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Original Article

Isolation of Antibacterial Depside Constituents from Indonesian Folious Lichen, *Candelaria fibrosa*

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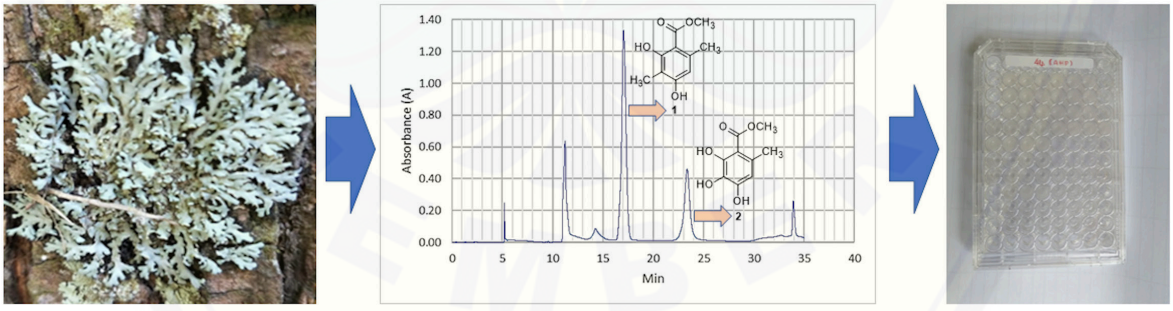
Graphical Abstract



Candelaria fibrosa

Abstract: There is an urgent need for novel drug leads, especially for microbial infections due to continuing emergence of drug resistance. Natural products are the backbone of modern medicine and the lichens have an important role to play in the discovery of novel drugs. Indonesia is gifted with a diverse array of lichens, which remain underexplored for medicinal applications. In this study, we have collected a lichen, *Candelaria fibrosa,* and conducted phytochemical and bioactivity studies. Using high performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy, we have isolated and

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characterised two depside compounds, atraric acid (7) and methyl 3-hydroxy orsellinate (8). These two depsides were reported from this lichen species for the first time. The evaluation of the crude methanol extract against Gram-positive bacteria, *Staphylococcus aureus*, indicated insignificant activity. However, the isolated compounds have been previously reported to possess low antimicrobial activity against common pathogenic bacteria (*Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa*) but to show significant anti-legionellosis.

Keywords: *Candelaria fibrosa*, atraric acid, methyl 3-hydroxy orsellinate, antibacterial, *Staphylococcus aureus.*

Introduction

Microbes including bacteria, fungi and virus cause serious infectious diseases. The infections are more rampant in the tropics and affects millions of people worldwide [1]. Bacterial infectious diseases have been one of the more serious health burdens since the early civilization of human kind [2]. Before the discovery of modern antibiotics, a rod-shaped Gram-negative bacterium *Yersinia pestis* could cause deadly bubonic plague [3] and could be transmitted to humans *via* numerous pathways. The discovery of penicillin founded the modern antibiotic era in fighting such bacterial infections, saving many lives worldwide [2]. The first antibiotic was discovered from micro-fungi in the form of secondary metabolites as part of a defence system against other lower-class organisms [2]. This discovery triggered an extensive exploration of the fungi kingdom including its symbiotic form, lichenised fungi [4].

Antibiotics have been used massively for treating many infectious diseases and their misuse has resulted in the rapid increase of antibiotic resistance, which has become a major public health threat [5]. It is estimated that by 2050, there will be 10 million deaths per year due to antibiotic-resistant infections [6]. Such public health threat is enhanced manifold in the tropical countries including Indonesia due to tropical climate conducive for microbial growth. The antibiotics failure will lead to catastrophic consequences to people worldwide. Therefore, there is urgent need to find novel antimicrobial drugs for treating both antibiotic resistant superbugs and microbial infections that don't have standard treatment regimens.

Novel and effective antimicrobial drugs can be developed from natural products especially from plants, fungi and lichens. Out of 1562 new drug leads developed in between 1981-2014, 73 % were discovered from natural products and 23 % of these natural leads were antimicrobials [7]. Lichen bioprospecting began in the 1940s and more than 1000 secondary metabolites were identified from this symbiont biomass [4]. Prospective antibacterial constituents have been reported from various lichen studies, which indicated a diverse antibacterial activity against gram-negative, gram-positive and mutant bacteria (Table 1, Fig. 1). Therefore, due to their niche habitat, it is likely that lichens could form the basis for future antibacterial drug discovery.

The Indonesian archipelago presents a diverse array of vegetation, from coastal to mountainous, and from desert to rain forest vegetations, with lichens an integral component of the ecosystem [15]. The indigenous Indonesian population have relied on these rich vegetations throughout generations for their food, shelter and medicine. Gifted with mega flora and fauna, Indonesia has potential to be the medicinal treasure chest of Asia-pacific region. Despite the world's second largest and most unique biodiversity covering the archipelago, research studies into the medicinal applications of secondary metabolites of the Indonesian lichens and plants are limited [15]. Reported cases of the use of lichens are limited to those with large biomass such as *Usnea sp,* which has been used traditionally to treat inflammation [16]. The studies on selected Indonesian lichens such as *Parmelia aurulenta* Tuck., *Parmelia cetrata* Ach., *Parmelia dilatata* Vain., *Physcia cf. millegrana* Degel., and *Usnea misaminensis* [16-19] have revealed several metabolites with various biological activities including anti-cancer, anthelmintic and antibacterial activities. Inspired by the diverse lichens and their preliminary biological activities, we have recently ventured into in-depth phytochemical and antibacterial screening focusing on one Indonesian foliose lichen, *Candelaria fibrosa* (Fr.) Müll. Arg. We have collected, extracted, isolated its secondary

Table 1. Reported antibacterial activities of major secondary metabolites of lichens

Constituents	MIC								
	BS	EC	EF	MRSA	MT	PA	SA	SE	SM
Usnic acid 1 [8-10]	1.2 mg/mL			25 mg/mL			7.5 mg/mL		
Anziaic acid 2 [11]	6 mg/mL	12 mg/mL							
Acremonidin E 3 [12]					50 mg/mL				
Lobaric acid 4 [13]	88 µM						39.6 µM		
Lobastin 5 [13]	44 µM						35.2 µM		
Divaricatic acid 6 [14]	7.0 mg/mL	>256	16.0 mg/mL	32.0 mg/mL		128.0 mg/mL	64.0 mg/mL	16.0 mg/mL	32.0 mg/mL

* µM unit; BC: *Bacillus cereus*, SA: *Staphylococcus aureus*, PS: *Pseudomonas aeruginosa*, BS: *Bacillus subtilis*, MT: *Mycobacterium tuberculosis*, SE: *Staphylococcus epidermidis*, SM: *Streptococcus mutans*, EF: *Enterococcus faecium*, *Escherichia coli*

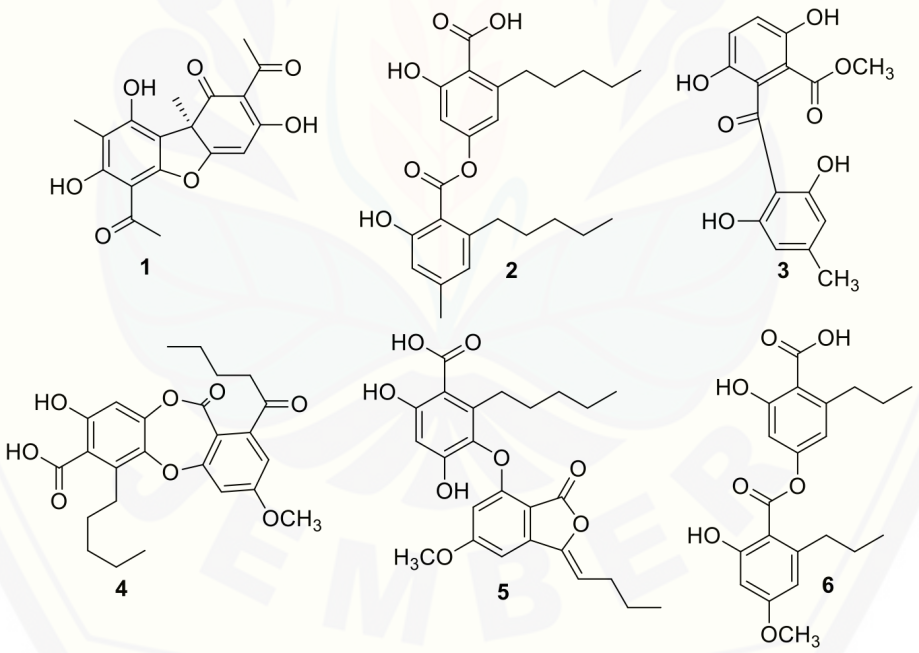

Figure 1. Selected antimicrobial compounds from lichen

metabolites, and evaluated their antibacterial activity against *Staphylococcus aureus*.

Material and methods

Chemicals and reagents

Analytical grade methanol and *n*-hexane were used in the extraction and fractionation of the lichen. HPLC grade acetonitrile and water were used as solvent system for separating compounds using High-Performance Liquid Chromatography (HPLC). Deuterated acetone-D_6 was used in obtaining the Nuclear Magnetic Resonance spectral data. Calcium chloride, magnesium chloride, dimethyl sulfoxide (DMSO) and

Mueller Hinton Broth media were purchased from Merck.

Lichen collection and sample preparation

Lichen was collected from the bark of *Swietenia mahogany* trees from Klocing, Bondowoso Regency, East Java, Indonesia. A voucher sample was deposited in the DUDRG laboratory Faculty of Pharmacy, University of Jember under accession number B4. A sample copy was identified by lichenologist, Mrs. Ludmilla Fitri Untari, in the Faculty of Biology, Gadjah Mada University.

Extraction and isolation of compounds

Dried powdered lichen (17.231 g) was soaked in methanol (250 mL) followed by stirring at 200 rpm for 24 h. The supernatant was collected by filtration and the extraction was repeated three times. The pooled supernatant was vacuum dried to produce 1.9093 g of crude extract. A portion (0.5 g) of extract was redissolved in *n*-hexane (10 mL), filtered and the supernatant was vacuum dried. The dried fraction was dissolved in methanol (4 mL), filtered with PTFE membrane filter (0.45 µm) and loaded onto a CECIL high-performance liquid chromatography (HPLC) system for secondary metabolite separation. Analytical and semi-preparative HPLC comprised a CECIL CE4300 detector, a CE4040 degasser and a CE4104 pump, which were controlled by CE4900 and PowerStream software. The analytical column used was a reverse-phase symmetry (4.6 x 150 mm, 4 µm, 100 Å) while the semi-preparative column used was a YMC J'sphere ODS-M80 (10 x 250 mm 4 µm 80 Å). Solvent A was 0.1 % formic acid in water and solvent B was 0.1 % formic acid in acetonitrile. Analytical chromatography was developed using a gradient elution was used for separating compounds with 0 to 40 % solvent B timed for 25 minutes and 40 to 50 % for 5 minutes with a flow rate of 1 mL/min. The chromatogram was recorded at λ 254 nm. Semi preparative chromatography was conducted using analytical gradient method with flow rate at 2 mL/min. A total of 29 injection blocks of 100 µL produced atraric acid (7) (5.0 mg) and methyl 3-hydroxy orsellinate (8) (4.0 mg) at retention times of *Rt* 16 and 23 minutes, respectively. Separately, fractions containing compound 7 and 8 were left in fume hood for two days followed by freeze drying for 24 h to obtain fluffy white solid.

Characterization of compounds

Pure compounds isolated from the lichen through repeated HPLC purification process were subjected to mass and NMR experiments. 1D-NMR spectra (^{1}H and ^{13}C NMR) and 2D-NMR spectra (gCOSY, gHSQC, gHMBC, zTOCSY) were recorded at 500 and 125 MHz, respectively, on a Bruker Avance 500 MHz. Electrospray Ionisation Mass Spectrometry (ESIMS) spectra were obtained from a Shimadzu LC-2010 mass spectrometer in electrospray positive and negative ionization modes (ESI-MS). The chemical shifts of proton and carbon of the two isolated compounds were compared with the known compounds recorded in the literature [17,20].

Antibacterial assay

Antibacterial activity was evaluated using a microdilution method [21]. *Staphylococcus aureus* ATCC 6538 was used in the antibacterial evaluation. Bacterial suspensions of *S. aureus* were prepared by loading two to three bacterial colony into cation adjusted Mueller Hinton Broth (CAMHB) media. The turbidity of bacterial suspension was then adjusted to 0.5 Mc Farland standard using spectrometry under λ 625 nm. The bacterial suspension was diluted (100-fold) to obtain 1 x 10^{6} CFU/mL bacterial suspension. Bacterial inhibition was measured by loading 50 µL bacterial suspension with crude extract solution (50 µL, 100 µg/mL in 1 % DMSO in CAMHB) or positive control (50 µL, 1 µg/mL gentamicin in CAMHB) or negative control (50 µL, 1 % DMSO in CAMHB) into microplate wells. CAMHB (100 µL) was also loaded into wells as a blank or media control. The mixture of crude extract with media and gentamicin with media was generated as the test control. The microplate was then incubated for 20 h at 37ºC followed by absorbance measurement at λ 625 nm. Absorbances for antibacterial assays were measured on a Molecular Devices

BIORAD-BenchMark M550 microplate reader. Antibacterial activity was measured as percentage inhibition at 50 µg/mL using the equation below (P: negative control; Q: media control, R: test sample; S: test control):

$$\% \text{ inhibition} = \left(1 - \frac{(\text{Abs. R} - \text{Abs. S})}{(\text{Abs. P} - \text{Abs. Q})}\right) \text{x} 100\%$$

Results and discussion

This study forms part of a lichen bioprospecting project in Indonesia. *C. fibrosa* (Fig. 2A) was discovered during a field trip in a mountainous range in Bondowoso Regency above 1500 metres above sea level (masl). The lichen grows on the bark of *Swietenia mahogany*. Due to limited crude extract yield, a short extract clean-up and semi-preparative HPLC protocols were employed using the methods described by us previously [17]. The chromatogram was recorded at λ 254 nm and the HPLC chromatograms of the crude extract of *C. fibrosa* are shown in Fig. 2B. A total of 29 injection blocks of 100 µL showed five chromatographic peaks. Of these five peaks, we have isolated two major compounds, atraric acid (7) (5.0 mg) and methyl 3-hydroxyorsellinate (8) (4.0 mg), which eluted at retention times of *t*R 16 and 23 minutes, respectively (Fig. 2B). These two depside compounds are reported here for the first time from lichen species.

Compound 7 was isolated as white amorphous crystalline (5.0 mg, 1.1 mg/g dried lichen sample). Analysis of the ^{1}H NMR spectrum revealed resonances typical for this compound class with a sharp singlet at δ 12 assigned to the C2 hydroxyl group that was bound to hydrogen. The resonance at δ 2.3 was assigned to the C6 methyl substituent. This methyl moiety is often substituted with other alkyl groups with two carbon fold addition such as propyl, pentyl, and heptyl groups. Another key feature is the aromatic proton attached to C3 and C5, which is indicated as a distinct peak at δ 6.0-6.2. In this case, the protons occurred as a doublet with meta-coupling constant around 2 *Hz*. The proton and carbon chemical shifts of this compound are given in Table 2. The mass spectra, and the 1D- and 2D-NMR spectra including ^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC and gHMBC of this compound is given in the supplementary figures S1-S5. The ESIMS spectrum of 1 indicated the molecular ion peak at *m/z* 197 with suggested molecular formula of $C_{10}H_{12}O_4$. Overall, these spectroscopic and spectrometric data analyses suggested the molecular structure of compound 1 as atraric acid.

Compound 8 appeared as white amorphous crystalline (4.0 mg, 0.8 mg/g dried lichen sample). Guided by structure 7, we elucidated the structure of methyl 3-hydroxyorsellinate 8 using mass and NMR spectra. The ESI-MS of 8 showed the molecular ion peak at *m/z* 198 with molecular formula of $C_9H_{10}O_5$. We found the structure was similar to atraric acid 7 with methyl group at C3 position substituted by a

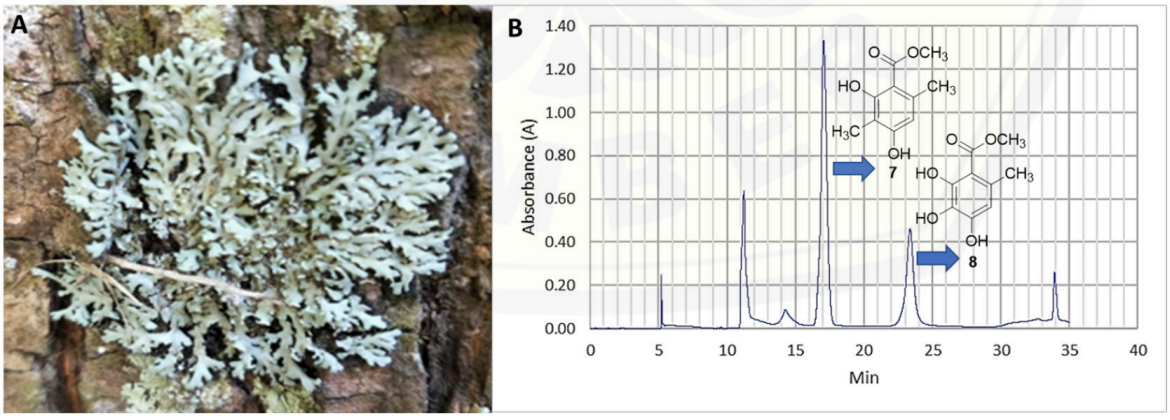

Figure 2. Two major compounds isolated from Indonesian folious lichen, *Candelaria fibrosa* (A), (B) HPLC chromatogram with the structures of atraric acid 7 (retention time of 16 min) and methyl 3-hydroxyorsellinate 8 (retention time of 23 min)

Table 2. ^{1}H and ^{13}C NMR data of atraric acid (1) and methyl 3-hydroxy orsellinate (2)

C/H atom	Atraric acid (1)		Methyl 3-hydroxy orsellinate (2)	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
C1	-	103.86	-	104.70
C2	-	160.16	-	167.12
C3	-	108.54	-	108.09
C4	-	163.21	-	165.81
C5	6.37	110.61	6.39	111.45
C6	-	139.67	-	152.39
C7	-	172.68	-	171.47
C3-CH_3	2.03	7.23	-	-
C6-CH_3	2.43	23.40	2.54	23.96
C7-OCH_3	3.91	51.31	3.99	52.04

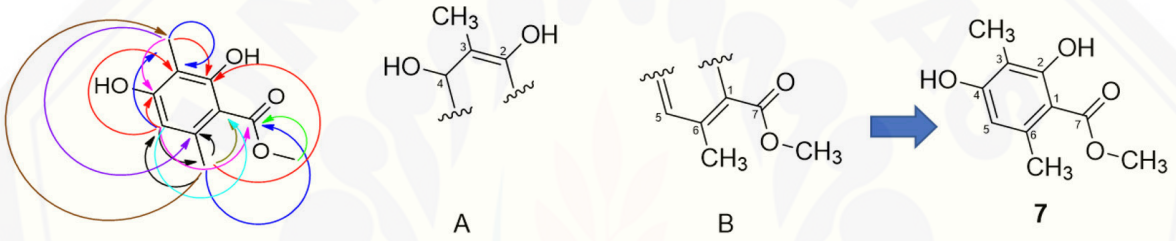

Figure 3. Proton-carbon correlation in atraric acid 7 molecule. Key features of depsides in constructing fragments A and B of atraric acid 7

hydroxyl group. The proton and carbon chemical shifts of this compound is given in Table 1. The mass spectra, and the 1D- and 2D-NMR spectra including ^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC and gHMBC of this compound is given in the supplementary figures S6-S10.

The compound identification was confirmed by comparing their chemical shift with those already reported in the literature on the respective compounds. These two compounds have been previously isolated from an angiosperm plant, stem bark of *Lonchocarpus atropurpureus* Benth [22,23]. Common secondary metabolites of lichen can be grouped into the depside, depsidone and dibenzofuran in which the depsidone is generated from intramolecular cyclisation of polydepsides with the last being a fused ring of two or more depside monomers [18]. As a molecular building block, depside and its derivatives are the most common lichen constituents. The key structural fragment features of these depside compounds can be constructed using the HMBC spectral corelation. For example, key fragments A and B forms the molecular structure of atraric acid (1) (Fig. 3).

Several lichens were previously known to possess potential antibiotic constituents [4], especially from *Cladonia* genus. We have tested crude *C. fibrosa* extract for its antibacterial activities against *Staphylococcus aureus* using microdilution assay. We found that *C. fibrosa* extract was ineffective antibacteria against *Staphylococcus aureus* (% inhibition of -33.2 ± 3.3 % ($p<0.05$) at a concentration of 50 µg/mL) compared to a positive control, gentamicin (Table 3). Interestingly, atraric acid isolated from other plant species has been previously reported to possess antibacterial activities [22,24] (Table 3).

The reported literature suggests that lichens are rich in antibacterial secondary metabolites and has potential for new drug discovery project, especially in Indonesia, where the lichens grow abundantly due to tropical climate. Tropical climate supports 80 % of the world's biodiversity

Table 3. Antibacterial activity of *C. fibrosa* crude extract and atraric acid (7)

Sample	MIC (µg/mL)								
	SA	EC	BC	PA	LP	LB	LM	LL	LD
Crude extract at 50 ug/mL	-33.2	NA	NA	NA	NA	NA	NA	NA	NA
Compound 7 [22,24]	156	NA	1250	625	12.0	24.0	21.3	6.7	21.3
Gentamicin at 1 ug/mL	99.3	98.3	NA	98.5	NA	NA	NA	NA	NA

BC: *Bacillus cereus*, SA: *Staphylococcus aureus*, PS: *Pseudomonas aeruginosa*, LP: *Legionella pneumophila*, LB: *Legionella bozemanii*, LM: *Legionella micdadei*, LL: *Legionella longbeachae*, LD: *Legionella dumoffii*

and at least 41 tropical diseases caused by endemic bacteria, viruses, parasites, and fungi [25]. Microbial infectious diseases are of increasing concern, not only in Indonesia but worldwide, as the geographic range of tropical diseases is expanding due to population explosion, deforestation and loss of biodiversity, climate change, urbanization, and change in agricultural practices. While there are many natural product-based solutions for these tropical diseases, the mechanism of action of active compounds within the natural product-based medicines, especially from Indonesia, remain largely unknown. Given that biodiscovery in Indonesia has received less attention, it is crucial for the university and the government to join hands and explore the chemotherapeutic agents from Indonesian mega- and micro- flora. Due to lack of financial capacity and high-end equipment such as MS and NMR, bio-exploration of Indonesian lichens can be best carried out using the techniques and technologies described by us previously [26] in collaboration with the universities in Australia, where first author has conducted and discovered numerous antimicrobial compounds [16-19,27].

Conclusion

Indonesia is rich in lichen diversity and we have identified number of lichens from East Java regions. The phytochemical and pharmacological studies on *C. fibrosa* revealed two major constituents, atraric acid 7 and methyl 3-hydroxy orsellinate 8, which were reported for the first time from the lichen species. Antibacterial activity test revealed the crude extract containing these secondary metabolites was ineffective against the pathogenic strain of *Staphylococcus aureus*. On the other hand, atraric acid 7 was previously reported to inhibit number of *Legionella* strains. This finding suggest that the lichens produce secondary metabolites that could selectively protect them against bacteria and other environment-related pathogens as well as the fungivores. There is need to study other lichens grown in the archipelago of Indonesia for their secondary metabolites.

Supplementary information

Figure S1. ^{1}H-NMR spectrum of compound 1 in acetone-D_6; Figure S2. ^{13}C-NMR spectrum of compound 1 in acetone-D_6; Figure S3. gCOSY spectrum of compound 1 in acetone-D_6; Figure S4. gHSQC spectrum of compound 1 in acetone-D_6; Figure S5. gHMBC spectrum of compound 1 in acetone-D_6; Figure S6. ^{1}H-NMR spectrum of compound 2 in acetone-D_6; Figure S7. ^{13}C-NMR spectrum of compound 2 in acetone-D_6; Figure S8. gCOSY spectrum of compound 2 in acetone-D_6; Figure S9. gHSQC spectrum of compound 2 in acetone-D_6; Figure S10. gHMBC spectrum of compound 2 in acetone-D_6; Figure S11. Low resolution mass spectra of compound 1 (top), compound 2 (bottom).

Author contributions

Conceptualization, research design and methodology, A.S.N.; validation, A.S.N., I.R.D., L.N.F., C.P.Z.S.P., B.T., P.W., and P.A.K.; formal analysis, A.S.N., I.R.D., L.N.F., C.P.Z.S.P., H.W., and P.A.K.; investigation, A.S.N., L.N.F., C.P.Z.S.P., I.R.D., H.W. and P.A.K.; resources, A.S.N. and P.A.K.; data curation, A.S.N., I.R.D., L.N.F., C.P.Z.S.P., and H.W.; writing-original draft preparation, A.S.N., I.R.D., L.N.F., C.P.Z.S.P.,

A.N.W.P., B.T., P.W. and P.A.K.; writing-review and editing, A.S.N., L.N.F., C.P.Z.S.P., A.N.W.P., P.W. and P.A.K.; visualization, A.S.N., L.N.F., and C.P.Z.S.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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