured with colorimetric quantification using a microplate reader in absorption at λ 405 nm, which correlated contrary to the inhibition activity of heme polymerization by antimalarial compounds. The result of the hematin standard curve measurement is shown in Figure 1, with a coefficient of determination (R2) value of 0.9907.

The IC_{50} values of the compounds, n-hexane extract, and negative and positive controls are shown in Table 2. According to the results, the IC_{50} values for compounds 1–4, *n*-hexane extract, and chloroquine phosphate were 7.70 ± 0.11, 9.48 ± 0.09, 7.88 ± 0.10, 8.36 ± 0.56, 2.46 ± 0.04, and 1.59 ± 0.03 mg/mL, respectively. These results indicated that all the isolated compounds and the extract were less active in inhibiting β-hematin crystal formation than the positive control, chloroquine diphosphate. However, the n-hexane extract had greater activity than the isolated compounds. The IC50 values of the compounds varied according to the difference in functional groups, where compound 4 had greater activity than compound 2, according to the number of hydroxyl groups. Based on previous research (Fitriastuti et al., 2017; Ignatushchenko et al., 1997), the proposed interaction between the β-hematin and 1 was described in Figure 4, which displayed significant interactions between the carbonyl oxygen and heme iron as well as the carboxylate side-groups of heme and the hydroxyl group of 1.

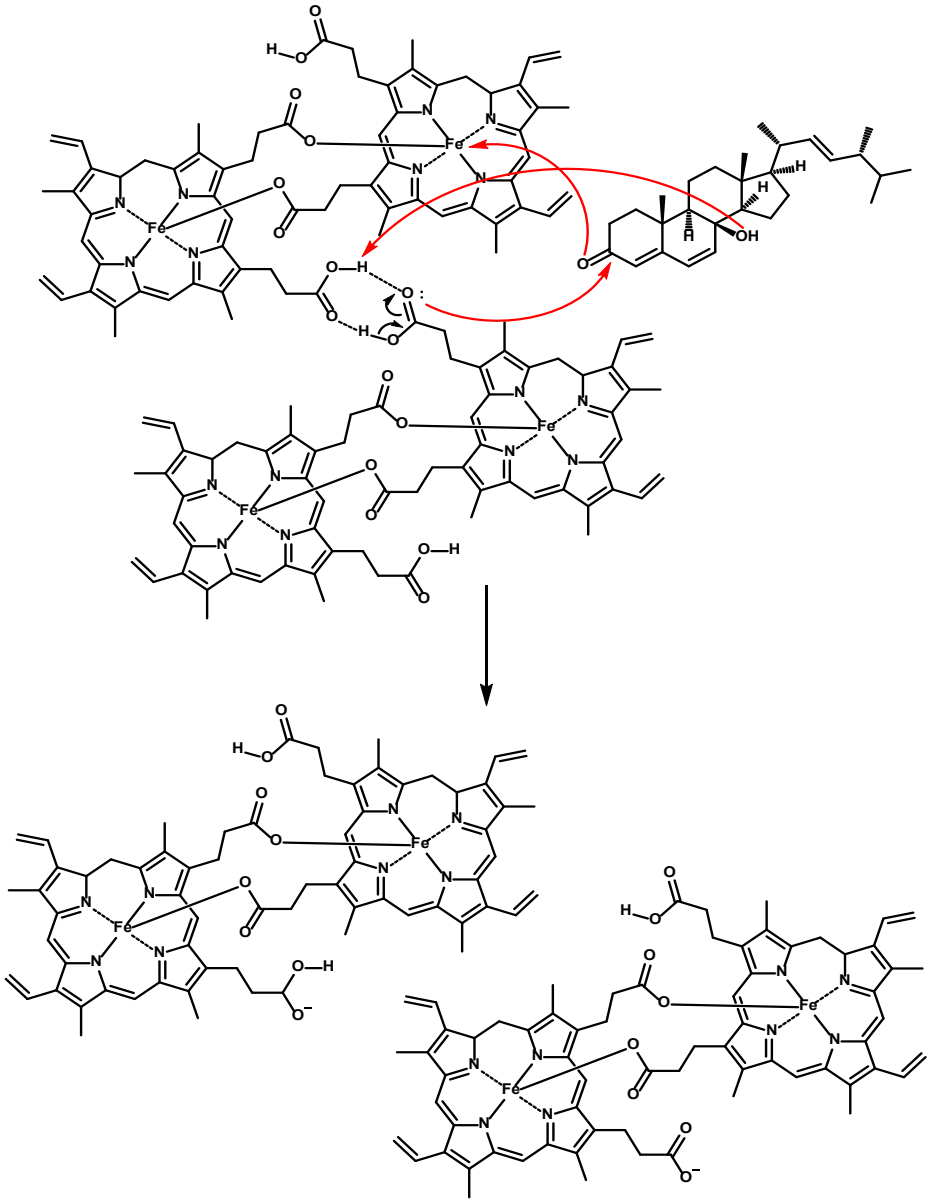

Figure 4. Proposed interaction between β-hematin and compound 1 (Ignatushchenko et al., 1997; Fitriastuti et al., 2017).

CONCLUSION

Isocyathisterol (1), β-sitosterol (2), ergosterol-5,8-proxide (3), and cerevisterol (4) have been isolated from *Stemphylium* sp. MAFF 241962, the endophytic fungus of *T. sureni.* The finding of phytosterol, β-sitosterol (2), might indicate a significant role of HGT between endophytic fungi and the host plant, but further investigation is still needed. The heme polymerization inhibition activity of compounds 1-4 showed moderate activity, with IC_{50} values of 7.70 ± 0.11, 9.48 ± 0.09, 7.88 ± 0.10, and 8.36 ± 0.56 mg/mL, respectively, compared to the positive control, chloroquine diphosphate, with IC_{50} value of 1.59 ± 0.03 mg/mL. This result provides new leads about the antimalarial properties of compounds 1-4. To optimize the compounds' potential as antimalarial agents, additional antimalarial activity assay, such as in vitro assays against Plasmodium falciparum, need to be performed.

ACKNOWLEDGMENTS

The authors are grateful to Sofa Fajriah at the Research Center for Chemistry, National Innovative and Research Council, Indonesia for NMR measurements.

AUTHOR CONTRIBUTIONS

Risyandi Anwar, Galih Bayu Pratama, Sofa Fajriah, and Unang Supratman assisted in conducting the experiments, performed the spectral analysis, and wrote the manuscript. Risyandi Anwar, Galih Bayu Pratama. Desi Harneti, Azmi Azhari, and Yoshihito Shiono designed and conducted all experiments, as well as wrote the manuscript. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there are no competing interests.

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Supplementary Data

Fungal Species Identification

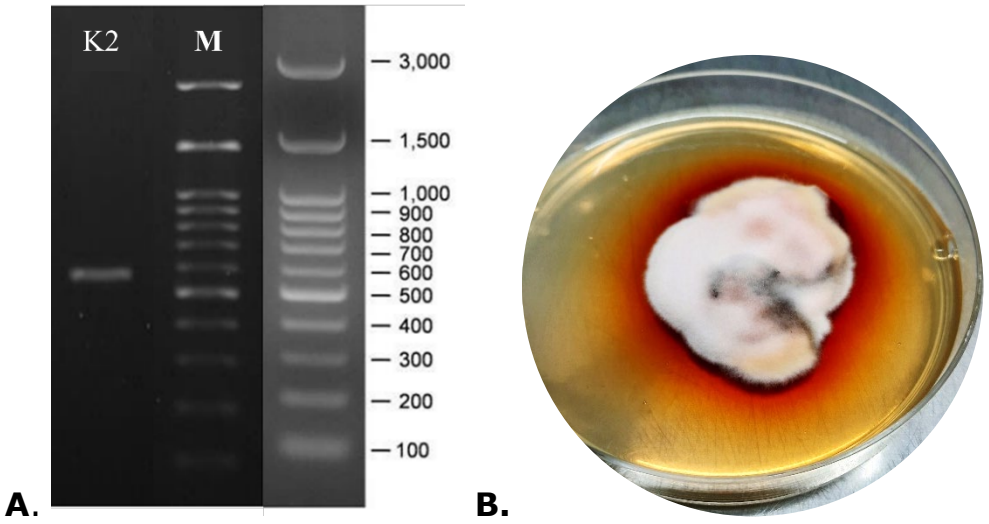

Figure S1. (A) The Gel Photo – PCR Product of Endophytic Fungal K2; (B) The Physical Appearance of Endophytic Fungal K2 in PDA

Table S1. The Sequence Assembly Result – PCR Product of Endophytic Fungal K2

Endophytic Fungal K2 Sequence Assembly (570bp)					
1	TGAACCTGCG	GAGGGATCAT	TACACAATAT	GAAAGCGGGT	TGGGACCTCA
51	CCTCGGTGAG	GGCTCCAGCT	TGTCTGAATT	ATTCACCCAT	GTCTTTTGCG
101	CACTTCTTGT	TTCCTGGGCG	GGTTCGCCCG	CCACCAGGAC	CAAACCATAA
151	ACCTTTTTGT	AATTGCAATC	AGCGTCAGTA	AACAATGTAA	TTATTACAAC
201	TTTCAACAAC	GGATCTCTTG	GTTCTGGCAT	CGATGAAGAA	CGCAGCGAAA
251	TGCGATACGT	AGTGTGAATT	GCAGAATTCA	GTGAATCATC	GAATCTTTGA
301	ACGCACATTG	CGCCCTTTGG	TATTCCAAAG	GGCATGCCTG	TTCGAGCGTC
351	ATTTGTACCC	TCAAGCTTTG	CTTGGTGTTG	GGCGTCTTTG	TCTCTCACGA
401	GACTCGCCTT	AAAATGATTG	GCAGCCGACC	TACTGGTTTC	GGAGCGCAGC
451	ACAATTCTTG	CACTTTGAAT	CAGCCTTGGT	TGAGCATCCA	TCAAGACCAC
501	ATTTTCTTAA	CTTTTGACCT	CGGATCAGGT	AGGGATACCC	GCTGAACTTA
551	AGCATATCAA	TAAGCGGAGG			

NMR Data Compound 1 – 4

Table S2 NMR Data of Compound **1** compared with isocyathisterol (Liu *et al.*, 2014)

Position	Compound 1 *		isocyathisterol *	
	^{13}C NMR δ_C	^{1}H NMR δ_H (ΣH, m, *J* = Hz)	^{13}C NMR δ_C	^{1}H NMR δ_H (ΣH, m, *J* = Hz)
1	35.1	2.06 (1H, m); 2.12 (1H, m)	35.1	1.70 (1H, m); 2.11 (1H, m)
2	33.7	2.41 (1H, m); 2.45 (1H, m)	33.7	2.44 (1H, brdd, 18.2, 5.1) 2.61 (1H, ddd, 18.2, 5.5)
3	199.9	-	199.7	-
4	125.2	5.70 (1H, s)	125.2	5.72 (1H, s)
5	163.4	-	163.3	-
6	128.0	6.06 (1H, d, 9.8)	128.0	6.09 (1H, d, 9.8)
7	139.3	6.13 (1H, d, 9.8)	139.3	6.16 (1H, d, 9.8)
8	71.8	-	71.8	-
9	53.1	1.47 (1H, m)	53.1	1.44 (1H, dd, 12.9, 2.8)
10	36.2	-	36.2	-
11	17.9	1.24 (2H, m)	17.8	1.88 (1H, dddd, 13.0, 13.0, 3.0)
12	40.8	1.28 (1H, m) 2.09 (1H, m)	40.8	1.28 (1H, m); 2.09 (1H, m)
13	44.1	-	44.1	-
14	57.0	1.39 (1H, m)	57.0	1.39 (1H, dd, 13.1, 6.9)
15	22.7	1.48 (1H, m); 1.61 (1H, m)	22.3	1.49 (1H, m); 1.63 (1H, m)
16	28.2	1.23 (1H, m); 1.70 (1H, m)	28.1	1.32 (1H, m); 1.73 (1H, m)
17	56.4	1.11 (1H, m)	56.5	1.12 (1H, dd, 9.6, 9.6, 9.6)
18	14.5	1.00 (3H, s)	14.4	1.02 (3H, s)
19	18.9	1.31 (3H, s)	18.9	1.33 (3H, s)
20	39.8	2.33 (1H, m)	39.7	2.03 (1H, m)
21	20.6	0.97 (3H, d, 6.5)	20.6	0.99 (3H, d, 6.6)
22	135.3	5.12 (1H, dd, 15.2, 8.2)	135.3	5.13 (1H, dd, 15.3, 8.4)
23	132.2	5.16 (1H, dd, 15.2, 8.2)	132.2	5.22 (1H, dd, 15.3, 7.7)
24	42.8	1.82 (1H, m)	42.8	1.85 (1H, m)
25	33.1	1.25 (1H, m)	33.1	1.46 (1H, m)
26	20.0	0.80 (3H, d, 6.7)	20.0	0.82 (3H, dd, 9.3, 6.8)
27	19.7	0.80 (3H, d, 6.8)	19.6	0.84 (3H, d, 6.8)
28	17.7	0.89 (3H, d, 6.8)	17.6	0.91 (3H, d, 6.8)

Note: *$CDCl_3$, ^{1}H NMR 500 MHz, ^{13}C NMR 125 MHz

Table S3. NMR Data of Compound **2** compared with β-sitosterol (Chaturvedula & Prakash, 2012)

Position	Compound 2 *		β-sitosterol **	
	^{13}C NMR δ_C	^{1}H NMR δ_H (ΣH, m, *J* = Hz)	^{13}C NMR δ_C	^{1}H NMR δ_H (ΣH, m, *J* = Hz)
1	37.3		37.5	
2	31.7		31.9	
3	71.8	3.51 (1H, m)	72.0	3.53 (1H, tdd, 4.5, 4.2, 3.8)
4	42.3		42.5	
5	140.8		140.9	
6	121.7	5.35 (1H, m)	121.9	5.36 (1H, t, 6.4)
7	31.7		32.1	
8	31.9		32.1	
9	50.2		50.3	
10	36.5		36.7	
11	21.1		21.3	
12	39.8		39.9	
13	42.3		42.6	
14	56.8		56.9	
15	24.3		26.3	
16	28.3		28.5	
17	56.1		56.3	
18	11.9	0.99 (3H, s)	12.0	0.99 (3H, s)
19	19.8	0.66 (3H, s)	19.0	0.68 (3H, s)
20	36.2		36.3	
21	18.8	0.90 (3H, d, 6.0)	19.2	0.93 (3H, d, 6.5)
22	34.0		34.2	
23	26.1		26.3	
24	45.9		46.1	
25	29.2		29.4	
26	19.1	0.83 (3H, d, 4.0)	19.0	0.83 (3H, d, 6.4)
27	19.4	0.81 (3H, d, 6.0)	19.6	0.81 (3H, d, 6.4)
28	23.1		23.3	
29	12.0	0.80 (3H, t, 7.2)	12.0	0.84 (3H, t, 7.2)

Note: *$CDCl_3$, ^{1}H NMR 500 MHz, ^{13}C NMR 125 MHz; **$CDCl_3$, ^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz

Table S4. NMR Data of Compound **3** compared with ergosterol-5,8-peroxide (Ioannou *et al.*, 2009).

Position	Compound 3 *		ergosterol -5,8-peroxide**	
	^{13}C NMR δ_C	^{1}H NMR δ_H (ΣH, m, *J* = Hz)	^{13}C NMR δ_C	^{1}H NMR δ_H (ΣH, m, *J* = Hz)
1	34.7	1.92 (1H, m); 1.68 (1H, m)	34.7	1.93 (1H, m); 1.67 (1H, m)
2	30.1	1.82 (1H, m); 1.52 (1H, m)	30.7	1.82 (1H, m); 1.52 (1H, m)
3	66.5	3.95 (1H, tt, 10.5, 5.0)	66.5	3.95 (1H, tt, 11.3, 5.0)
4	36.9	2.10 (1H, m); 1.89 (1H, m)	36.9	2.10 (1H, m); 1.89 (1H, m)
5	82.2	-	82.2	-
6	135.5	6.22 (1H, d, 8.5)	135.5	6.22 (1H, d, 8.5)
7	130.8	6.48 (1H, d, 8.5)	130.7	6.48 (1H, d, 8.5)
8	79.4	-	79.4	-
9	51.1	1.47 (1H, m)	51.1	1.48 (1H, m)
10	37.0	-	36.9	-
11	23.4	1.49 (1H, m); 1.20 (1H, m)	23.4	1.50 (1H, m); 1.20 (1H, m)
12	39.4	1.94 (1H, m); 1.22 (1H, m)	39.3	1.94 (1H, m); 1.22 (1H, m)
13	44.6	-	44.5	-
14	51.7	1.55 (1H, m)	51.7	1.54 (1H, m);
15	20.7	1.58 (1H, m); 1.38 (1H, m)	20.7	1.58 (1H, m); 1.38 (1H, m)
16	28.7	1.75 (1H, m); 1.34 (1H, m)	28.9	1.75 (1H, m); 1.34 (1H, m)
17	56.2	1.18 (1H, m)	56.1	1.19 (1H, m)
18	12.9	0.79 (3H, s)	12.8	0.79 (3H, s)
19	18.2	0.86 (3H, s)	18.2	0.86 (3H, s)
20	39.7	2.00 (1H, m)	39.8	1.99 (1H, m)
21	20.9	0.97 (3H, d, 6.5)	20.9	0.97 (3H, d, 6.6)
22	135.2	5.11 (1H, dd, 15.2, 8.2)	135.4	5.10 (1H, dd, 15.2, 8.2)
23	132.3	5.20 (1H, dd, 15.2, 8.2)	132.4	5.17 (1H, dd, 15.2, 8.0)
24	42.8	1.80 (1H, m)	43.0	1.81 (1H, m)
25	33.1	1.46 (1H, m)	33.2	1.44 (1H, m)
26	19.7	0.79 (3H, d, 6.7)	19.6	0.79 (3H, d, 6.7)
27	20.0	0.81 (3H, d, 6.8)	20.1	0.81 (3H, d, 6.8)
28	17.6	0.89 (3H, d, 6.8)	18.0	0.89 (3H, d, 6.8)

Note: *$CDCl_3$, ^{1}H NMR 500 MHz, ^{13}C NMR 125 MHz; **$CDCl_3$, ^{1}H NMR 600 MHz, ^{13}C NMR 75 MHz

Table S5. NMR Data of Compound **4** compared with cerevisterol (Jiang *et al.*, 2018)

Position	Compound 4 *		cerevisterol **	
	^{13}C NMR δ_C	^{1}H NMR δ_H (ΣH, m, *J* = Hz)	^{13}C NMR δ_C	^{1}H NMR δ_H (ΣH, m, *J* = Hz)
1	32.5		32.9	
2	30.7		30.8	
3	67.5	4.07 (1H, m)	67.7	4.06 (1H, m)
4	39.3		39.2	
5	75.4		75.9	
6	73.5	4.84 (1H, d, 6.5)	73.6	3.60 (1H, m)
7	114.2	5.25 (1H, m)	117.5	5.36 (1H, m)
8	145.8		144.0	
9	43.5		43.4	
10	37.4		37.1	
11	22.1		22.0	
12	39.2		39.2	
13	43.9		43.7	
14	55.0		54.7	
15	22.9		22.9	
16	28.0		27.9	
17	56.1		56.0	
18	12.4	0.58 (3H, s)	12.3	0.58 (3H, s)
19	18.3	1.05 (3H, s)	18.8	1.07 (3H, s)
20	40.5		40.4	
21	19.7	1.02 (3H, d, 6.7)	19.6	1.00 (3H, d, 6.6)
22	135.5	5.16 (1H, m)	135.4	5.16 (1H, dd, 7.2, 15)
23	132.3	5.21 (1H, m)	132.2	5.20 (1H, dd, 7.2, 15)
24	42.9		42.8	
25	33.2		33.1	
26	20.1	0.81 (3H, dd, 9.3; 6.8)	19.9	0.80 (3H, d, 6.6)
27	21.2	0.83 (3H, dd, 9.3; 6.8)	21.1	0.82 (3H, d, 6.6)
28	17.6	0.91 (3H, d, 6.8)	17.6	0.90 (3H, d, 7.2)

Note: *$CDCl_3$, ^{1}H NMR 500 MHz, ^{13}C NMR 125 MHz; **$CDCl_3$, ^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz

Compound 1 Structure Elucidation

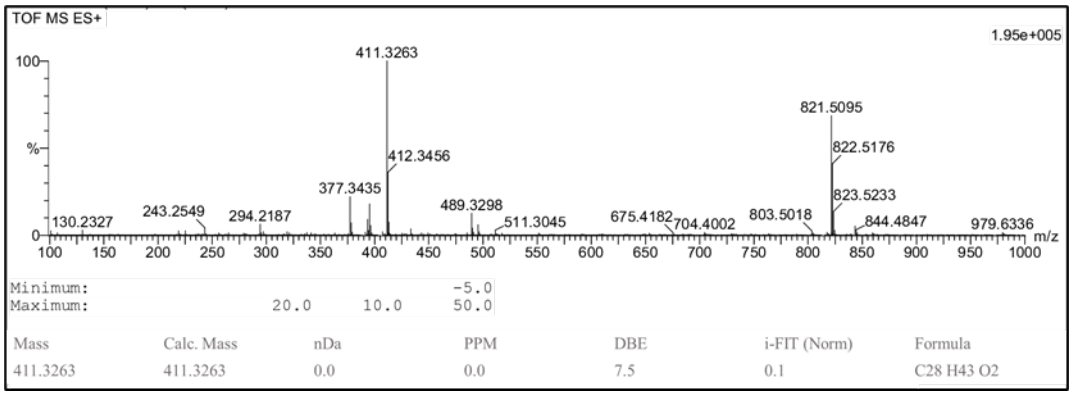

Figure S2. **HR-TOFMS spectrum of compound 1.**

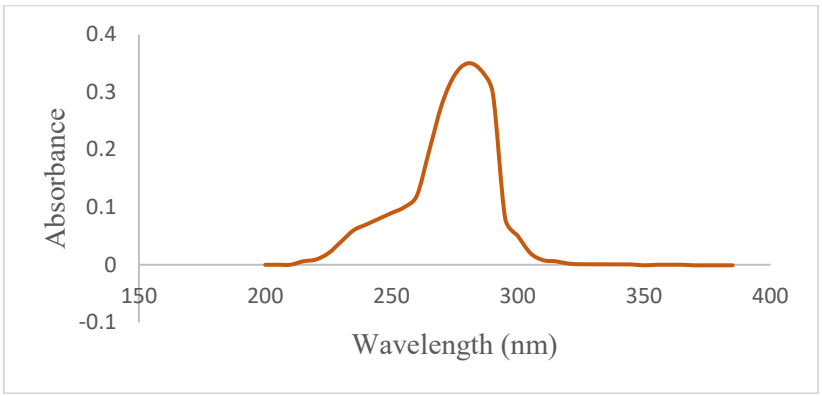

Figure S3. **UV spectrum of compound 1**

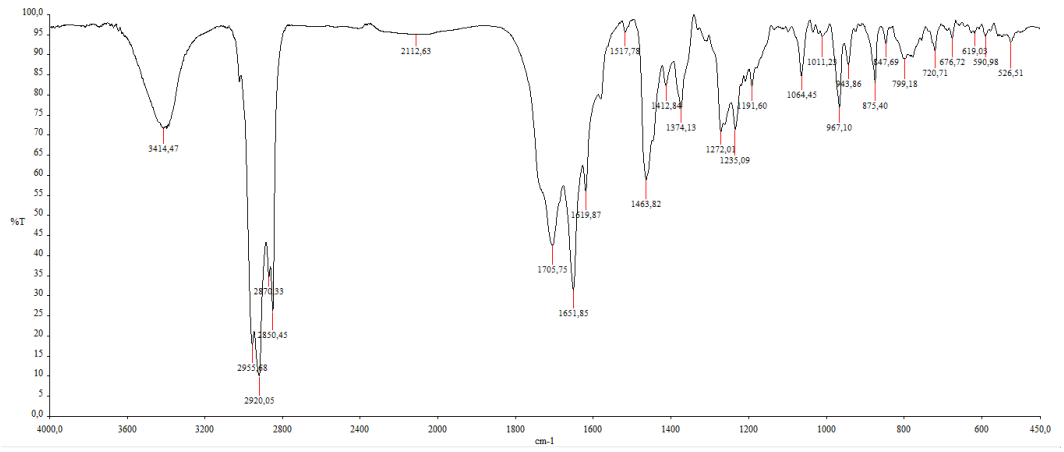

Figure S4. **IR spectrum of compound 1.**

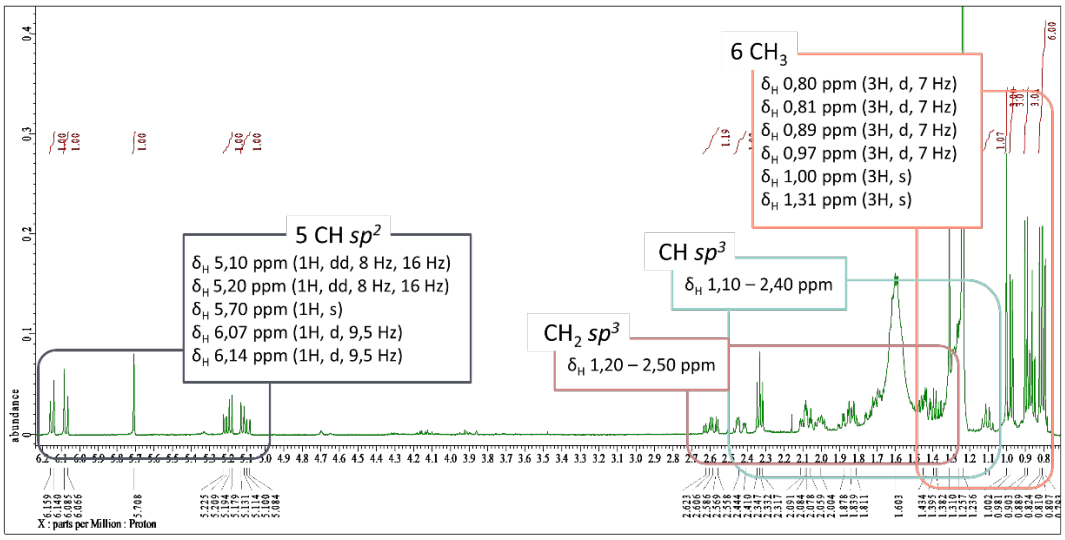

Figure S5. ^{1}H-NMR spectrum of compound 1 (500 MHz, $CDCl_3$).

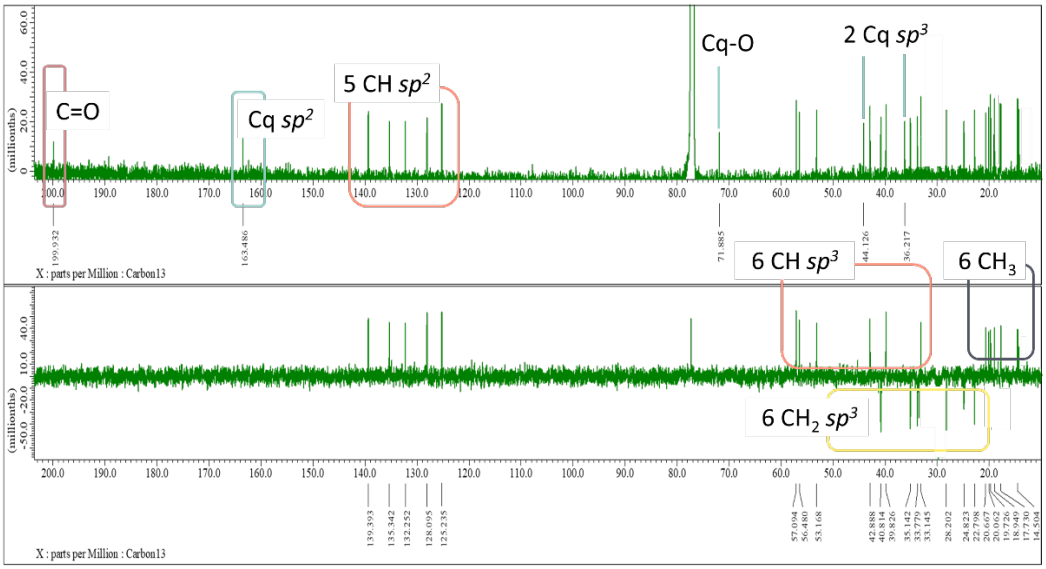

Figure S6. ^{13}C-NMR and DEPT-135 spectrum of compound 1 (125 MHz, $CDCl_3$).

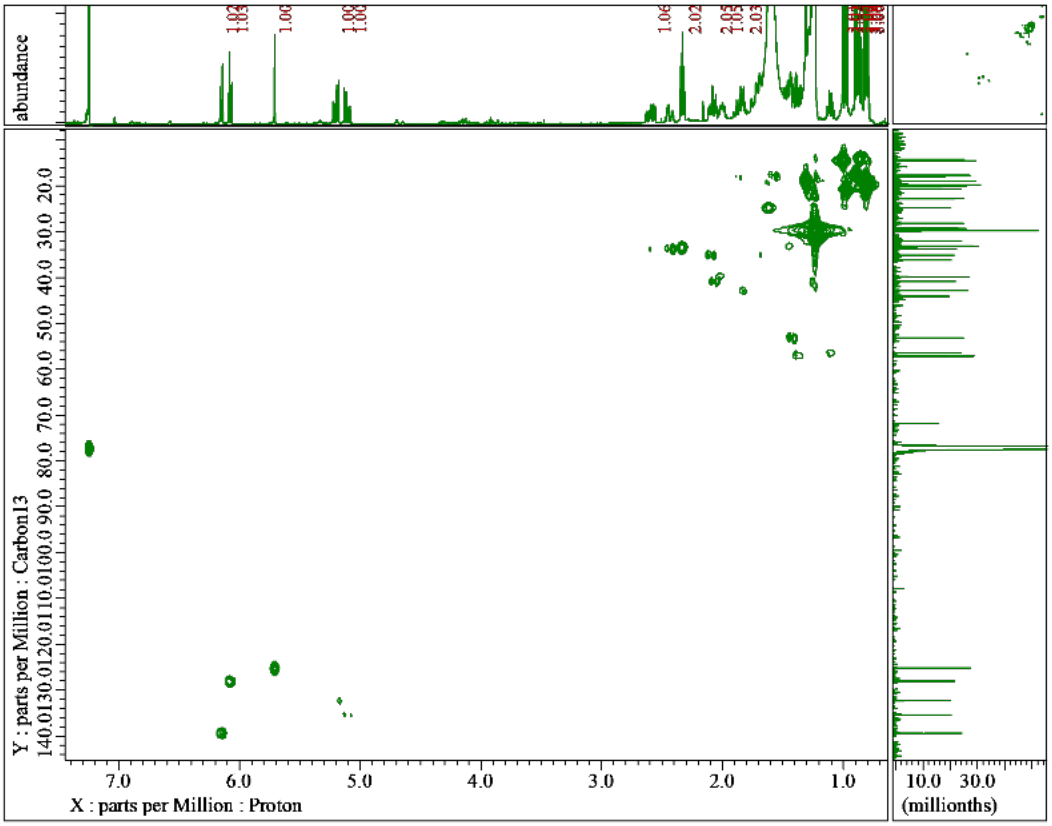

Figure S7. HMQC correlation of compound 1.

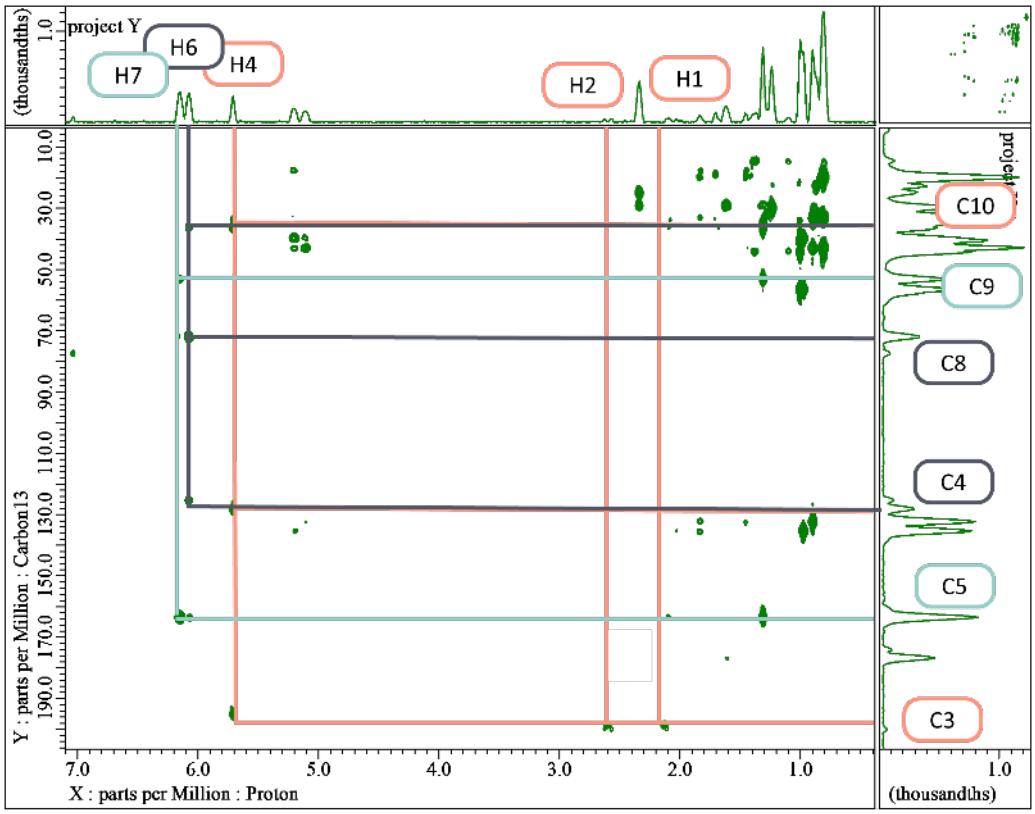

Figure S8. HMBC correlation of compound 1.

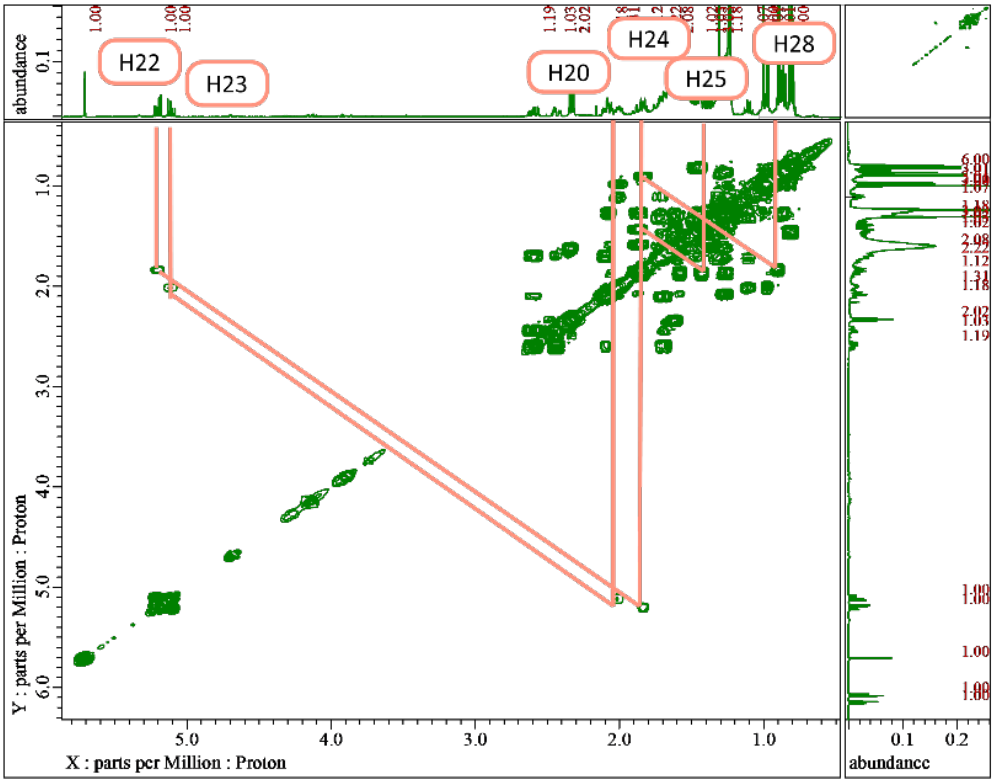

Figure S9. ^{1}H-^{1}H COSY correlation of compound **1**.

Compound 2 Structure Elucidation

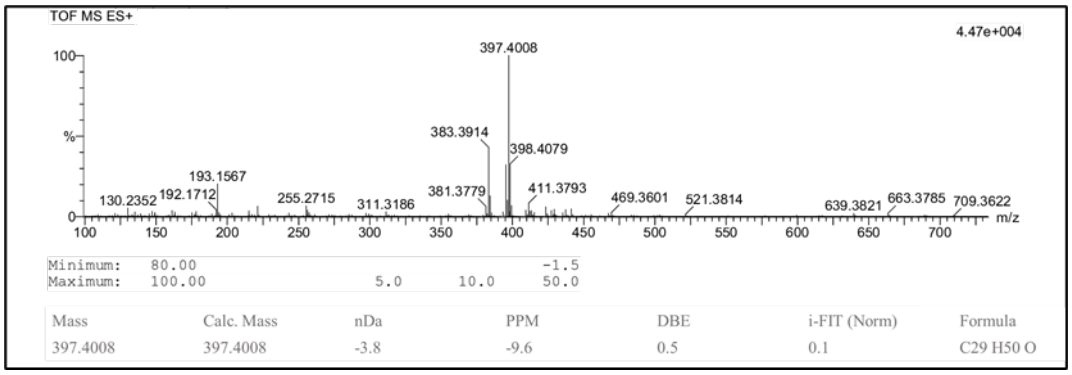

Figure S10. HR-TOFMS spectrum of compound 2.

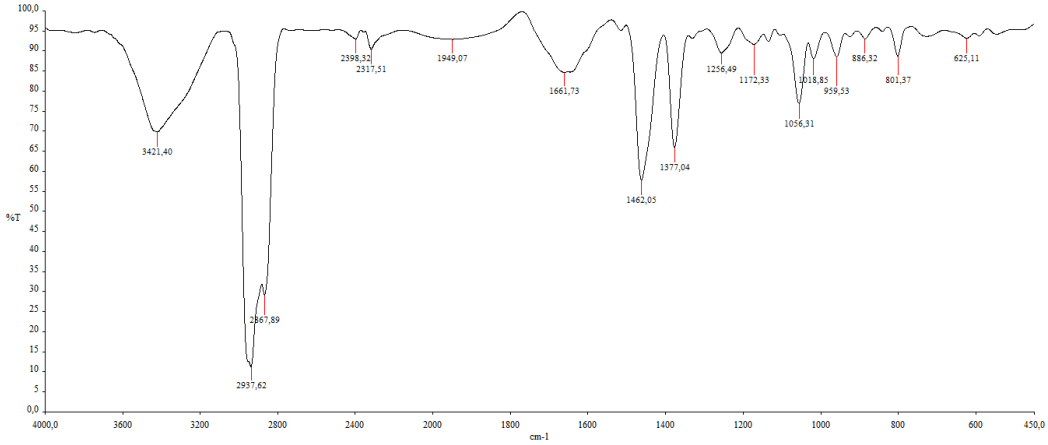

Figure S11. IR spectrum of compound 2.

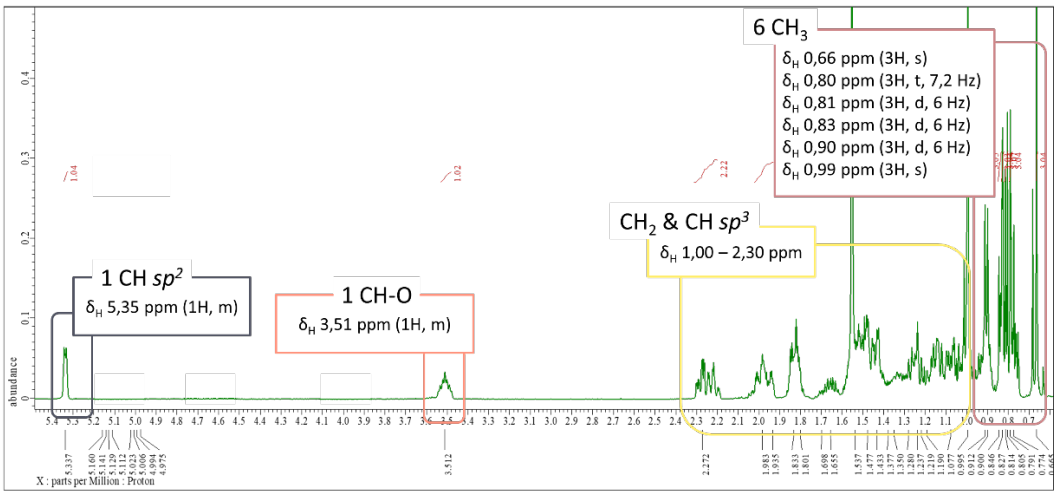

Figure. S12. ^{1}H-NMR spectrum of compound 2 (500 MHz, $CDCl_3$).

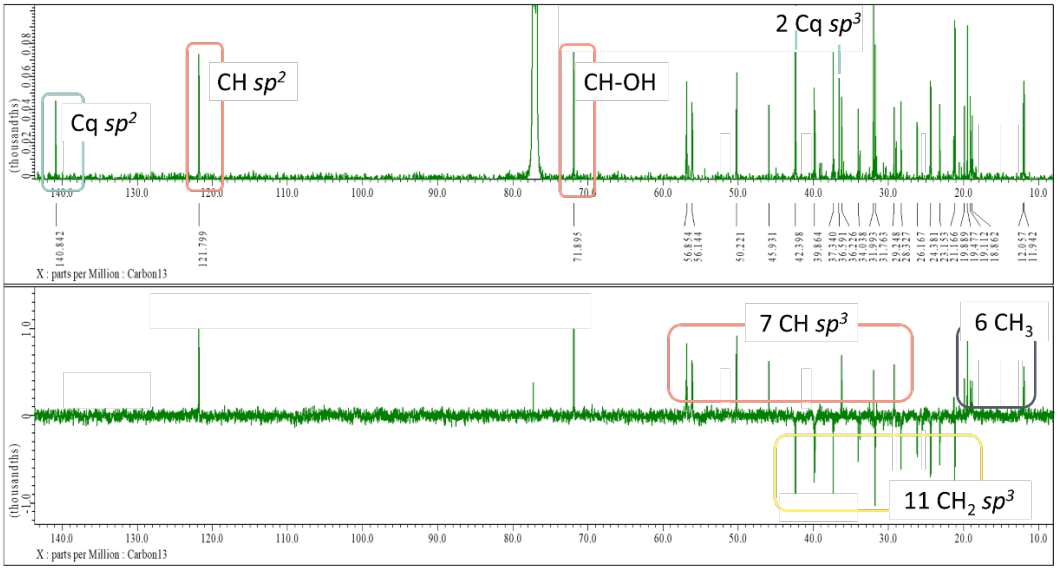

Figure S13. ^{13}C-NMR and DEPT-135 spectrum of compound 2 (125 MHz, $CDCl_3$).

Compound 3 Structure Elucidation

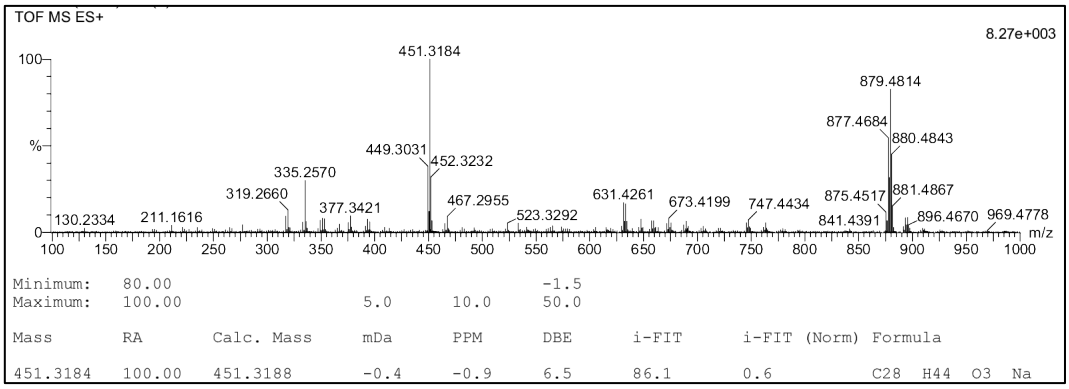

Fig. S14 HR-TOFMS spectrum of compound **3**.

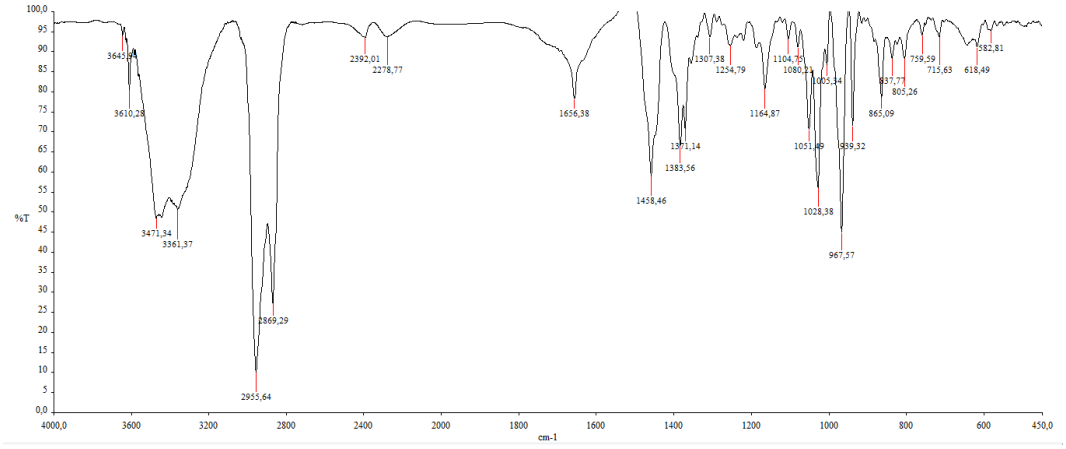

Fig. S15 IR spectrum of compound **3**.

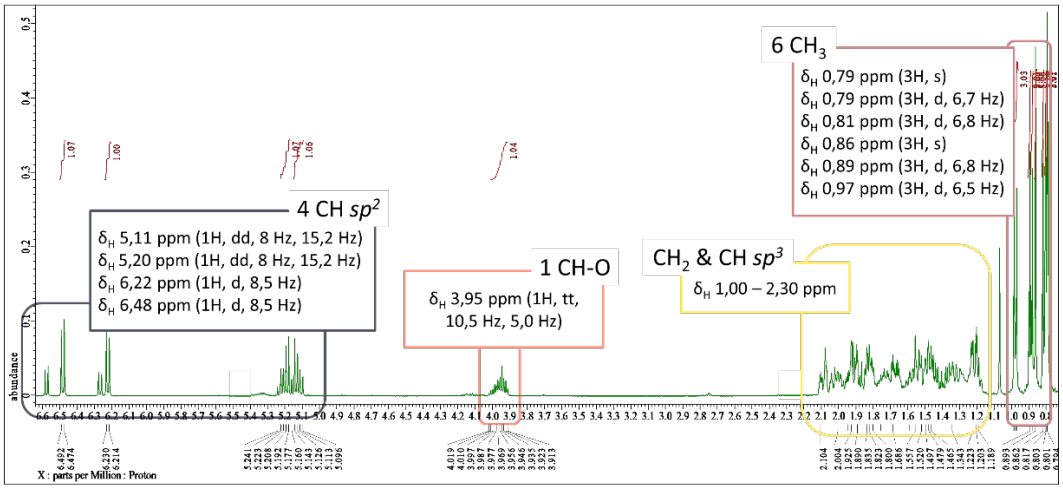

Fig. S16 ^{1}H-NMR spectrum of compound **3** (500 MHz, $CDCl_3$).

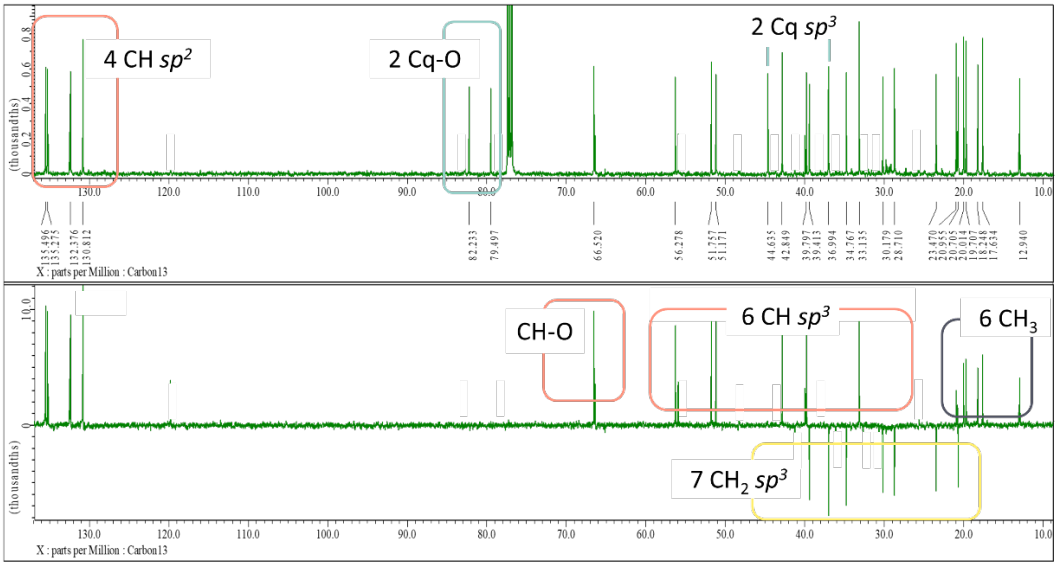

Fig. S17 ^{13}C-NMR and DEPT-135 spectrum of compound **3** (125 MHz, $CDCl_3$).

Compound 4 Structure Elucidation

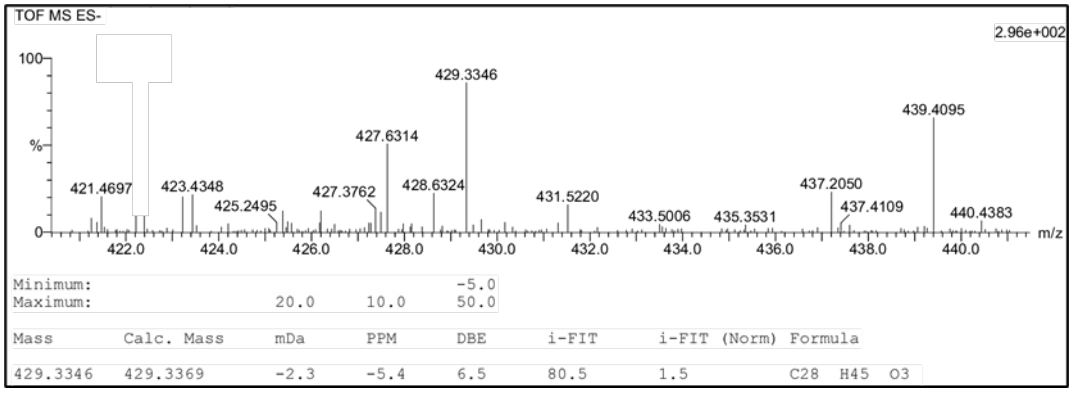

Fig. S18 HR-TOFMS spectrum of compound **4**.

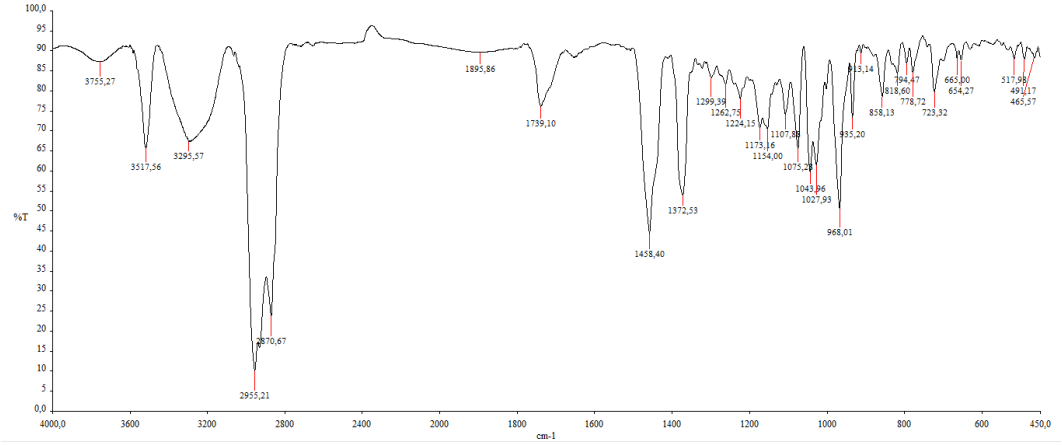

Fig. S19 IR spectrum of compound **4**.

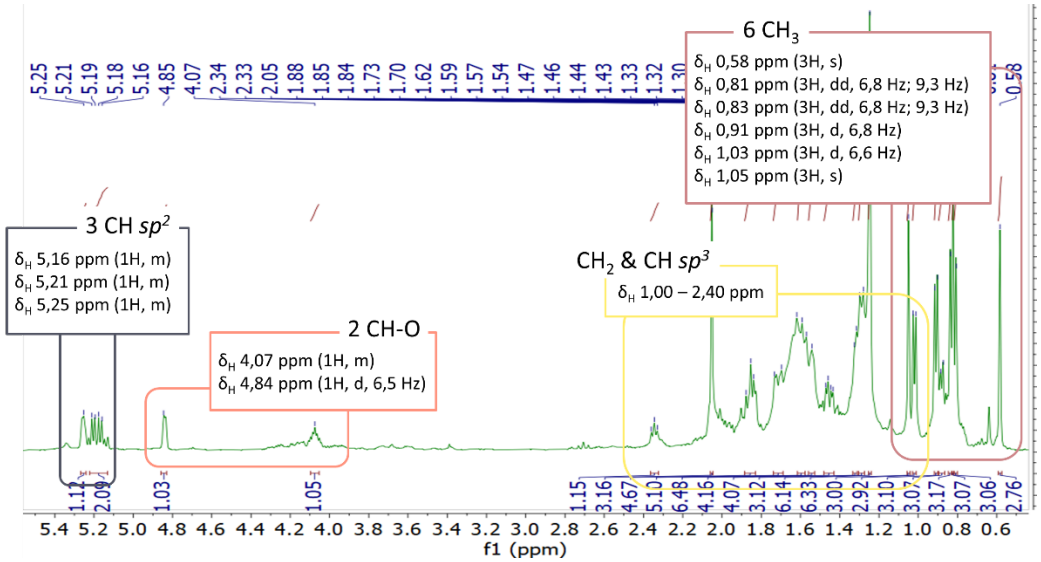

Fig. S20 ^{1}H-NMR spectrum of compound **4** (500 MHz, $CDCl_3$).

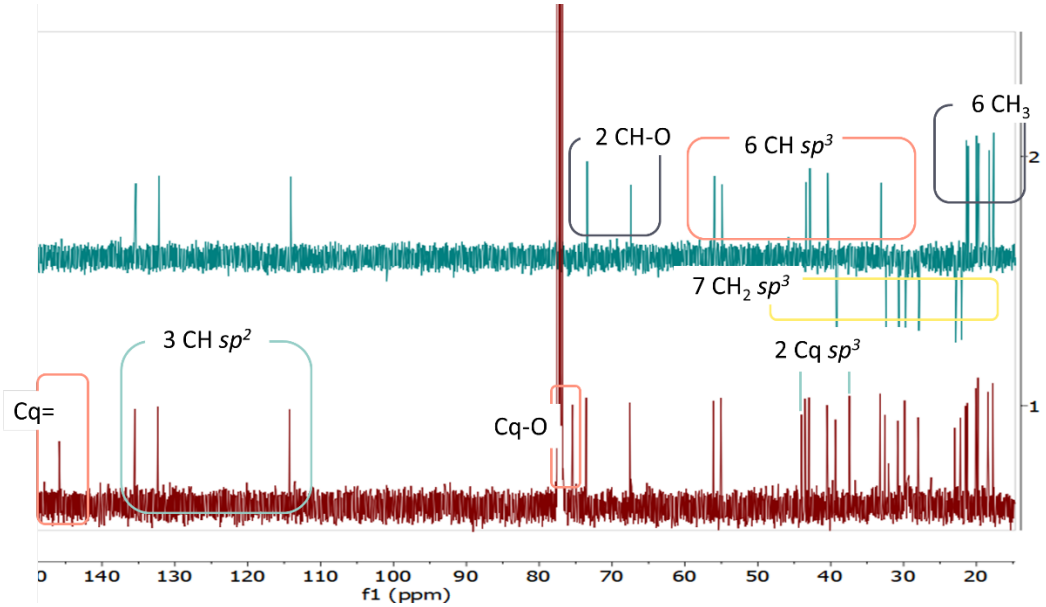

Fig. S21 ^{13}C-NMR and DEPT-135 spectrum of compound **4** (125 MHz, $CDCl_3$).

Heme Polymerization Inhibition Activity of Compound 1 – 4

Table S6. The IC_{50} values of compound **1** – **4**, *n*-hexane extract, and positive control chloroquine diphosphate according to HPIA assay

Samples	Concentration (mg/mL)	Percentage of inhibition	IC_{50} (mg/mL)
Isocyathisterol (**1**)	10	62.73 ± 0.51	7.70 ± 0.11
	5	37.05 ± 0.84	
	2.5	16.86 ± 2.02	
	1.25	4.24 ± 1.11	
	0.63	-5.32 ± 2.04	
β-sitosterol (**2**)	10	50.51 ± 0.52	9.48 ± 0.09
	5	26.78 ± 0.96	
	2.5	11.43 ± 2.15	
	1.25	5.15 ± 2.11	
	0.63	-5.09 ± 2.83	
Ergosterol-5,8-peroxide (**3**)	10	55.89 ± 0.92	7.88 ± 0.10
	5	42.82 ± 1.17	
	2.5	27.72 ± 1.40	
	1.25	15.35 ± 0.96	
	0.63	8.57 ± 1.69	
Cerevisterol (**4**)	10	54.08 ± 2.40	8.36 ± 0.56
	5	40.57 ± 3.13	
	2.5	24.90 ± 5.28	
	1.25	13.69 ± 4.47	
	0.63	8.04 ± 5.67	
n-hexane extract	5	81.21 ± 0.21	2.46 ± 0.04
	2.5	61.86 ± 0.56	
	1.25	41.61 ± 0.08	
	0.63	21.79 ± 0.02	
	0.31	1.03 ± 2.04	
Chloroquine diphosphate	5	95.63 ± 0.05	1.59 ± 0.03
	2.5	72.54 ± 0.01	
	1.25	52.99 ± 0.28	
	0.63	34.53 ± 0.33	
	0.31	20.54 ± 0.98	

Supplement

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	1
		Introduction	
Background and objectives	2a	Scientific background and explanation of rationale	2-3
	2b	Specific objectives or hypotheses	3
		Methods	
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	3-5
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	-
Participants	4a	Eligibility criteria for participants	4
	4b	Settings and locations where the data were collected	5
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	5
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	3, 7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	-
Sample size	7a	How sample size was determined	-
	7b	When applicable, explanation of any interim analyses and stopping guidelines	-
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	4-5
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	5
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	-
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	4-5
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	5
	11b	If relevant, description of the similarity of interventions	-
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	8
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	-

Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	8
	13b	For each group, losses and exclusions after randomisation, together with reasons	-
Recruitment	14a	Dates defining the periods of recruitment and follow-up	-
	14b	Why the trial ended or was stopped	-
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	9, Table 2
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	9, Figure 1
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	8-9, Table 1, Figure 2
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	-
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	-
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	-
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	14
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	15
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	11-13
Other information			
Registration	23	Registration number and name of trial registry	3
Protocol	24	Where the full trial protocol can be accessed, if available	Figure 1
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Acknowledgements

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.