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Amphidinol C, a major polyketide from an Irish strain of the dinoflagellate *Amphidinium carterae*

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ABSTRACT

An Irish strain of the dinoflagellate *Amphidinium carterae* was previously shown to produce antibacterial amphidinol derivatives of unknown masses. Inspection of the major metabolites present in a bulk culture of this strain led to the isolation and structure elucidation of a new amphidinol derivative named amphidinol C featuring an unprecedented tetrahydropyran ring between the positions C-7 and C-11. The structure was determined using extensive analyses of NMR and MS data and comparison with data of analogues. The new ring was proposed to stem from a nucleophilic substitution of the sulphate present on the side chain of Amphidinol B. The major metabolites isolated were tested for their antibacterial and antifungal activities and Amphidinol C showed moderate fungicidal activity against yeast and filamentous fungi at 8–16 μg mL^{-1}.

1. Introduction

Microalgae have become a hotbed for natural product researchers in the search for new and bioactive metabolites. The large diversity of chemical structures isolated from microalgae makes them ideal candidates for drug discovery. Dinoflagellates, in particular, are known for their ability to produce a variety of secondary metabolites which are sometimes toxic and/or can have a wide range of biological and pharmaceutical applications (Gallardo-Rodriguez et al., 2012; Kobayashi and Kubota, 2010). The focus on microalgae for metabolite production is also linked to the ability of large scale cultures allowing the production of metabolites in sufficient amount for biodiscovery (Morsy et al., 2006). Dinoflagellates of the genus *Amphidinium* occur both in temperate and tropical waters and they have been found to produce a large array of highly complex metabolites of polyketide origin such as amphidinols, amphirionins, karatungiols and other macrolides (Echigoya et al., 2005; Kobayashi, 2008; Kumagai et al., 2015; Martinez et al., 2019; Nuzzo et al., 2014; Washida et al., 2006). Amphidinols (AMs) are long and polycyclic polyhydroxy polyketides which have proven to show potent antifungal, ichthyotoxic, hemolytic, cytotoxic, antiprotozoan or antidiatom activities (Minamida et al., 2014; Morales-Amador et al., 2021). The structure of the first amphidinol was reported in 1991 from the dinoflagellate *Amphidinium klebsii* and almost 30 amphidinols have since been isolated from various *Amphidinium* spp (Satake et al., 1991; Wellkamp et al., 2020).

Amphidinium carterae is a widely distributed species of dinoflagellate which has a large variation in amphidinol composition reported to be strain specific (Wellkamp et al., 2020). Previous studies on methanolic fractions containing amphidinol derivatives from the Irish strain LACW11 of *A. carterae* indicated strong activity against *S. aureus* and *E. faecalis* (Barone et al., 2021). One of the major compound was characterised by LC-MS with a molecular mass corresponding to a dehydrated derivative of amphidinol A. The strain was then cultured at large scale and a deep chemical investigation was undertaken with a focus on this new mass. Herein, we describe the isolation and structure elucidation of the new amphidinol C (**1**) (Fig. 1). Amphidinol C was tested against a panel of Gram-Positive and Gram-negative bacteria as well as yeast and filamentous fungi.

2. Results and discussion

Compound **1** was obtained as an off white amorphous solid (1.2 mg)

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from 7.0 g of freeze-dried biomass of *A. carterae*. The molecular formula of **1** $C_{69}H_{124}O_{23}$ was deduced from the observation of the $[M + Na]^+$ adduct at *m/z* 1343.8415. The compound was found to belong to the amphidinol family by the presence of two characteristic methyl doublets at δ_H 0.94 (d, $J = 7.0$ Hz, 3 H, H-67) and 0.96 (d, $J = 6.5$ Hz, 3 H, H-66) in its ^{1}H NMR spectrum and 25 oxygenated carbons together with 9 unsaturated carbons corresponding to 4 olefins and a ketone with a characteristic signal at δ_C 211.3 (C-13) among the 69 non-equivalent carbons observed in the ^{13}C NMR spectrum of **1**. Comparison of the ^{1}H NMR spectrum of **1** with those of previously reported amphidinols revealed a high level of similarity with the spectra of amphidinol A (**2**) and B (**3**) recently isolated from another strain of *A. carterae*,(Cutignano et al., 2017). These two derivatives were described themselves as shorter analogues of amphidinol-18 and -19 (Nuzzo et al., 2014). Most regions of the ^{1}H NMR spectra of **1-3** were superimposable apart from the signals corresponding to the northern part of the molecule and more precisely the C-7/C-11 subunit. As the molecular formula suggested the loss of a molecule of water compared to AM-A (**2**), we first assumed that **1** was a dehydrated product of AM-A that could lead to a conjugated ketone at C-13. However, the ^{13}C NMR spectrum of **1** did not evidence any additional olefin compared to AM-A and B and the chemical shift of the signal corresponding to the ketone at C-13 was not consistent with a conjugation. Due to a series of overlap of signals in the ^{1}H NMR spectrum, we assigned all spin coupled systems using COSY, HSQC, H2BC and HMBC experiments. The deshieldings of the signals corresponding to the C-7 and C-11 carbons at δ_C 79.4 and 75.6 compared to the corresponding signals in AM-A (**2**) suggested a substitution on the oxygens of both alcohols. Large splittings of the signals of the geminal diastereotopic protons H-8, H-9 and H-10 were consistent with the presence of a ring between these two positions. As the HMBC spectrum did not evidence any clear H-7/C-11 nor H-11/C-7 correlation, we had to rely on the NOESY spectrum to give additional proof of the presence of this ring. The low value of the $^3J_{HC}$ correlations between these two positions C-7 and C-11 can be explained by the conformation of the *cis*-2,5-disubstituted ring of the tetrahydropyran ring. This value could be much higher for the *trans* relative configuration present in the other two tetrahydropyran rings of **1**. Interestingly, an absence of HMBC correlations was also noticed for the characellides with a similar *cis*-2,5-disubstituted tetrahydropyran ring (Afoullouss et al., 2019). Gratifyingly, the observation of key H-7/H-11/H-9b nOes came as confirmations of the tetrahydropyran ring. At the same time, the H-7/H-11 NOE revealed a *cis* diaxial orientation for H-7 and H-11 protons. A NOE was observed between H-29/H-31 which was indicative of an *E* configuration around the double bond at C-30/C-31 while a coupling constant of 15.5 Hz between H-52 and H-53 was reminiscent of the same *E* configuration for this second olefin. When comparing our NMR data in CD_3OD with some NMR data of AM-B, we noticed that AM-B had been previously reported with some discrepancies observed in the numbering of the carbon chain and we therefore bring some corrections in the table below (Table 1) (Cutignano et al., 2017).

Due to the low natural abundance of amphidinols, it is usually not straightforward or even possible to determine their relative/absolute configurations by spectroscopic methods. Even though computational methods have made enormous improvements in recent years, the technique is not fully reliable for complex molecules like amphidinols. Organic synthesis is then the only alternative route to assess with confidence the configurations of the stereogenic centers. In the amphidinol family, amphidinol-3, with a planar structure first reported in 1991 (Satake et al., 1991) and a spatial arrangement proposed in 1999 (Murata et al., 1999), has been targeted for total synthesis by the group of Oishi. Their immense efforts culminated recently in the total synthesis of this polyketide that led to successive revisions of the relative/absolute configurations of all stereogenic centers (Wakamiya et al., 2020, 2018). The planar structures of amphidinol 3 and amphidinol A and B being identical between the positions C-19 and C-55 and the chemical shifts of the NMR signals of compound **1** and AM-3 being comparable for this region, we propose the same configurations for this core part of all analogues of AM-A (Fig. 1) (Wakamiya et al., 2020). Combinatorial syntheses of the C-1/C-14 subunit of AM-3 concluded of *R* configurations for the hydroxyl groups at positions C-2, C-6 and C-10 (BouzBouz and Cossy, 2001; Oishi et al., 2008). However, the hydroxyls are located in positions C-2, C-7 and C-11 for AM-A and as their absolute configuration is currently not assigned, we can only propose a 2 *R* configuration using biosynthetic analogies. Additionally, labelling experiments have shown that the C-1/C-14 moiety is formed though the addition of repeating acetate units which would indicate that the stereochemistry of the hydroxyls should be conserved along the polyol chain (Houdai et al., 2001). The hydroxyl positions of AM-3 differ from those of **1** and any definitive conclusion would then be premature at that stage. However, respecting the biosynthetic logics, we could presume that the three remaining hydroxyl at C-7, C-11 and C-15 being separated by three methylenes should have the same configurations leading to *syn* relative configurations (Fig. 1).

The bioactivity of amphidinol C (**1**) was tested against a panel of pathogens (Table 2). Only antifungal activity was confirmed at the doses tested, returning minimum fungicidal concentrations of 8 and 16 μg mL^{-1} against *Aspergillus fumigatus* and *Candida albicans*, respectively. These activities are similar to those obtained for other amphidinols against similar fungi (Martinez et al., 2019; Morsy et al., 2008; Nuzzo et al., 2014; Satake et al., 2017).

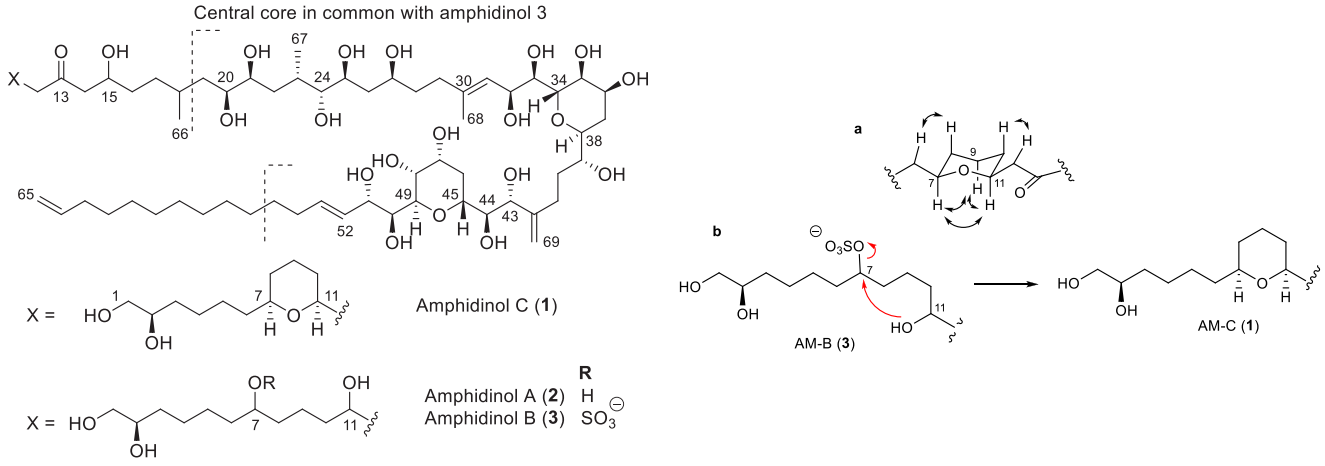

Fig. 1. Chemical structures of the new amphidinol C (**1**), and the known amphidinols A (**2**) and B (**3**). a. Key nOes for the first tetrahydropyran ring between C-7 and C-11 for **1**; b. proposition of transformation of AM-B (**3**) into AM-C (**1**).

Table 1
^{1}H NMR (600 MHz) and ^{13}C NMR data (150 MHz) for Amphidinol C (**1**) and B (**3**) (Cutignano et al., 2017) in CD_3OD.

AM-C (**1**)						AM-B (**3**)					
pos.	δ_C	δ_H, mult., *J* in Hz	pos.	δ_C	δ_H, mult., *J* in Hz	pos.	δ_C	δ_H, mult., *J* in Hz	pos.	δ_C	δ_H, mult., *J* in Hz
1a	67.5	3.47, dd 11.0, 5.0;	**36**	67.5	3.97 m	**1a**	67.1	3.44, dd, 11.0, 6.0	**36**	67.2	4.00, m
1b		3.41, dd 11.0, 6.5				**1b**		3.51, m			
2	73.4	3.56, m	**37**	30.5	1.79, m	**2**	73.0	3.61, m	**37**	30.2	1.82, m
3a	34.6	1.48, m	**38**	75.8	3.49 m	**3**	34.2	1.41, m	**38**	75.5	3.55, m
3b		1.35, m									
4a	26.8	1.44, m	**39**	74.7	3.60 m	**4a**	26.6	1.52, m	**39**	74.0	3.64, m
4b		1.40, m				**4b**		1.43, m			
5a	26.9	1.44, m	**40a**	32.5	1.97, br d 13.5	**5**	25.8	1.48, m	**40a**	32.1	2.00, m
5b		1.35, m	**40b**		1.56, m				**40b**		1.60, m
6a	37.6	1.46, m	**41**	27.9	2.42, m	**6a**	35.2	1.70, m	**41**	27.7	2.45, m
6b		1.40, m			2.09, m	**6b**		1.50, m			2.15, m
7	79.4	3.31, m	**42**	151.5	–	7	80.3	4.39, m	**42**	151.3	–
8a	32.7	1.59, m 1.17, qd 11.5, 3.5	**43**	76.9	4.19, d 9.0	**8a**	35.3	1.70, m	**43**	76.4	4.22, d 8.8
8b						**8b**		1.50, m			
9a	24.7	1.83, m	**44**	75.2	3.35 m	**9a**	21.9	1.60, m	**44**	75.1	3.38, m
9b		1.58, m				**9b**		1.49, m			
10a	32.7	1.61, m	**45**	70.5	4.04 m	**10a**	38.4	1.55, m	**45**	70.4	4.07, m
10b		1.19, qd 13.0, 4.5				**10b**		1.48, m			
11	75.8	3.79, br dd 10.0, 9.0	**46**	31.6	2.09, m	**11a**	68.6	4.09, m	**46**	31.5	2.12, m
					1.55, m	**11b**					1.60, m
12	50.9	2.61, d 9.0	**47**	67.4	4.05, m	**12**	51.6	2.65, m	**47**	69.2	4.07, m
13	211.3	–	**48**	68.7	4.04, m	**13**	211.6	–	**48**	68.6	4.07, m
14	51.8	2.62, m	**49**	80.7	3.75, d 10.0	**14**	51.6	2.65, m	**49**	80.4	3.78, m
15	69.4	4.00, m	**50**	71.9	3.96, m	**15**	68.8	4.07, m	**50**	71.9	3.99, m
16a	35.8	1.55, m	**51**	74.3	4.36, dd 8.0, 3.0	**16a**	35.5	1.50, m; 1.40, m	**51**	74.1	4.39, m
16b		1.40 m				**16b**					
17a	33.3	1.61, m	**52**	128.6	5.60, dd 15.5, 8.0	**17a**	33.0	1.62, m; 1.15, m	**52**	128.1	5.63, dd 15.0, 7.5
17b		1.12, m				**17b**					
18	30.9	1.67, m	**53**	136.1	5.79, dt 15.5, 7.0	**18**	30.2	1.70, m	**53**	135.6	5.82, dt, 15.0, 6.5
19a	41.6	1.47, m	**54**	33.8	2.08, m	**19a**	41.8	1.50, m; 1.40, m	**54**	33.2	2.10, m
19b		1.36, m				**19b**					
20	73.4	3.52 m	**55**	30.6	1.40, m	**20**	73.3	3.55, m	**55**	30.8	1.44, m
21	72.8	3.51 m	**56–61**	30.8	1.31, m	**21**	72.6	3.50, m	**56–61**	30.6	1.35, m
22	38.5	1.68, m	**62**	30.4	1.30, m	**22**	38.3	1.50, m; 1.40, m	**62**	30.6	1.33, m
		1.39, m									
23	31.3	2.14, m	**63**	35.1	2.05, q 7.5	**23**	31.0	2.18, m	**63**	34.4	2.08, m
24	77.3	3.36, dd 7.0, 2.0	**64**	140.4	5.80, m	**24**	77.2	3.39, m	**64**	135.8	5.81, m
25	72.5	3.67, m	**65a**	114.7	4.98, br d 16.5	**25**	72.5	3.71, m	**65a**	114.3	5.01, m
			65b		4.91, br d 10.5				**65b**		4.94, br d 10.7
26a	42.0	1.98, br d 14.0	**66**	21.2	0.96, d 6.5	**26a**	41.7	2.02, m	**66**	20.8	0.99, d 6.9
26b		1.51, m				**26b**		1.56, m			
27	71.7	3.87, m	**67**	14.0	0.94, d 7.0	**27**	71.4	3.90, m	**67**	13.7	0.97, d 7.0
28a	36.7	1.68, m	**68**	17.4	1.75, s	**28a**	36.7	1.71, m; 1.63, m	**68**	17.0	1.78, s
28b		1.59, m				**28b**					
29a	36.8	2.21, m	**69a**	113.2	5.07, br s	**29a**	36.4	2.24, m; 2.17, m	**69a**	112.7	5.10, s 5.01, s
29b		2.12, m	**69b**		4.98, br s	**29b**			**69b**		
30	139.3	–				**30**	139.2	–			
31	126.5	5.48, br d, 9.0				**31**	126.0	5.52, d 8.5			
32	68.0	4.56, br d, 9.0				**32**	67.6	4.58, dd 8.5, 1.5			
33	72.5	3.68, br d, 9.0				**33**	72.4	3.70, m			
34	79.3	3.96, br d, 9.0				**34**	79.2	3.99, m			
35	69.0	4.04, m				**35**	67.2	4.07, m			

Microalgae and their associated compounds present a promising source of novel therapeutics and anti-microbial agents. Their ability to be cultured in laboratories at large scales gives them enormous advantages over the isolation of natural products from more typical marine organisms like sponges which have in the past been major producers of marine drugs. Amphidinols have been reported to have antifungal, cytotoxic and haemolytic activity with AM3 being the most active of all the analogues (Echigoya et al., 2005; Martinez et al., 2019; Nuzzo et al., 2014). Their activity comes from their pore-forming ability of liposomes containing membrane sterols (Gopal et al., 1997; Hieda et al., 2021). Amphidinol C (**1**), a dehydrated analogue of amphidinol A (**2**) was the major amphidinol present in the strain LACW11 of *A. carterae*. Strain variation in relation to concentration and differences in analogue production has been reported which suggest the continued importance of further chemical and genetic investigations into this species of dinoflagellate. This compound showed a quite specific and moderate activity towards the fungus *Aspergillus flavus*.

3. Experimental

3.1. General procedures

Optical rotations were recorded on a Unipol L1000 (Schmidt+Haensch) polarimeter equipped with a 10-cm cell. CD spectra were acquired using Jasco J-810 (CD) spectrometer. UV measurements were obtained by extraction of the DAD signal of UHPLC Dionex Ultimate 3000 (Thermo Scientific). NMR experiments were performed on a 600 MHz spectrometer equipped with a cryoprobe (Varian). Chemical shifts (δ in ppm) are referenced to the carbon (δ_C 49.0) and residual protons (δ_H 3.31) signals of CD_3OD. High-resolution mass spectra were obtained from a mass spectrometer Agilent 6540 in ESI(+). Semi-Preparative purifications were performed using an HPLC-UV equipped

Table 2
MIC and MCF for amphidinol C (**1**) against bacteria (Gram-positive and Gram-negative), yeast and filamentous fungi strains. ATCC = American Type Culture Collection, MRSA = Methicillin Resistant Staphylococcus Aureus, CA = Community Acquired, LA = Livestock-Acquired, HA=Healthcare-Acquired. Concentrations are expressed in μg/mL.

Strain	MIC	MFC
Aspergillus flavus ATCC 204304	4	8
Candida albicans ATCC 90028	16	16
Staphylococcus aureus ATCC 25293	> 32	–
CA-MRSA USA 300	> 32	–
HA-MRSA HU25	> 32	–
LA-MRSA ATCC BAA-2313	> 32	–
Staphylococcus epidermidis ATCC 35894	> 32	–
Enterococcus faecalis ATCC 29212	> 32	–
Escherichia coli ATCC 25922	> 32	–
Pseudomonas aeruginosa ATCC 27853	> 32	–

with a Jasco PU-2087 pump and UV-2075 detector (Tokyo, Japan).

3.2. Microalgal culture

A batch culture of *Amphidinium carterae* LACW11 isolated from the northwest of Ireland was prepared in 5 sterile 10 L glass bottle (30 L final volume culture) fitted with a 2-port vented cap at a starting concentration of 0.5 mg/mL (wet biomass) in f/2 medium without silicate. The culture was incubated for 30 days at 20 ± 1 °C under ca. 60–80 μmol/m^2/s illumination provided by LED panels (white light) and a 14:10 light:dark photoperiod. Aeration through a 0.22 μm filter airline was provided at a rate of 210 mL/min. On day 30, the cells were harvested by centrifugation at 2000 rpm for 5 min to collect the biomass, which was then desalted with 1 mL of 0.5 M ammonium formate prior to over-night freeze-drying (Scanvac. MillRock, Kingston, NY, USA) and subsequent storage at − 20 °C. A voucher with code LACW11 is maintained at IT Sligo.

3.3. Extraction and isolation

The freeze-dried biomass (7 g) was extracted with MeOH (5 × 80 mL) aided with sonication (5 min) and centrifuged (10 min × 4500 rpm). The extract was further fractionated by polarity, separating the most polar compounds (hydrophilic) from the most non-polar compounds (lipophilic). The extract (2.62 g) of the microalga was fractionated by elution with a gradient of H_2O-CH_3OH from 100:0–0:100, using C-18 reverse phase VLC method. Fraction 4 (97.0 mg) and 5 (1.35 g) were found to contain our compounds of interest. Fraction 5 was further fractionated via RP-C_{18} VLC by eluting methanol:water 80:20 *v/v* until complete elution of amphidinol derivatives monitored by LC-MS. Fraction 4 and the eluent from fraction 5 was combined to a mass of 149 mg and were separated into subfractions by using reversed phase semi-preparative HPLC (C_{18} column 5 μm, 250 ×10 mm Xselect) with gradient of solvent A: H_2O, solvent B:CH_3CN. 0–40 min:70–90% B resulting in 19 peaks. Peak 16 (6.7 mg) was repurified using analytical HPLC (T3 column 5 μm, 4.6 ×10 mm Xselect HSS) resulting in the isolation of compound **1** (1.2 mg).

3.4. Bacterial strains

Microbiological assays were carried out using *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *Candida albicans* ATCC 90028 and *Aspergillus flavus* ATCC 204304. The strains were grown in selective agar media (Bile Aescuilin Azide (BEA) for *E. faecalis*, Mannitol Salt Agar (MSA) for *S. aureus*) then incubated at 37 °C overnight prior to carrying out the bioassays. Sabouraud Dextrose Agar (SDA) and overnight incubation at 35 °C were used for *C. albicans*. All media were manufactured by Oxoid (Basingstoke, UK).

3.5. Determination of minimum inhibitory concentrations (MIC)

The susceptibility tests were carried out using the broth microdilution assay with kanamycin as positive bacterial break point control according CLSI. Also, MICs for C. albicans ATCC 90028 and Aspergillus flavus ATCC 204304 were analysed using amphotericin b as quality control according to CLSI M27-A2 and CLSI M38-A3.

One colony of ATCC strain was inoculated in 5 mL of BHI (Brain Heart Infusion broth) for bacterial strains and RPMI-1640 for *C. albicans* 4–6 h prior to carrying out a dilution at a final concentration of 10^5 cfu/mL for inoculation of the wells of a 96-well plate in 0.1 mL of sterile Muller-Hinton broth (MHB and RMPI-1640). Prior to inoculation, scalar dilutions (1:2) of the test compound were performed from the 1st to the 11th wells of a row of a 96-well plate with a final volume of 100 μL of MHB. The 12th well was used as a growth control. Control wells of MHB and DMSO solvent were also included. Triplicate wells were used for all the samples and the plates were incubated at 37° C for 14–18 h. Wells inoculated with C. *albicans* were incubated at 35 °C. The MIC points were determined after 24 h and confirmed after 48 h. Nitro blue-tetrazolium (1 mg/mL) was then added to each well and incubated for 30 min to detect the MIC points.

3.6. Determination of minimum bactericidal and fungicidal concentration

A volume of 50 μL from all the wells above the MIC point were inoculated in Muller-Hinton agar media. The plates were further incubated for 24 h and 48 h for bacterial and yeast, respectively. The Minimum bactericidal/fungicidal concentrations were then identified as the lowest concentration of compound returning no growth. All analyses were performed in triplicates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phytol.2022.08.001.

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