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44-Methylgambierone, a new gambierone analogue isolated from *Gambierdiscus australes*

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ABSTRACT

A new analogue of gambierone, 44-methylgambierone, was isolated from the benthic dinoflagellate *Gambierdiscus australes* collected from Raoul Island (Rangitahua/Kermadec Islands). This molecule has been previously reported as maitotoxin-3. The structure of 44-methylgambierone was elucidated using 1D- and 2D-nuclear magnetic resonance spectroscopy and mass spectrometry techniques. The nine-ring polyether backbone (A–I) and functional groups (carbonyl, terminal diol, 1,3-diene and monosulphate) are the same for both compounds with the addition of an olefinic methyl group being the only modification in 44-methylgambierone.

Introduction

Species from the epiphytic, benthic dinoflagellate genus *Gambierdiscus* produce a complex array of secondary metabolites that show varying levels of toxicity. These include ciguatoxins (CTXs) [1], maitotoxins (MTXs) [2–5], gambieric acid [6], gambierol [7], gambieroxide [8] and most recently, gambierone (**1**; Fig. 1) [9]. CTXs have been demonstrated to bioaccumulate and biotransform up food webs from herbivorous fish grazing on the coral or macroalgae supporting benthic *Gambierdiscus*, to omnivorous and carnivorous fish that predate on them. CTXs are thought to be the main toxin group responsible for ciguatera fish poisoning (CFP), with intoxications manifesting as a wide array of symptoms including gastrointestinal discomfort (e.g. nausea and diarrhoea), neurological impairment (e.g. parathesia and dysaesthesia) and/or cardiovascular complications (e.g. hypotension and bradycardia). However, the role of these other bioactive metabolites produced by *Gambierdiscus*, in CFP intoxications, remains uncertain as it is not yet understood whether these compounds bioaccumulate to significant levels in fish flesh, although they have been consistently found in visceral tissues [10,11]. Therefore, characterising new secondary metabolites produced by *Gambierdiscus* is required to determine if they also play a role in human intoxication events.

Many of the secondary metabolites produced by *Gambierdiscus* have ladder-shaped cyclic polyether backbones. The length of the cyclic backbone can be as few as eight rings in gambierol, nine rings in gambieric acid and gambierone, or as many as 32 rings in MTX-1. Maitotoxins-1, -2 and -4 are all large mono- or di-sulphated compounds with molecular weights greater than 3250 Da. In 1994 Lewis and Holmes reported a smaller molecular weight compound and named it MTX-3, although the structure was not characterised at the time [3]. Based off our interpretation of the published ionspray mass spectra a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for monitoring putatively assigned MTX-3 in a range of sample types, including *Gambierdiscus* culture extracts [12].

In the present study, the LC–MS/MS method was used to track the purification of putative MTX-3 from *G. australes* cultures. The purified compound was structurally characterised by nuclear magnetic resonance spectroscopy (NMR) and liquid chromatography mass spectrometry (LC–MS) demonstrating that it was a new gambierone analogue; 44-methylgambierone (**2**; Fig. 1).

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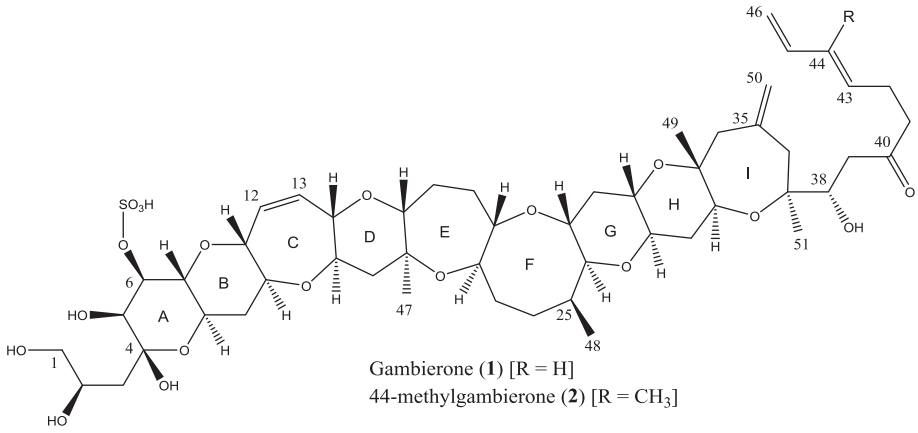

Fig. 1. Structures of gambierone (**1**) and 44-methylgambierone (**2**).

Results and discussion

44-Methylgambierone (**2**; Fig. 1) was purified from a total of 120 L of four monoculture *Gambierdiscus australes* isolates (CB-C, CB-F, 3b and CAWD246). The pelleted cells were extracted twice with aqueous methanol and the resulting extract was subjected to liquid–liquid partitioning, orthogonal solid-phase clean-up and preparative liquid chromatography. The yield of **2** was estimated as 1.7 mg when using high performance liquid chromatography with UV/visible detection (HPLC-UVD) and calibrating against a structurally-related molecule, yessotoxin (Supplementary Information, Figs. S1 and S2). Key structural features, similarities to gambierone (**1**; Fig. 1), and position of the additional methyl group were determined by LC–MS and 1D- and 2D-NMR analytical techniques.

LC–MS analysis of **2** using both negative and positive electrospray ionisation (ESI) revealed a $[M-H]^-$ ion at *m*/*z* 1,037.2 and a $[M+H]^+$ ion at *m*/*z* 1,039.2 (Fig. 2). Additional ions observed in the +ESI spectrum represented water loss ions (*m*/*z* 1,021.2, 1,003.2, 985.2) and a $[M-SO_3+H]^+$ ion (*m*/*z* 959.2) plus sequential water loss ions (*m*/*z* 941.2, 923.2, 905.2, 887.2). Fragmentation via collision induced dissociation in −ESI mode revealed a single dominant fragment ion representing a bisulphate anion (*m*/*z* 96.8; Fig. 3).

High-resolution ESI time-of-flight MS (HR-ESI-TOFMS) analysis of purified **2** gave $[M-H]^-$ *m*/*z* 1,037.4651, which is consistent with compound **2** having the deprotonated molecular ion $C_{52}H_{77}O_{19}S$ (calculated 1,037.4780 Da; Δ +12.9 mDa).

Structural insights for **2**, and similarities with **1**, were gained through use of several complementary techniques. The UV/visible absorption spectrum for **2** (Supplementary Information, Fig. S1), which had an absorbance maximum at 231 nm, was characteristic of a conjugated diene like the 1,3-diene found in **1**. When **2** was oxidised with periodic acid and analysed by LC–MS, a 32 Da loss was observed and suggested the presence of a terminal diol, which is also found in **1** (Fig. 4). Reduction experiments and LC–MS analysis showed that **2** reacted with two moles of sodium borohydride causing an increase in the molecular weight of 4 Da. This was attributed to the reduction of the carbonyl (C-40) and the hemiketal (C-4). This data, in conjunction with the 14 Da mass difference, suggested that **2** possesses a similar structure to **1**, with the addition of a methylene in the hydrocarbon side chain or that a proton was exchanged for a methyl group.

Detailed analysis of the 1D and 2D NMR spectral data including COSY, TOCSY, HSQC, HMBC and H2BC spectral data, supplemented by a series of 1D-SELTOCSY experiments and higher resolution SHSQC and SHMBC spectra recorded across crowded regions of the HSQC and HMBC spectra (Supplementary Information, Figs. S3–S35) established the chemical shifts of all the ^1H and ^{13}C NMR signals of **2** (Table 1). This revealed remarkable similarities in the ^1H and ^{13}C signal assignments for the atoms of the nine-ring polyether backbone (A–I) and the C-40 carbonyl signal (^{13}C signal at 211.8 ppm) of **2** with those reported for **1** by Rodriguez et al. (Table 1) [9]. Stereochemical relationships were elucidated in ROESY and NOESY experiments and a series of 1D-SELROESY and SELNOESY experiments.

Structural similarities between **1** and **2** included the presence of a $HOCH_2$-CHOH-CH_2– side chain group attached to ring A at C-4, which was confirmed by ^{13}C signals at 68.1 and 70.5 ppm for C-1 and C-2 respectively, and the chemical shifts and multiplicity of the H-1 methylene protons (3.47 ppm, dd, 11.2, 4.6 Hz; 3.41 ppm, m) and the H-2 methine proton (4.10 ppm, m) signals. The protons of the terminal CH_2OH group also showed HMBC correlations to C-2 (70.5 ppm) and C-3 (40.1 ppm). Confirmation of the sulphate group's attachment to C-6 was achieved by the H-6 signal at 4.71 ppm. The chemical shift and coupling constants (dd, 9.7, 3.1 Hz) of this signal corresponded closely to those reported for the equivalent proton signal of **1** (4.70 ppm, dd, 10.0, 3.2 Hz) [9]. SELTOCSY spectra determined for the H-6 signal of **2** with mixing times of 80, 160 and 240 msec identified the shifts of the H-5 to H-13 signals as reported in Table 1 also corresponded closely to those reported for **1** [9].

The attachment of methyl groups to C-47, C-48, C-49 and C-51 was substantiated by ^1H signals at 1.21, 1.00, 1.19 and 1.13 ppm respectively, which showed HSQC correlations to ^{13}C signals at 17.0, 13.8, 17.2 and 21.0 ppm respectively. Each methyl group also displayed sets of HMBC correlation that aligned with the ^{13}C shifts of the adjacent carbon atoms (Table 1).

The signal-to-noise ratio of the HSQC spectrum of **2** was such that in addition to ^1J correlations low level ^2J correlations were observed for many of the atoms of **2**. For example, the C-45 signal (142.9 ppm) showed a low level ^2J correlation to the pair of H-46 protons (4.90 and 5.09 ppm), C-41 (44.2 ppm) and C-42 (23.6 ppm) showed mutual medium level ^2J correlations to the H-41 and H-42 protons (2.59 and 2.39 ppm respectively), while C-15 showed a low level ^2J correlation to the adjacent H-14 proton (3.81 ppm). The latter ^2J correlation distinguished the H-14 resonance from that of the H-11 (3.78 ppm), and differentiated between their HSQC correlated carbon signals which occurred at 83.4 and 82.7 ppm respectively. Some of the low level ^2J correlations observed in the HSQC spectra for **2** are annotated in the deposited spectra (Supplementary Information, Figs. S20–S22).

Based on significant variations in the chemical shifts and couplings of some of the olefinic protons associated with the terminal

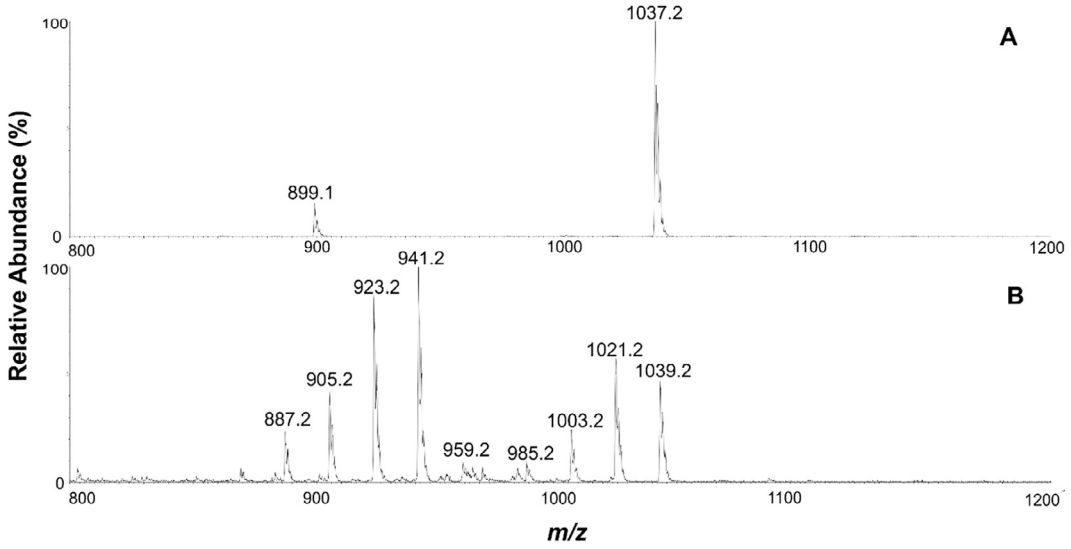

Fig. 2. Full scan mass spectrum of 44-methylgambierone (**2**; *m*/*z* 800–1200) in (A) −ESI mode showing the $[M-H]^-$ ion (*m*/*z* 1,037.2); and (B) +ESI mode showing the $[M+H]^+$ (*m*/*z* 1,039.2), $[M-H_2O+H]^+$ (*m*/*z* 1,021.2), $[M-2H_2O+H]^+$ (*m*/*z* 1,003.2), $[M-SO_3+H]^+$ (*m*/*z* 959.2) and a series of sulphite plus water loss ions (*m*/*z* 941.2, 923.2, 905.2 and 887.2).

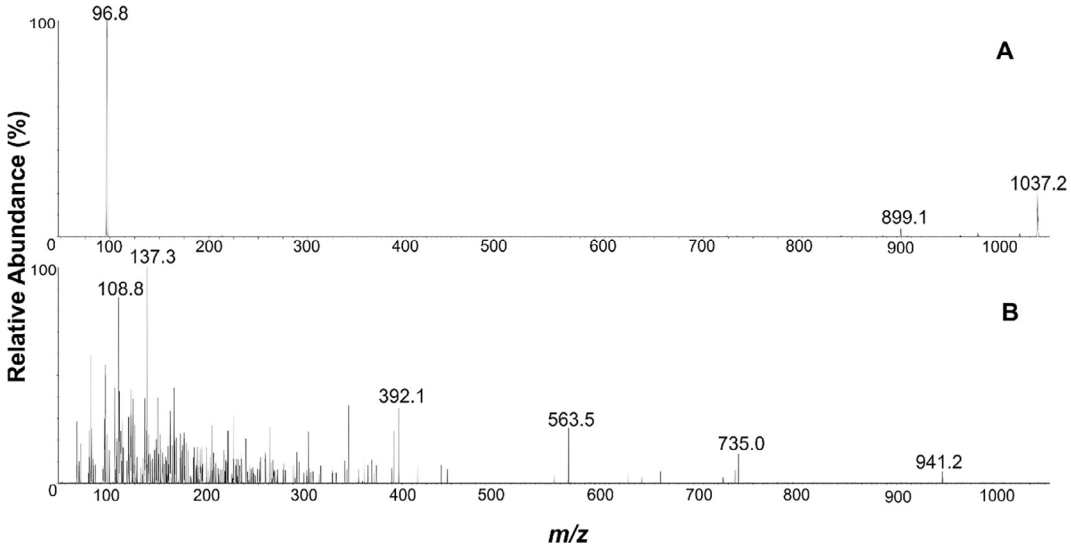

Fig. 3. Collision induced dissociation (CE 50 V) tandem MS spectra (*m*/*z* 48–1,050) of purified **2** generated from (A) the $[M-H]^-$ parent ion (*m*/*z* 1,037.2) in −ESI mode; and (B) the $[M+H]^+$ parent ion (*m*/*z* 1,039.2) in +ESI mode.

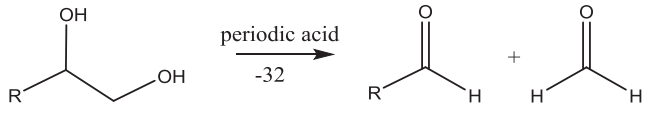

Fig. 4. Periodic acid oxidation reaction of **2** suggesting the presence of a terminal diol.

43,45-diene functionality of **2**, compared to those for **1**, it was evident that one of the olefinic protons had been replaced by a methyl group. Detailed analysis of the ^1H and ^{13}C chemical shifts and proton signal multiplicities determined for atoms of the 43,45-diene system revealed that the methyl group's carbon atom was attached to C-44 and that its ^1H and ^{13}C NMR signals occurred at 1.75 ppm and 12.0 ppm respectively. The attachment of a methyl group to C-44 was confirmed by the occurrence of the H-43 signal of **2** (5.45 ppm) as a well-defined triplet (J = 7.4 Hz) arising from its coupling to the pair of H-42 methylene protons at 2.39 ppm. Whereas the equivalent H-43 signal of **1** (5.70 ppm) appears as a doublet of triplets arising from couplings to H-44 and the pair of H-42 methylene protons (J = 15.0, 7.0 Hz respectively). The protons of the 44-methyl group showed HMBC correlations to ^{13}C signals which occurred at 132.6, 136.3 and 142.9 ppm representing C-43, C-44 and C-45 respectively.

Resolution enhancement processing of the ^1H NMR spectrum of **2**, and correlations observed in the COSY and TOCSY spectra of **2**, showed that the 44-methyl group exhibited long range doublet and triplet couplings to H-43 (J = 1.2 Hz) and the pair of H-42 protons (J = 0.6 Hz) respectively. ROESY, NOESY, SELROESY and SELNOESY correlations determined for H-43 (5.45 ppm), H-45 (6.34 ppm) and H-46 (4.90 and 5.09 ppm) olefinic protons and the olefinic methyl group (1.75 ppm) substantiated the conclusion that **2** was the 44-methyl analogue of **1** (Fig. 5) and established there was a *trans* relationship between H-43 and the 44-methyl

Table 1
^{13}C NMR (201 MHz) and ^{1}H (800 MHz) chemical shifts (ppm), multiplicity and coupling constants coupling constants (Hz) for gambierone (**1**) and 44-methylgambierone (**2**) generated in CD_3OD.

Atom	Gambierone[a]		44-Methylgambierone	
	δ_C	δ_H (multi, *J* in Hz)	δ_C	δ_H (multi, *J* in Hz)
1	67.9	3.47 (dd, 11.0, 4.5), 3.42 (m)	68.1	3.47 (dd, 11.2, 4.6), 3.41 (m)
2	70.3	4.11 (m)	70.5	4.10 (m)
3	39.6	2.01 (m), 1.70 (dd, 14.5, 10.0)	40.1	2.01 (m), 1.70 (14.6, 10.1)
4	101.0	–	101.1	–
5	73.1	4.21 (d, 3.2)	73.3	4.21 (d, 3.1)
6	77.9	4.70 (dd, 10.0, 3.2)	78.0	4.71 (dd, 9.7, 3.1)
7	77.4	3.37 (t, 10.0)	77.6	3.37 (t, 9.9)
8	68.0	3.77 (m)	68.3	3.77 (m)
9	37.8	2.17 (m), 1.58 (q, 12.0)	38.2	2.17 (m), 1.58 (q, 11.5)
10	79.8	3.35 (m)	79.9	3.35 (m)
11	82.6	3.79 (m)	82.7	3.78 (m)
12	133.2	5.64 (12.5, 2.5)	133.1	5.63 (dd, 12.6, 2.4)
13	133.5	5.75 (12.5, 2.5)	133.5	5.75 (dd, 12.6, 2.4)
14	83.2	3.81 (m)	83.4	3.81 (m)
15	80.0	3.44 (m)	80.1	3.43 (m)
16	47.4	1.99 (m), 1.49 (t, 11.0)	47.7	1.99 (dd, 12.2, 5.3), 1.49 (br t 11.6)
17	76.7	–	76.9	–
18	87.0	3.00 (dd, 11.0, 2.5)	87.1	3.01 (dd, 11.1, 2.8)
19	25.3	1.78 (m), 1.62 (m)	25.7	1.78 (m), 1.63 (m)
20	34.2	1.95 (m), 1.80 (m)	34.5	1.95 (m), 1.75 (m)
21	87.6	3.54 (m)	87.7	3.54 (m)
22	75.9	3.54 (m)	76.1	3.55 (m)
23	32.7	1.82 (m), 1.64 (m)	33.1	1.81 (m). 1.64 (m)
24	29.5	1.92 (m), 1.77 (m)	29.8	1.92 (m), 1.78 (m)
25	35.6	2.19 (m)	35.9	2.19 (m)
26	86.1	3.11 (m)	86.2	3.11 (m)
27	77.9	3.51 (m)	78.1	3.51 (m)
28	39.8	2.20 (m), 1.33 (q, 11.3)	40.0	2.19 (m), 1.33 (q, 11.4)
29	70.2	3.12 (td, 9.5, 4.3)	70.4	3.11 (t,d, 9.6, 4.2)
30	78.3	2.94 (ddd, 11.5, 9.5, 4.5),	78.4	2.94 (ddd, 11.9, 9.6, 4.5)
31	34.9	1.90 (m), 1.55 (q, 11.5)	35.2	1.88 (m), 1.55 (q, 11.7)
32	72.6	3.77 (m)	72.8	3.77 (m)
33	77.4	–	77.6	–
34	54.2	2.35 (d, 12.0), 2.14 (d, 12.0)	54.4	2.35 (br d, 12.0), 2.14 (br d, 12.0)
35	144.0	–	143.8	–
36	43.3	2.54 (d, 14.0), 2.22 (d, 14.0)	43.6	2.54 (br d, 14.0), 2.22 (br d, 14.0)
37	80.0	–	80.1	–
38	73.5	4.06 (dd, 8.7, 3.5)	73.8	4.06 (dd, 9.6, 2.7)
39	45.8	2.62 (dd, 12.0, 3.0), 2.60 (dd, 12.0, 9.0)	46.2	2.60 (m, 2H)
40	212.2	–	211.8	–
41	43.7	2.61 (t, 7.0)	44.2	2.59 (t, 7.4)
42	27.3	2.33 (q, 7.0)	23.6	2.39 (q, 7.4)
43	134.9	5.70 (dt, 15.0, 7.0)	132.6	5.45 (br t, 7.4)
44	133.4	6.08 (dd, 15.0, 10.5)	136.3	–
45	138.9	6.29 (dt, 17.0, 10.4)	142.9	6.34 (ddd, 17.4, 10.5, 0.8)
46	115.9	5.08 (dd, 17.0, 1.8), 4.94 (dd, 10.3, 1.8)	111.5	5.09 (br d, 17.4), 4.90 (br d, 10.5)
47	16.2	1.20 (3H, s)	17.0	1.21 (3H, s)
48	13.3	1.00 (3H, d, 7.3)	13.8	1.00 (3H, d, 7.0)
49	16.7	1.19 (3H, s)	17.2	1.19 (3H, s)
50	119.0	4.98 (br s), 4.86 (br s)	118.9	4.97 (br s), 4.85 (br s)
51	20.6	1.13 (3H, s)	21.0	1.13 (3H, s)
52			12.0	1.75 (td, 1.2, 0.6)[b]

[a] Values from Rodriguez et al. (2015) [9].
[b] Visible when processed with resolution enhancement.

group across the 43(44)-double bond. All of the ring and side chain protons of **2** displayed positive ROESY and SELROESY correlations, as did the ring A–I protons of **2** in NOESY and SELROESY experiments. In addition, negative NOESY and SELNOESY correlations were observed for the H-41–H-46 protons and the 44-methyl group.

While the NMR data presented in Table 1 does not establish the absolute configuration of **2**, it is reasonable, based on the close correspondence of the ring A–I chemical shifts observed for **1** and **2**, to propose that the absolute configuration of **2** corresponds to that proposed by Rodriguez et al., 2015 [9].

Conclusion

44-Methylgambierone (**2**), was isolated from four monocultures of the benthic dinoflagellate *Gambierdiscus australes*, collected from Raoul Island (Rangitahua/Kermadec Islands). The structure of **2** has many similarities with gambierone; a single carbonyl, 1,3-diene, a terminal diol and monosulphate, and these features were used to confirm the core structural characteristics of **2**. Using NMR, the nine-ring backbone (A–I) was confirmed to be the same as **1** and the addition of an olefinic methyl on C-44 accounted for the 14 Da difference in molecular weight. Future work will be

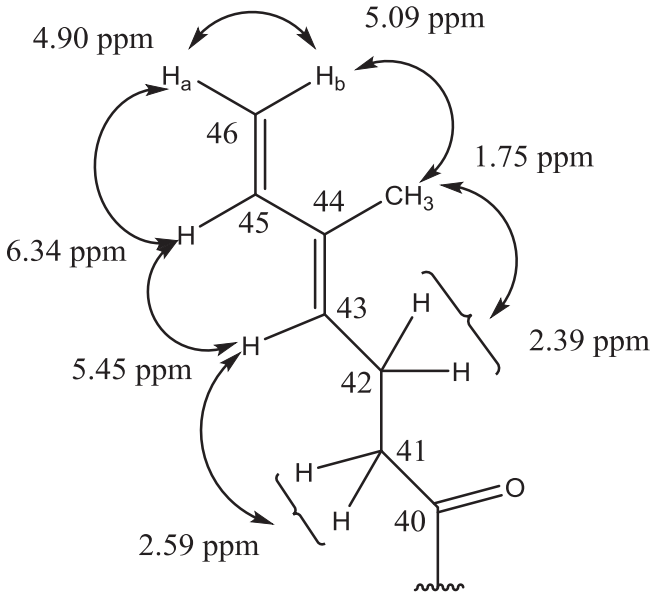

Fig. 5. ROESY correlations observed for H-43, H-45, H-46 and the 44-methyl group.

conducted to accurately quantify the mass yield of **2** using qNMR and allow the determination of acute toxicity on purified material.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tetlet.2019.01.043.

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