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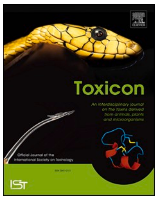

Production and stability of Oxygen-18 labeled Caribbean ciguatoxins and gambierones

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ABSTRACT

Ciguatoxins (CTXs) and gambierones are ladder-shaped polyethers associated with ciguatera poisoning and *Gambierdiscus* spp. Several of these compounds contain carbonyl or hemiketal groups, which have the potential to exchange with ^{18}O-labeled water under acidic conditions. The effects of solvent composition and acid on the rate of exchange and on the stability of the labels at various pH values were assessed to optimize the incorporation of ^{18}O into Caribbean ciguatoxin-1 and -2 (C-CTX1/2), gambierone, and 44-methylgambierone. LC–HRMS results showed that ^{18}O-labeling occurred at the hydroxy group of the hemiketal at C-56 in C-CTX1/2, and at the hydroxy group of the hemiketal at C-4 and the ketone at C-40 in gambierones. Labeling occurred very rapidly (complete in <30 min) for C-CTX1/2, and more slowly (complete in ca. 16 h) for both gambierones. Labeled C-CTX1/2 was reduced with sodium borohydride to produce ^{18}O-labeled C-CTX3/4. The incorporated ^{18}O labels in the gambierones and C-CTXs were retained in aqueous solvent mixtures under neutral conditions in a short-term stability study, demonstrating that these ^{18}O-labeled toxins have the potential to be used in isotope dilution and metabolism studies.

1. Introduction

Ciguatera poisoning is caused by the consumption of ciguatoxin-contaminated seafood including commercially relevant fish harvested from tropical and sub-tropical regions. Ciguatoxins (CTXs) production has been linked to the benthic dinoflagellate genera *Gambierdiscus* and *Fukuyoa* with toxins moving into marine food webs via herbivory fish. Several precursor Pacific CTXs have been identified in these genera (Chinain et al., 2010), and have been shown to undergo biotransformation into the more toxic CTXs found in fish flesh (Ikehara et al., 2017).

Ciguatoxins are large ladder-shaped polyether compounds with molecular masses of 1000–1150 Da. They are odourless, tasteless and heat-stable compounds that are potent voltage-gated sodium channel activators, and very low levels are required to induce a toxic effect (Friedman et al., 2008). While no regulatory limit for CTXs has been established, the guidance levels of 0.01 μg/kg CTX1B and 0.1 μg/kg Caribbean CTXs (C-CTXs) in fish flesh suggested by the US Food and Drug Administration has also been endorsed by the European Food Safety Authority (EFSA Panel on Contaminants in the Food Chain, 2010; Food and Agriculture Organization of the United Nations and World Health Organization, 2020; Food and Drug Administration, 2021). Detection of CTXs at these levels using mass spectrometry can be difficult due to significant matrix effects, low recovery, poor ionization efficiency, and in-source fragmentation, which all contribute to poor sensitivity (Harwood et al., 2017; Suzuki et al., 2017).

Gambierdiscus and *Fukuyoa* spp. produce a wide array of ladder-shaped polyethers including gambierol (Satake et al., 1993a), gambieric acids (Morohashi et al., 2000), maitotoxins (Murata et al., 1994), CTXs (Satake et al., 1993b, 1996) and gambierones (Rodríguez et al., 2015). Gambierone and 44-methylgambierone are members of a class of sulfated polyethers identified across several species of these genera and in *Coolia tropicalis* (Murray et al., 2020, 2021; Tibiriçá et al., 2020). Gambierones exhibit CTX-like effects on sodium channels, although at a

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much lower potency than CTXs, and have minimal toxicity following intraperitoneal injection in mouse bioassays (Boente-Juncal et al., 2019; Murray et al., 2020, 2021; Rodríguez et al., 2015). While the diversity and distribution of gambierones across *Gambierdiscus* and other benthic dinoflagellate genera is not fully understood, the presence of known gambierones in several species suggests they have potential to be used as biomarkers for environmental monitoring of *Gambierdiscus* dominance in coral reef systems (Murray et al., 2020; Yon et al., 2021).

LC−MS analysis of complex samples, including fish tissues, can result in performance issues associated with matrix effects and sample preparation that can be improved through the use of isotopically labeled analytes as internal standards (Haddad et al., 2019; Stokvis et al., 2005). Furthermore, isotopically labeled analytes can be used to investigate in vitro and in vivo metabolism, where the use of mass spectrometry can provide accurate metabolite tracing and potentially identify pathways associated with metabolism (Mutlib, 2008). There is limited availability of CTX standards, especially those associated with the Caribbean region. Several algal CTXs can be isolated from toxin-producing *Gambierdiscus* isolates, but this will not be feasible for C-CTXs until C-CTX-producing algae can be identified and brought into culture. With their complex structures and laborious synthesis schemes (Inoue et al., 2006; Sasaki et al., 2021), availability of standards relies on isolation from incurred fish tissue. This limits the possibilities for incorporating isotopic labeling during laboratory synthesis, or by modification of culture conditions such as that used in the production of ^{18}O-labeled yessotoxins (Yamazaki et al., 2012). At this time, there are no commercially available isotopically labeled CTX standards. An alternative strategy for stable isotope labeling would be to utilize acid-catalyzed oxygen exchange. In this case, carbonyl groups exchange oxygen with water by the reversible formation of hydrates (Theodorou et al., 2014). For example, ^{18}O-labeling of peptides on their carboxyl-containing amino acid residues has proven to be a successful method for isotopic labeling (Niles et al., 2009).

In this study, we investigated the feasibility of oxygen exchange with C-CTXs and gambierones using ^{18}O-labeled water. The aim of this work was to identify suitable reaction conditions for label incorporation and the extent of labeling for these marine toxins. The labeling kinetics and label stability were analyzed to evaluate their potential for use in analytical and biological studies.

2. Material and methods

2.1. Chemical and reagents

Acetonitrile and formic acid (~98%) were LC–MS grade from Fisher Scientific (Ottawa, ON, Canada). Ammonium acetate (LC–MS grade, ~98%), sodium borohydride (~98%) and *m*-aminophenylboronic acid–agarose (mAPBAG) aqueous gel suspension were from Millipore–Sigma (Oakville, ON, Canada). Glass-distilled dichloromethane was from Caledon Laboratories (Georgetown, ON, Canada). Additional MeOH, dichloromethane, and hexane used in preparation of fish materials was HPLC grade from Fisher Scientific (Waltham, MA, USA). $H_2^{18}O$ (97 atom-% ^{18}O) was from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Distilled water was ultra-purified to 18.2 MΩ·cm using a Milli-Q water purification system (Millipore–Sigma). All solvent mixtures were prepared by volume. Gambierone (19.9 μg/mL in MeOH) was from CIFGA (Lugo, Spain) and 44-methylgambierone (25 μg/mL in MeOH) was from Cawthron Institute (Nelson, New Zealand).

2.2. Preparation of semi-purified C-CTX1/2

Due to the current lack of reference materials for C-CTXs, semi-purified C-CTX1/2 was prepared from C-CTX-laden fish muscle tissue (*Sphyraena barracuda* and *Scomberomorus cavalla*), and a small aliquot of the semi-pure toxin isolate was used in this study. Briefly, fish previously collected near St. Thomas, U.S. Virgin Islands that had been confirmed to be toxic via an in vitro ouabain-veratrine dependent mouse neuroblastoma assay, and the presence of C-CTX-1/2 verified using LC–MS/MS analysis (described elsewhere (Robertson et al., 2014)), were homogenized, combined, and subsequently extracted in MeOH (2 mL/g) three times. Extracts were dried by rotary evaporation at 50 °C, then reconstituted in 80% aqueous MeOH and twice partitioned with hexane (1:1). The methanolic phase was subsequently adjusted to 60% aqueous MeOH and partitioned three times with CH_2Cl_2 and dried at 40 °C by rotary evaporation. The residue was reconstituted in CH_2Cl_2 and loaded onto an open column packed with silica gel (BDH, 120 g, 60 Å, 60–200 μm; VWR, Suwanee, GA, USA) and eluted by gravity. The column was preconditioned with approximately 5 bed volumes of CH_2Cl_2. The C-CTX1/2 pool was eluted in 95:5 CH_2Cl_2–MeOH, dried and then loaded onto a prepacked silica cartridge (5 g/20 mL, Strata® SI-1 Silica, 55 μm, 70 Å; Phenomenex, Torrance, CA, USA) that had been preconditioned with CH_2Cl_2. The C-CTX1/2 was eluted with 4% MeOH in CH_2Cl_2 and dried. The residue was further fractionated by semi-preparative HPLC on a Luna PFP(2) column (150 × 10 mm, 5 μm; Phenomenex, Torrance, CA, USA) at 30 °C with a mobile phase composed of water (A) and methanol (B) with gradient elution (3.0 mL/min) as follows: 0–1 min, 60% B; 1–15 min, 60–100% B; 15–22 min, 100% B; followed by re-equilibration at 60% B. Fractions containing C-CTX1/2 were pooled and verified by LC–HRMS analysis (Kryuchkov et al., 2020).

2.3. ^{18}O-exchange experiments

Experiment 1 (preliminary investigation)

Aliquots (10 μL) of *S. barracuda* extract, gambierone and 44-methylgambierone were prepared separately by evaporation under N_2 at 35 °C in glass vials. The residues were each dissolved in 8:4:1 MeCN–$H_2^{18}O$–formic acid (10–50 μL). The solutions were vortex-mixed for 1 min and allowed to stand at ambient temperature for 48 h, then transferred to a vial insert and analyzed by LC–HRMS.

Experiment 2 (labeling kinetics)

Gambierone and 44-methylgambierone (10 μL of each) were added into the same vial and evaporated under N_2 at 35 °C. An aliquot of *S. barracuda* extract (10 μL) was aliquoted into a separate vial and also evaporated under N_2 at 35 °C. The residues were dissolved in 40:9:1 MeCN–$H_2^{18}O$–formic acid (50–100 μL) and vortex-mixed for 1 min. The solutions were transferred to vial inserts and placed in the LC autosampler at 25 °C and analyzed repeatedly for 17 h by LC–HRMS.

Experiment 3 (borohydride reduction of ^{18}O-labeled C-CTXs and gambierones)

Semi-purified C-CTX1/2 (2 μL) and gambierone (10 μL) were evaporated under N_2 at 35 °C in separate vials and prepared according the procedure described in *Experiment 1* and allowed to stand for 2 h and 24 h, respectively. $NaBH_4$ (1 mg) was added to the solutions and allowed to react for 10 min. The reaction was terminated by addition of 10% formic acid (10 μL) and the solution filtered through a PVDF filter (0.22 μm, Canadian Life Sciences; Peterborough, ON, Canada) at 6010×*g*. The filtrate was transferred to a vial insert and analyzed by LC–HRMS.

Experiment 4 (removal of acid from ^{18}O-labeled C-CTX1/2 by partitioning)

Semi-purified C-CTX1/2 (2.5 μL) was evaporated under N_2 at 35 °C and dissolved in 45:45:2 MeCN–$H_2^{18}O$–formic acid (92 μL). The solution was vortex-mixed for 1 min and allowed to stand at ambient temperature for 2 h. The aqueous content of the solution was increased by the addition of $H_2^{18}O$ (20 μL) and transferred to a glass conical test tube. Dichloromethane (CH_2Cl_2) (100 μL) was added and the solution was vortex-mixed for 1 min and centrifuged at 260×*g* for 3 min to separate the two layers. The CH_2Cl_2 layer was removed and evaporated under N_2 at 40 °C, dissolved in 100 μL of MeCN, and transferred to a vial insert for LC–HRMS analysis.

Table 1
Parameters evaluated and the overall level of ^{18}O incorporation into C-CTX1/2.

Trial	Acid type	Acid (%)	$H_2{}^{18}O$ (%)	Temperature (°C)	Extent of labeling (%) $[M + NH_4]^+$
1	Trifluoroacetic acid	5	40	25	30.6
2	Trifluoroacetic acid	2.5	20	40	35.2
3	Formic acid	5	20	40	60.8
4	Formic acid	2.5	40	25	73.8

2.4. Partial factorial study of [^{18}O]C-CTX1/2 labeling parameters

A two-level partial factorial design was applied to the labeling of CTXs by modifying the conditions described in *Experiment 1*. The parameters evaluated included: the proportion of ^{18}O-water, the type and concentration of the acid used, and temperature. A low and a high level was used for each parameter as described (Table 1). Aliquots (10 μL) of *S. barracuda* extract were prepared separately by evaporation under N_2 at 35 °C in glass vials. The residues were each dissolved in MeCN–$H_2{}^{18}O$–acid (10 μL) according to Table 1. The solutions were vortex-mixed for 1 min and allowed to stand at ambient temperature for 48 h, then transferred to a vial insert and analyzed by LC−HRMS to determine the extent of labeling for each trial.

2.5. LC–HRMS analysis

Analyses were performed according to Kryuchkov et al. (2020) with some modifications. An Agilent 1200 LC was equipped with a binary pump, temperature-controlled autosampler (10 °C) and column compartment (40 °C) (Agilent Technologies, Mississauga, ON, Canada) coupled to a Q Exactive HF Orbitrap mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) with a heated electrospray ionization probe (HESI-II). Chromatographic separation was on an F5 column (100 mm × 2.1 mm, 1.7 μm; Phenomenex, Torrance, CA, USA) using gradient elution. The mobile phase was composed of water (A) and 95:5 acetonitrile–water (B), each containing 5 mM ammonium acetate (pH 6.8). The gradient employed varied for gambierones and C-CTXs. The gradient for C-CTXs was as follows: 0–18 min, 30–60% B; 18–18.1 min, 60–99% B; 18.1–22 min, 99% B; followed by a 4-min re-equilibration at 30% B. The gradient for gambierones was as follows: 0–18 min, 10–80% B; 18–18.1 min, 80–99% B; 18.1–22 min, 99% B; followed by an 8 min re-equilibration with 10% B. The flow rate was 0.3 mL/min with an injection volume of 5.0 μL.

Full-scan acquisition was performed with a range of m/z 1000–1250 for C-CTXs in positive polarity, and m/z 800–1400 for gambierones with positive and negative polarity switching. The spray voltage of the source was ±4500 V, with a capillary temperature of 400 °C. The sheath and auxiliary gas were set at 45 and 10, respectively, with a max spray current of 100 μA. The probe heater temperature was set at 250 °C and the S-Lens RF level was set to maximum (100). The mass resolution was set at 120 000 with an AGC target of 5×10^6 and a maximum injection time of 512 ms per scan.

Product-ion spectra were acquired using parallel reaction monitoring (PRM) in positive mode with an isolation window of 1 Da. The mass resolution setting was set at 240 000 with an AGC target of 5×10^6 and a maximum injection time of 512 ms, with a normalized collision energy of 12 for C-CTXs, and a collision energy of 30 eV for gambierones.

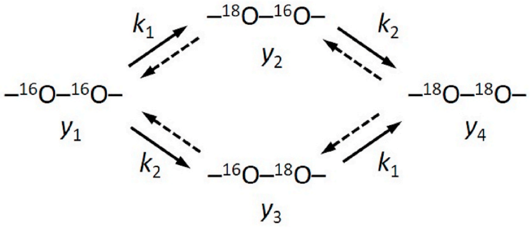
2.6. Isotope distribution analysis

Isotopic peak height profiles were collected from LC−HRMS spectra of the protonated or deprotonated molecule ($[M+H]^+$, $[M-H]^-$) as well as sodium ($[M+Na]^+$) or ammonium ($[M + NH_4]^+$) adducts of the compounds of interest. The observed isotopic profiles were used to extract the isotopic composition of oxygen-18 of the CTXs and gambierones. By knowing the identity of the analyzed compounds (their molecular formulae), we established the corresponding isotopic patterns using Fourier-transform-based methods described by Ipsen (2014) and implemented in R package ecipex (https://CRAN.R-project.org/package=ecipex). By specifying the number of exchangeable sites for incorporation of oxygen-18, and adopting natural isotopic composition of all other makeup elements in the molecules, the isotopic composition of oxygen at these sites was obtained using partial least-squares fitting. The NRC Isotopic Enrichment Calculator (Mallia et al., 2019) was modified to implement these calculations (Version December 2021; currently available at https://metrology.shinyapps.io/isotopic-enrichment-calculator with source code available from https://github.com/meijaj/isotopic-enrichment-calculator). Parsing mass spectra provides isotopic composition of oxygen at each of the labeling sites and the relative abundances of isotopologues having, in the case of gambierones, 0–2 oxygen-18 atoms incorporated. These results were then used to fit the kinetic model of oxygen-18 uptake as described below. The changes in the abundance of label at different locations in the molecule and the total extent of labeling were plotted in SigmaPlot (version 14.0) against time for *Experiment 2*.

2.7. Analysis of the isotopic label stability

C-CTX1/2 (10 μL) was evaporated under N_2 at 35 °C and reconstituted in 45:45:2 MeCN–$H_2{}^{18}O$–formic acid (92 μL). The solution was vortex-mixed for 1 min and allowed to stand at ambient temperature for 2 h. The aqueous content of the solution was increased by the addition of $H_2{}^{18}O$ (20 μL) and transferred to a glass conical test tube. CH_2Cl_2 (100 μL) was added and the solution was vortex-mixed for 1 min and centrifuged at 260×*g* for 3 min to separate the two layers. The CH_2Cl_2 layer was separated, evaporated under N_2 at 40 °C, and reconstituted in 100 μL of MeCN to afford a solution of ^{18}O-labeled C-CTX1/2. Buffers of various pH were assessed for their effects on label stability. Buffers (100 mM) were prepared at pH 3.0 (formate), pH 5.0 (acetate), pH 6.7 (ammonium acetate), pH 7.0 (phosphate) and pH 9.0 (ammonium bicarbonate). ^{18}O-labeled C-CTX1/2 (20 μL) was mixed with 1:1 MeCN–buffer (90 μL), placed in the HPLC autosampler at 25 °C, analyzed by LC−HRMS at regular intervals for 17 h, and the isotopic profile assessed with the NRC Isotope Enrichment Calculator to assess the stability of the isotope labeling of CTXs overnight.

Gambierone and 44-methylgambierone (10 μL) were aliquoted together into a vial and evaporated under N_2 at 35 °C. The residue was dissolved in 8:4:1 MeCN–$H_2{}^{18}O$–formic acid (100 μL) and allowed to stand at ambient temperature for 48 h. The solvent was evaporated under N_2 and the residue dissolved in $CHCl_3$ (250 μL) and prepared mAPBAG was added (100 μL, filtered) (Mudge et al., 2022). The suspension was shaken for 3 h at 850 rpm and ambient temperature. The $CHCl_3$ was removed with a micropipette and residual solvent was gently evaporated from the gel under N_2. The gambierones were eluted from the gel by adding 1:1 MeCN–H_2O (250 μL) and shaking the suspension at 850 rpm for 2 h. The resulting solution of ^{18}O-labeled gambierones was transferred to a glass vial with a micropipette and stored at −20 °C until the stability assessment. Strongly acidic conditions (HCl, 100 mM; pH 1.0) and various pH buffers (100 mM) were prepared at pH 3.0 (formate), pH 5.0 (acetate), pH 6.7 (ammonium acetate), pH 7.0 (phosphate) and pH 9.0 (ammonium bicarbonate) to assess the stability of ^{18}O-labeled gambierones. Aliquots of the solution of ^{18}O-labeled gambierones (10 μL) were combined with 1:1 MeCN–buffer (90 μL), placed in the HPLC autosampler at 25 °C, and analyzed by LC−HRMS at regular intervals for 17 h. The observed isotopic patterns were then evaluated with the NRC Isotope Enrichment Calculator to assess the stability of the isotope labeling of gambierones.



Scheme 1. The kinetic model for oxygen-18 exchange in gambierones.

2.8. Kinetic modeling and data fitting

The changes in the isotopic composition of gambierones were modelled using a network of reversible first-order reactions. The two sites of exchangeable oxygen give rise to four distinct isotopologues depending on the incorporation of oxygen-18 (Scheme 1): unlabeled gambierone (^{16}O–^{16}O or y_1), two mono-labeled gambierones (^{16}O–^{18}O and ^{18}O–^{16}O or y_2 and y_3), and bi-labeled gambierone (^{18}O–^{18}O or y_4).

The concentration profiles of these four isotopologues was modelled using a set of four ordinary differential equations with three parameters (eq. (1)) – forward rate constants corresponding to the uptake of oxygen-18 at each site (k_1 and k_2) and a scale parameter (R) which sets the magnitude of the two backward rate constants (k_1/R and k_2/R).

This mechanistic kinetic model was fitted to the parsed mass spectral data in R using a general-purpose quasi-Newton optimization method. For example, the observed proportion of mono-labeled gambierones at any given time is calculated from the model parameters as $[y_2(t) + y_3(t)]/[y_1(t) + y_2(t) + y_3(t) + y_4(t)]$. The kinetic model was fitted to parsed mass spectra by finding the best parameter values that minimized the squared differences between the observed and predicted proportions of non-labeled, mono-labeled, and bi-labeled gambierones across all time points.

2.9. Stability data fitting

Stability data from Section 2.6 were plotted in SigmaPlot (version 14.0) where 3-parameter exponential decay curves were fitted to the stability data with constraints to the variables y_0 and b, which were set to 0.2% (the natural abundance of ^{18}O), and >0 (eq. (2)), respectively.

$$\text{Extent of Labeling } (\%, \text{ stability}) = y_0 + a \times e^{(-b \times t)} \tag{2}$$

Observed half-lives ($t_{½}$) were calculated from the first order rate constants (b) obtained from fitting the stability data.

$$\begin{bmatrix} dy_1/dt \\ dy_2/dt \\ dy_3/dt \\ dy_4/dt \end{bmatrix} = \begin{bmatrix} -k_1 - k_2 & R^{-1}k_1 & R^{-1}k_2 & 0 \\ k_1 & -R^{-1}k_1 - k_2 & 0 & R^{-1}k_2 \\ k_2 & 0 & -k_1 - R^{-1}k_2k_1 & R^{-1}k_1 - k_1 - k_2 \\ 0 & k_2 & & \end{bmatrix} \times \begin{bmatrix} y_1(t) \\ y_2(t) \\ y_3(t) \\ y_4(t) \end{bmatrix} \tag{1}$$

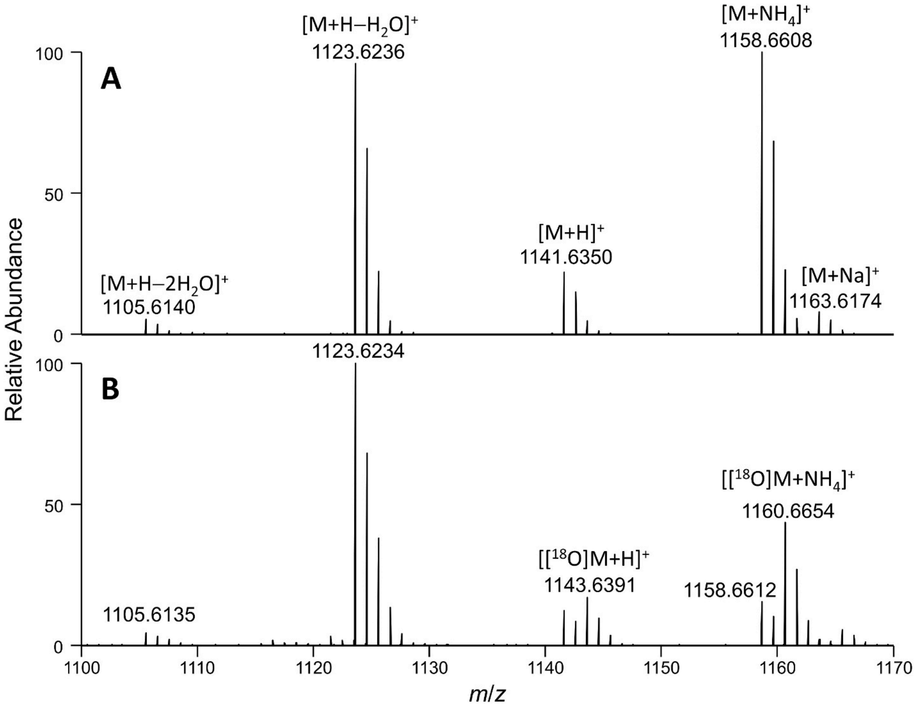


Fig. 1. Full-scan mass spectra in positive ionization mode for C-CTX1/2 in: (A) unlabeled control, and; (B) ^{18}O-labeled C-CTX1/2 after 48 h of reaction (*Experiment 1*), using an extract of *S. barracuda* containing C-CTX1/2.

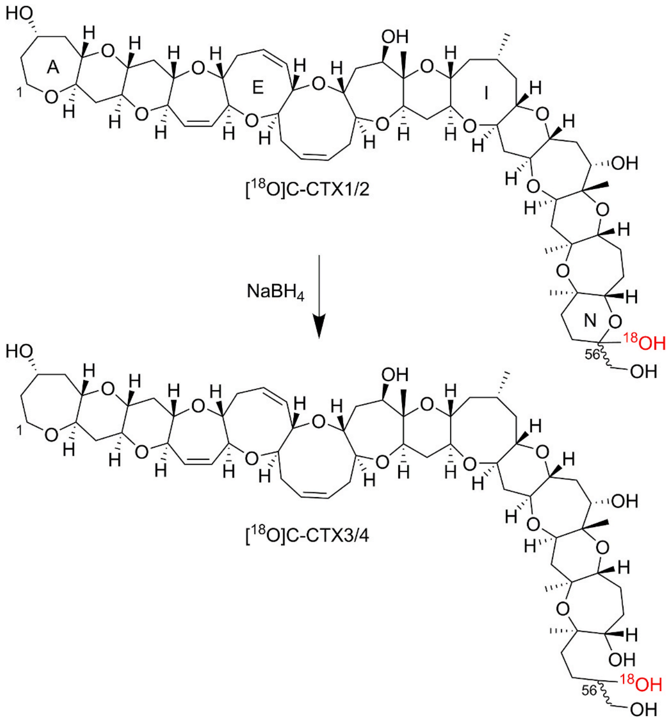


Fig. 2. Sodium borohydride reduction of ^{18}O-labeled C-CTX1/2 to produce labeled C-CTX3/4.

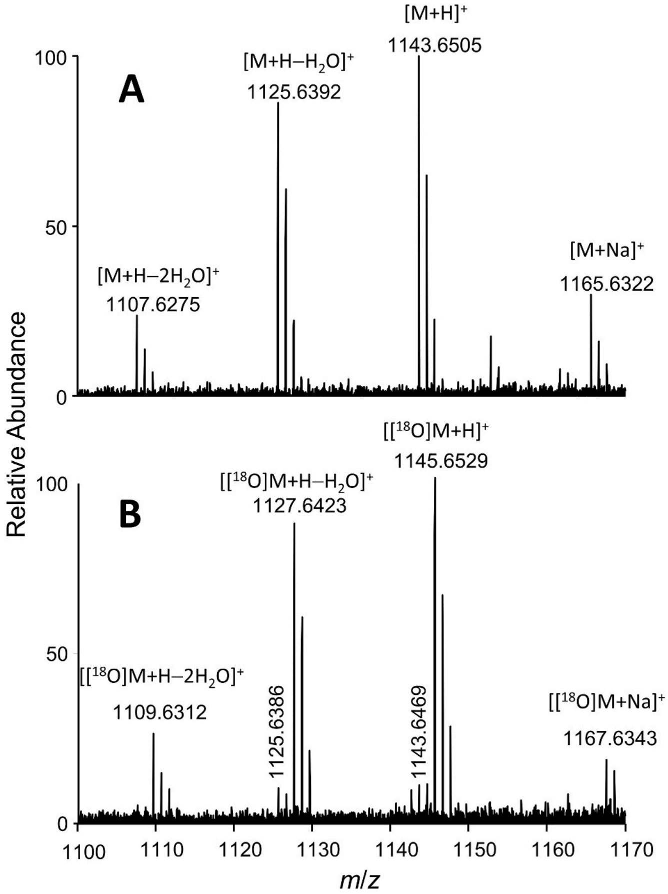


Fig. 3. Full-scan mass spectra in positive ionization mode for: (A) unlabeled C-CTX3/4, and; (B) ^{18}O-labeled C-CTX3/4 produced by sodium borohydride reduction of ^{18}O-labeled C-CTX1/2 (*Experiment 3*).

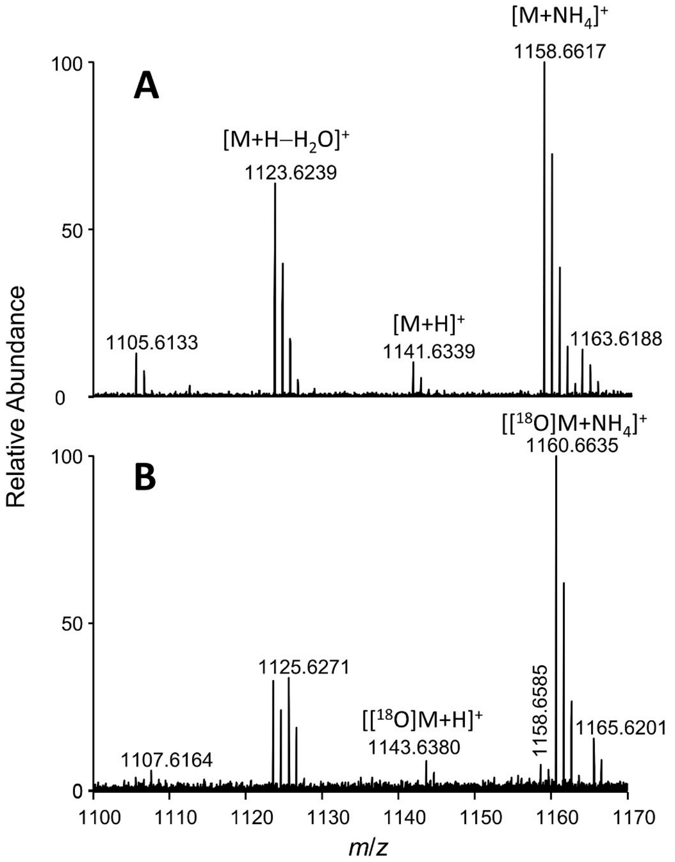


Fig. 4. Full-scan mass spectra in positive ionization mode for: (A) unlabeled C-CTX1/2, and; (B) ^{18}O-labeled C-CTX1/2 after CH_2Cl_2 partitioning to remove residual acid (*Experiment 4*).

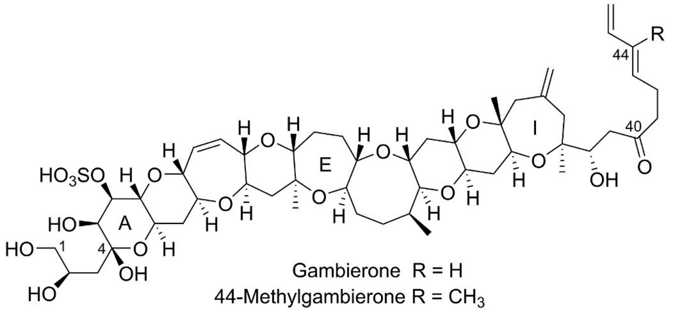


Fig. 5. Chemical structures of gambierone and 44-methylgambierone.

3. Results and discussion

3.1. ^{18}O-labeling of C-CTXs

The hemiketal on the N-ring at C-56 on C-CTX1/2 (Fig. 2) opens under acidic conditions, as suggested by the formation of a methyl ketal in acidified methanol (Estevez et al., 2020a), and by the apparent on-column epimerization during LC–HRMS (Kryuchkov et al., 2020). This potentially allows for exchange with $H_2{}^{18}O$ to produce ^{18}O-labeled C-CTXs. Preliminary work was performed with aliquots of fish extracts contaminated with C-CTXs and monitored using LC–HRMS. The extent of labeling of C-CTX1/2 was assessed by analyzing the isotope distribution of adduct ions in the full-scan mass spectra using the NRC Isotope Enrichment Calculator. Full-scan mass spectra (Fig. 1) obtained from *Experiment 1* revealed an apparent variation in ^{18}O-labeling in the

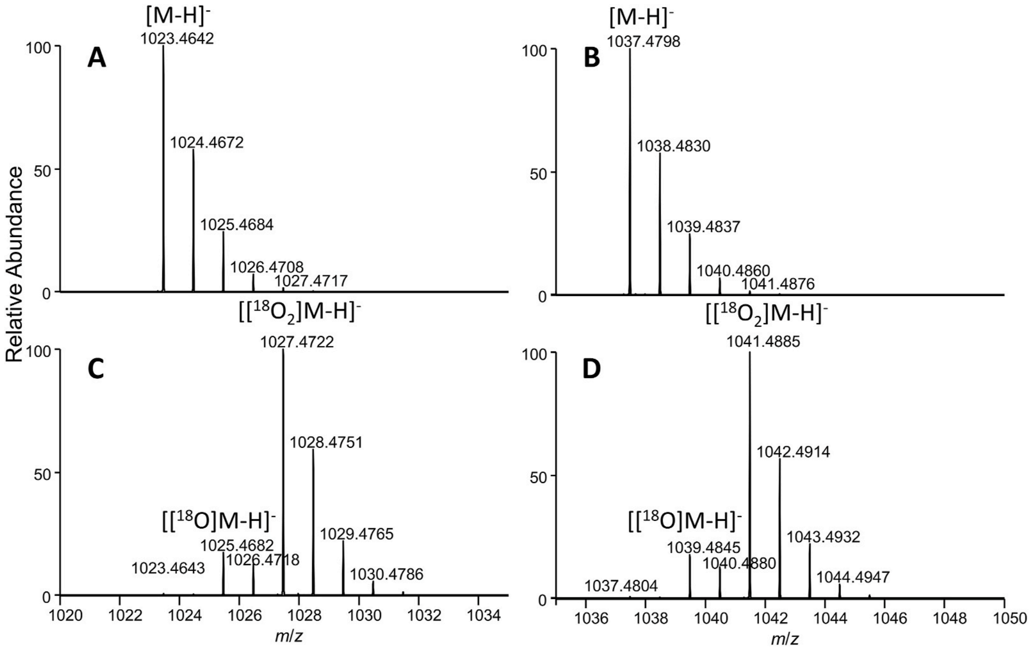


Fig. 6. Full-scan mass spectra in negative ionization mode for: unlabeled (A) gambierone and (B) 44-methylgambierone; and ^{18}O-labeled (C) gambierone and (D) ^{18}O-labeled 44-methylgambierone after 48 h of acid catalyzed reaction with $H_2{}^{18}O$ (*Experiment 1*).

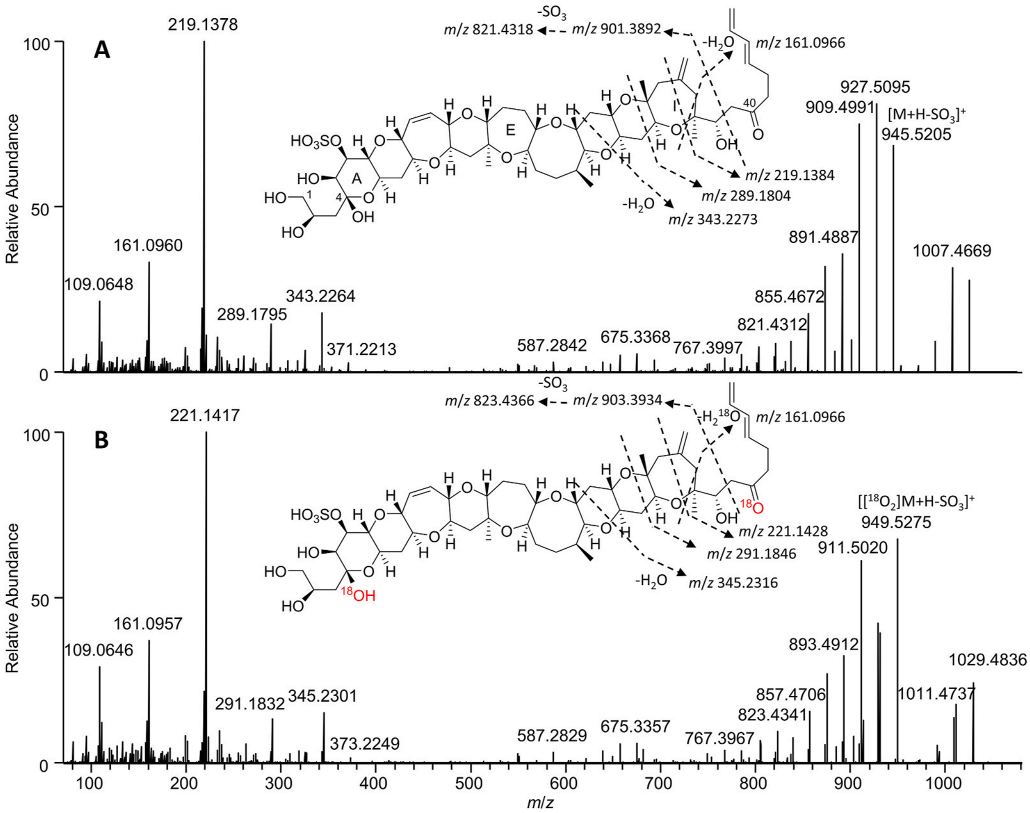


Fig. 7. Product-ion spectra in positive ionization mode of the protonated molecules of: (A) gambierone ($[M+H]^+$, *m/z* 1025.4750), and; (B) ^{18}O-labeled gambierone ($[M+H]^+$, *m/z* 1029.4844).

adducts, with the isotope distribution of $[[^{18}O]M + H]^+$ and the ammonium adduct indicating 52% and 71% labeling, respectively. The variance between the observed extent of labeling for the $[[^{18}O]M + H]^+$ and the ammonium adduct is most likely due to the facile loss of water in the electrospray ion source in positive mode. The loss of H_2O or $H_2{}^{18}O$ from the ammonium adduct interferes with the observed isotope distribution of the $[[^{18}O]M + H]^+$ ion. The labeling percentages were essentially identical when determined from the ammonium, sodium and potassium adducts (data not shown) in the full-scan spectrum, and were consistently higher than those determined from the corresponsing

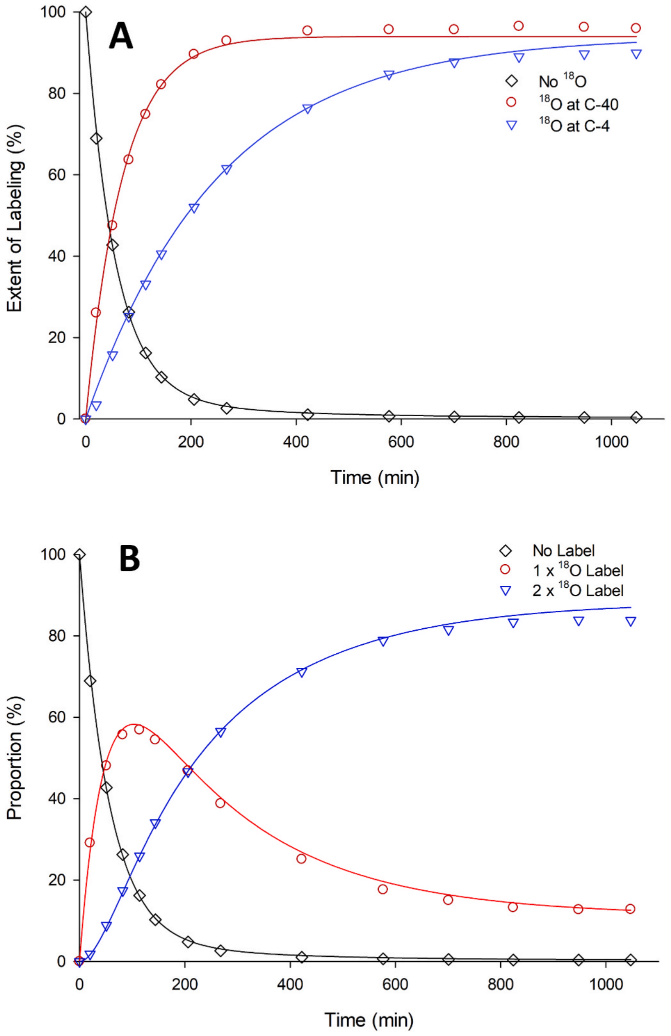


Fig. 8. ^{18}O-incorporation into gambierone with time. (A) Extent of labeling at each location, and; (B) proportion of gambierone molecules labeled at no, one, or both locations within the molecule (*Experiment 2*). The decay and growth curves were fitted to the data using a network of reversible first-order reactions.

$[M+H]^+$ ions, suggesting that the extent of labeling determined using the ammonium adduct should be used. The lower apparent labeling of the in-source water loss fragment at *m/z* 1123.6234 ($[M + H–H_2O]^+$ (Fig. 1) suggested that the labeling was at a location where it was readily lost from $[M+H]^+$ under ESI conditions. Due to the relatively low abundance of the $[[^{18}O]M + H]^+$ for the labeled CTX, it was not possible to obtain MS/MS spectra to confirm the location of label, but based on the presence of a hemiketal at C-56, the N-ring hemiketal is the only plausible location for the addition of the labeled oxygen.

The extent of labeling was relatively low after 48 h, therefore a partial factorial experiment was conducted to determine the factors responsible for the low extent of labeling observed in the preliminary experiment. These included a comparison of the proportion of ^{18}O-water, and the type and concentration of the acid used. The results comparing the average response of the low and high levels for each factor, as summarized in Table 1 and Fig. S1, indicated that lower percentages of labeling were observed with trifluoroacetic acid and higher acid concentration, and increased percentages with higher proportions of ^{18}O water, while temperature did not have a significant effect. Further investigations on the proportions of water were later found to have minimal effect on labeling, as it was present in excess relative to C-CTX1/2. Based on these findings, a 24-h kinetic study was performed to assess the rate of labeling (*Experiment 2*). Given the low ^{18}O-incorporation observed after 48 h and the limited supply of C-CTX1/2, these samples were placed in the autosampler at 25 °C and analyzed after 8 h, and every 4 h thereafter. At 8 h, labeling had reached 73% based on the ammonium adduct and did not change throughout the remainder of the experiment, therefore no kinetic modeling was possible for C-CTXs. These results suggested that ^{18}O-exchange with C-CTX1/2 might be fast, and that the low incorporation observed could be caused by back-exchange (loss of ^{18}O) prior to reaching the mass spectrometer. This would be consistent with the lower extent of labeling observed with TFA during the factorial study, as the stronger acid would be expected to promote faster back-exchange.

This hypothesis was tested by reducing the labeled C-CTX1/2 to C-CTX3/4 with sodium borohydride (Kryuchkov et al., 2020), which in the case of C-CTX1/2 labeled on the ketal at C-56, will result in a non-exchangeable ^{18}O-labeled hydroxy group at C-56 (*Experiment 3*; Fig. 2). For this work, semi-purified C-CTX1/2 without detectable levels of C-CTX3/4 was used in order to avoid interference by any unlabeled C-CTX3/4 present in the sample. Comparison of the full-scan mass spectra of naturally-occurring C-CTX3/4 in a fish extract and the borohydride-reduced $[^{18}O]$C-CTX1/2 (i.e. ^{18}O-labeled C-CTX3/4) indicated that the labeling of C-CTX1/2 had occurred very quickly, with greater than 90% ^{18}O-labeling observed after 2 h (Fig. 3). Furthermore, the resulting $[^{18}O]$C-CTX3/4 did not undergo back-exchange prior to detection. This supports the hypothesis that the measured ^{18}O-incorporation of C-CTX1/2 had been affected by back-exchange promoted by the presence of acid and exposure to unlabeled water in the chromatographic separation. That greater than 90% labeling was observed after 2 h of reaction suggested this reaction was very fast, with a half-life of less than 30 min. Furthermore, the product-ion spectrum of $[^{18}O]$C-CTX3/4 indicated that the location of label must be the open N-ring, as shown by the presence of unlabeled product ion at *m/z* 979.5395 and ^{18}O-labeled product ions at *m/z* 285.1943, 257.1530 and 227.1524 (Fig. S2). Therefore, C-CTX1/2 became irreversibly ^{18}O-labeled at the C-56 hydroxy group when reduced to ^{18}O-labeled C-CTX3/4 (Fig. 3), confirming the position of the label at C-56.

A procedure was developed to extract the labeled C-CTX1/2 by liquid–liquid partitioning (*Experiment 4*) with CH_2Cl_2 in the absence of unlabeled water, to separate it from the acid. This allowed the recovery of the labeled C-CTX1/2 under neutral conditions and resulted in a 91% incorporation of ^{18}O into C-CTX1/2, based on the ammonium adduct (Fig. 4).

3.2. *^{18}O-labeling of gambierones*

Gambierone and 44-methylgambierone have two locations in their structures potentially available for oxygen exchange under acidic conditions. These are the hemiketal located at C-4 on the A-ring and the ketone at C-40 on the aliphatic hydroxyketone side chain (Fig. 5). Initial investigations of both gambierones (*Experiment 1*; Fig. 6) resulted in 88% and 91% labeling at two positions in the molecules.

The product-ion spectrum of ^{18}O-labeled gambierone had several product ions in the high mass range that were 2 or 4 *m/z* higher compared to unlabeled gambierone, although the MS/MS data suggested that one of the labeled oxygen atoms was eliminated from the structure (Fig. 7). There were limited product ions indicative of cleavages in the A–E rings, making identification of the exact location of this easily eliminated label difficult. However, based on the structure of gambierone, it is most likely the A-ring hemiketal at C-4. The low-mass product ions at *m/z* 221.1417, *m/z* 291.1832 and *m/z* 345.2301 strongly suggested labeling on or after the I-ring, with the most probable location being the ketone at C-40. The product ion at *m/z* 161.0960 resulting from the cleavage between C-36 and C-37 in the I-ring was present in both the labeled and unlabeled spectra, suggesting that the water loss for this product ion occurred from the ketone position. Corresponding product ions were observed in the mass spectra of $[^{18}O_2]$44-methylgambierone and unlabeled 44-methylgambierone, suggesting that the

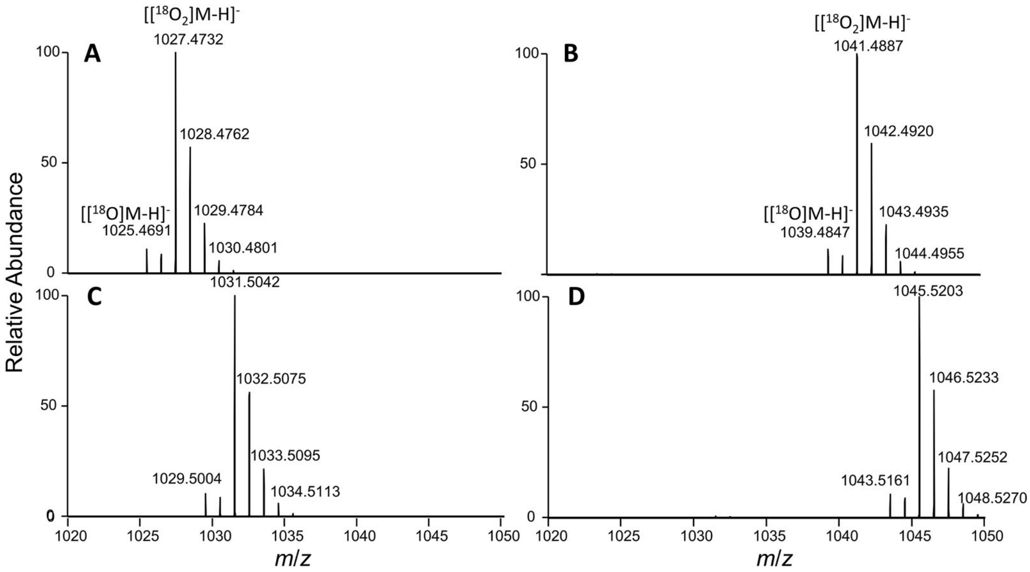


Fig. 9. Full-scan mass spectra in negative ionization mode for: (A) ^{18}O-labeled gambierone, and; (B) 44-methylgambierone, and after sodium borohydride reduction of; (C) ^{18}O-labeled gambierone, and; (D) 44-methylgambierone, to irreversibly incorporate stable isotopes.

Table 2
Stability of [^{18}O]C-CTX1/2 under several pH conditions, based on LC−HRMS measurement of the ammonium adduct [M + NH$_4$]$^+$, at the beginning and completion of a stability study (17 h) at 25 °C, with observed half-life estimates based on 3-parameter logarithmic decay curves.[a]

pH	buffer	Extent of labeling (%)		Half-life (h)
		initial	after 17 h	
3.0	formate	77.9	20.5	11
5.0	acetate	78.9	75.2	220
6.7	ammonium acetate	78.5	78.6	3900
7.0	phosphate	76.8	62.9	55
9.0	ammonium bicarbonate	77.8	20.6	11

[a] Constrained to $y_0 = 0.2\%$.

locations of the ^{18}O-labels in 44-methylgambierone were identical to those in gambierone (Fig. S3).

A kinetic study (*Experiment 2*) was performed to assess the rate of labeling for gambierones at 25 °C. After 13 h, the extent of labeling reached a maximum, with 96% and 89% labeling at locations 1 and 2, respectively. This corresponded to approximately 13% labeling at a single location, and 83% at both locations, in the two gambierones at the completion of the experiment (Fig. 8; Fig. S4). The labeling reactions followed first-order kinetics and were fitted to the network of reversible first-order reactions, with logarithmic decay and growth curves. The kinetics of isotope exchange were virtually identical for both compounds, with unlabeled gambierone and 44-methylgambierone incorporating oxygen-18 with half-lives of about 40 min, and with the two exchange sites being labeled with half-lives of approximately 50 and 190 min (Figs. 8 and S4). An additional kinetic study of 44-methylgambierone was monitored over a 2-h period to identify which position was exchanging at a faster rate by acquiring MS/MS data of the labeled products. Monitoring the product-ion spectrum of [[^{18}O]M + H]$^+$ at *m/z* 1041.4973 and the rate of formation of the product ion at *m/z* 235.1579 indicated that the ketone at C-40 exchanged faster than the hemiketal at C-4.

Sodium borohydride reduction was used to test for the possible effects of back-exchange during analysis due to the presence of acid, as was done for C-CTX1/2 (*Experiment 3*). The unreduced ^{18}O-labeled gambierones showed 94% labeling at both locations. Gambierones contain two functional groups that are potentially reducible with NaBH$_4$, the carbonyl at C-40 and the hemiketal at C-4, and these are also the expected sites for oxygen-18 labeling. Borohydride-reduced ^{18}O-labeled gambierones were observed to have 94% labeling at both locations (Fig. 9), suggesting that no detectable back-exchange had occurred prior to detection, in contrast to the situation for C-CTX1/2.

3.3. Isotopic stability of ^{18}O-labeled C-CTXs and gambierones

Due to the limited quantities of semi-purified C-CTX1/2 available,

Table 3
Effect of pH on ^{18}O-labeled gambierone and 44-methylgambierone in a 17-h stability study at 25 °C, and observed $t_{½}$ for back-exchange of the two labeled positions (C-4 and C-40).

pH	Gambierone						44-Methylgambierone					
	Half-life (h)		Extent of Labeling (%)				Half-life (h)		Extent of Labeling (%)			
	C-40	C-4	Initial		17 h		C-40	C-4	Initial		17 h	
			C-40	C-4	C-40	C-4			C-40	C-4	C-40	C-4
1.0[a]	0.4	0.02	91	91	15[a]	0[a]	0.4	0.02	91	91	16[a]	0[a]
3.0	260	9	91	91	87	17	270	8	91	91	87	16
5.0[b]	570	570	91	91	88	88	540	540	90	90	88	88
6.7[b]	3100	3100	91	91	90	90	2400	2400	91	91	90	90
7.0[b]	170	170	91	91	84	84	180	180	90	90	84	84
9.0	45	13	90	90	67	29	44	13	90	90	68	28

[a] Initial and final (45 min after preparation) analysis for pH 1.
[b] Slow back-exchange appeared consistent at both positions, but was too slow to measure accurately at these pH values in only 17 h.

the stability of the ^{18}O-labeling could only be followed at a few pH values and analyzed 3 to 4 times over a 17 h period at 25 °C. The labeled C-CTX1/2 used in the stability study had 78% labeling at a single location for C-CTX1/2. LC–HRMS analysis indicated an incorporation level of 78% for the ^{18}O-labeled C-CTX1/2 after 17 h at pH 6.7, indicating very high stability under neutral conditions, whereas all other pH conditions assessed resulted in some degree of back-exchange (Table 2). The fastest back-exchange was observed at pH 3 and 9, with ^{18}O-labeled C-CTX1/2 having half-lives of less than 24 h. Full-scan HRMS spectra at each pH after 17 h are shown in Fig. S5, and confirm considerable back-exchange under these conditions. Neutral conditions were also evaluated with phosphate buffer (100 mM). These data suggest that there may be some phosphate-catalyzed back-exchange of [^{18}O]C-CTX1/2, as after 17 h the extent of labeling dropped to 63% in the presence of phosphate buffer, which was prepared at a neutral pH similar to that of the ammonium acetate for which minimal back-exchange was observed. Due to limited quantities of C-CTX1/2, lower concentrations of phosphate buffer were not assessed. However, a previous study reported concentration-dependent general acid catalysis of the exchange of the carbonyl oxygen of acetone (Greenzaid et al., 1968). Based on these findings, ^{18}O-labeled C-CTX1/2 appears to be sufficiently stable under neutral conditions to be used for analytical measurements and possibly also for in vitro assays, although alternative neutral buffers may be necessary if the use of phosphate buffers proves problematic. While not assessed due to sample availability, it is probable that storage at lower temperatures would reduce the rate of back-exchange.

To evaluate the rate of back-exchange for ^{18}O-labeled C-CTX3/4, [^{18}O]C-CTX1/2 was reduced with sodium borohydride and mixed with formate (pH 3), ammonium acetate (pH 6.7) and ammonium bicarbonate (pH 9) buffers. The samples were analyzed after 5 h at ambient temperature and all were found to have >95% ^{18}O-label incorporation, showing that back-exchange was negligible, in contrast to [^{18}O]C-CTX1/2. This is because borohydride reduces the hemiketal at C-56 of C-CTX1/2 to an open-ring hydroxy group (Kryuchkov et al., 2020), where the ^{18}O-label is permanently affixed to the molecule and no longer able to undergo acid-catalyzed oxygen exchange.

As with C-CTXs, the stability of gambierones was assessed after the removal of residual acid. However, the procedure developed for separation of the C-CTXs from the acid catalyst was not appropriate for gambierones, because they do not partition efficiently into CH_2Cl_2 (Estevez et al., 2020b). Instead, the labeled gambierone was removed from the acidic solution using a recently developed boronate affinity technique with mAPBAG (Mudge et al., 2022). The resulting solution was a mixture of the two ^{18}O-labeled gambierones in 1:1 MeCN–water, thereby enabling the addition of buffered solutions to control the pH. The stability of the ^{18}O-incorporation was assessed at a range of pH values, from strongly acidic to weakly basic for gambierone and 44-methylgambierone. Comparisons of the full-scan spectra of the time zero control with spectra after 17 h in the various buffers are shown in Figs. S6 and S7. The extent of labeling at the two positions was determined from the LC–HRMS spectra with the NRC Isotope Enrichment Calculator and fitted to 3-parameter first-order decay curves at each pH (Figs. S8 and S9), and the results are summarized in Table 3. Back-exchange (loss of ^{18}O) was observed relatively quickly in strong acid and at pH 3 and 9, indicating that these are not suitable for storage or use of ^{18}O-labeled gambierones. Strong acid (0.1 M HCl) caused a rapid back-exchange at both locations on the gambierone structure, with the ^{18}O at C-40 exchanging almost instantaneously, with a half-life of around 1 min, while for the C-4 label the half-life was 22 min. This back-exchange was slower at pH 3 and 9, but loss of labeling was also observed. As was observed for C-CTXs, phosphate buffer appeared to have a catalytic effect on the stability of the label. Exchange was slower at pH 5 with acetate buffer (half-life ~550 h) than at pH 7 using phosphate (~175 h). The half-life estimated for ^{18}O-labeled gambierones in ammonium acetate at neutral pH was approximately 100–130 d. These experiments were performed at 25 °C and, although it is likely that stability would be improved at lower temperatures, the stability of the label may be sufficient for analytical measurements and in vitro assays.

There remains a paucity of CTX reference materials, which are necessary for reliable quantitation, identification and verification of these toxins in screening and monitoring work, and in fish from outbreaks worldwide. Recent work on the development of CTX reference materials has highlighted several difficulties associated with this work, including large sample requirements, low levels of CTXs in the fish flesh, and relatively low LC–MS instrument response (Gago-Martinez et al., 2021). Isotope-labeled internal standards provide a complementary approach for the development of sample preparation and quantitative procedures, as isotope dilution is an effective methodology for evaluating matrix effects, extraction efficiencies, and instrument response in high matrix materials (Haddad et al., 2019; Stokvis et al., 2005). This would require less standard for the developmental stages of LC−MS methods, thus reducing overall reference material needs. Future work will focus on using ^{18}O-labeled gambierones and CTXs to establish isotope dilution methodologies and determine the impact of matrix effects on LC−MS detection of these toxins in crude and semi-purified fractions from algae and fish.

Credit author statement

E. Mudge: Methodology, Investigation, Formal Analysis, Visualization, Writing – Original Draft, Writing – Review & Editing. **J. Meija**: Software, Formal Analysis, Visualization, Writing – Original Draft. **S. Uhlig**: Resources, Writing – Review & Editing. **A. Robertson**: Resources, Funding Acquisition, Writing – Review & Editing. **P. McCarron**: Supervision, Writing – Review & Editing. **C. Miles:** Conceptualization, Methodology, Writing – Review & Editing.

Ethical statement

The coauthors state that the work submitted has not been submitted for publication elsewhere. The manuscript was prepared and submitted in accordance with the ‘Ethics in publishing’ and ‘Ethical guidelines for journal publications’ standards provided on the journal website.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2022.03.005.

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