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Hydrolysis of hydroxybenzoate saxitoxin analogues originating from *Gymnodinium catenatum*

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ABSTRACT

The paralytic shellfish poisoning (PSP) toxin producer *Gymnodinium catenatum* produces several hydrophobic analogues of saxitoxin (STX). These are poorly studied due to their recent discovery and lack of standards. It was previously observed these hydrophobic analogues could be partially hydrolysed, loosing its benzoate moiety during alkaline oxidation to obtain fluorescent products measurable by HPLC analysis. The hydrolysis reaction was further explored to study two practical aspects. One was the indirect measurement of these compounds through its hydrolysis products: the decarbamoyl analogues of STX. The second one was to simplify standard production of decarbamoyl analogues, which are commonly found in contaminated shellfish products.

PSP analogues are unstable in alkaline media. The hydrolysis of benzoate analogues progressed rapidly with increasing base amount, but the decarbamoylgonyautoxin type hydrolysis products were short lived and converted into decarbamoylsaxitoxin type analogues. For a rapid estimation of the presence of these benzoate analogues in seafood, decarbamoylgonyautoxin type analogues can only be measured as decarbamoylsaxitoxin type equivalents.

For production of standards, complete hydrolysis of hydroxybenzoate decarbamoylsaxitoxin analogues can render decarbamoylsaxitoxin and decarbamoylneosaxitoxin. Obtaining decarbamoylgonyautoxins was not suitable resorting to decarbamoylgonyautoxin type hydroxybenzoate analogues due to the prolonged reaction times required for complete hydrolysis and their instability at high pH. Hydrolysis of sulphated-benzoate analogues was best suited for obtaining decarbamoylgonyautoxins due to the very rapid hydrolysis time required.

1. Introduction

Saxitoxin (STX) and its analogues are the agents of paralytic shellfish poisoning (PSP). The STX analogues known until recently were comprised only of hydrophilic compounds (Fig. 1) (Oshima, 1995). However, the discovery of three hydrophobic hydroxybenzoate analogues in Australian strains of the microalgae *Gymnodinium catenatum*, designated GC1 through GC3 (Negri et al., 2003), unfolded a new picture for the study of PSP toxins (PSTs). GC3 was found to be the 4-hydroxybenzoate ester derivative of decarbamoylsaxitoxin (dcSTX), while GC1 and GC2 were the epimeric 11-*O*-sulphate derivatives of GC3 (Fig. 1). In a Portuguese strain of *G. catenatum* it was later discovered many more benzoate analogues existed (Vale, 2008b).

The presence of GC analogues might represent an important fraction of the STX analogues produced by this microalgae (Negri et al., 2007). The fate of these compounds in filter feeder organisms predating upon *G. catenatum* around the world remains largely unknown. It was hypothesised that bivalves in general could metabolise these to decarbamoyl analogues. So far only data related to bivalves from the Portuguese coast is available (Vale, 2008a). From other regions no information is yet available.

This can represent a food safety issue given the growing tendency to abandon live animal assays for marine biotoxin testing, to be replaced for example by chemical assays, among others (Hess et al., 2006). HPLC methods can miss their presence because, in order to remove interfering compounds, solid-phase extraction (SPE) in C18 cartridges is often used (Lawrence, Niedzwiadek, & Menard, 2005). Assessment of these compounds in bivalves from worldwide is urgently needed. However, no commercial standards are available, and a complexity of GC analogues exists (Vale, 2008b). It was earlier found that during pre-column oxidation partial hydrolysis of GC toxins to the corresponding decarbamoyl analogues occurred quickly due to the alkaline media used for oxidation (Fig. 1). At the time of that research only peroxide oxidation, which uses strong alkaline media, was briefly studied to demonstrate the complete hydrolysis and thus understand why a complex fluorescent profile was obtained with simultaneous presence of oxidation products corresponding to the classic decarba-

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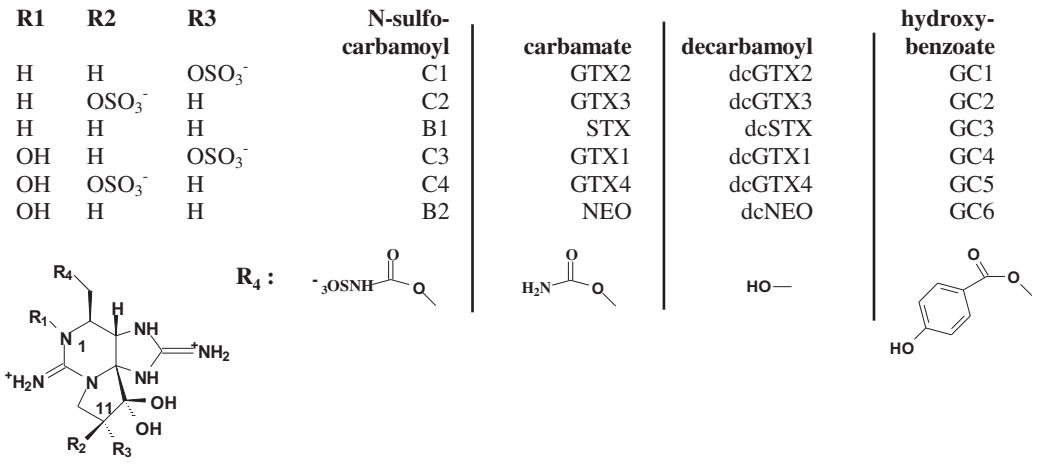

Fig. 1. Structures of dinoflagellate toxins associated with paralytic shellfish poisoning syndrome. After alkaline hydrolysis GC3 and GC6 should render dcSTX and dcNEO, respectively, while GC1/2 and GC4/5 should render dcGTX2/3 and dcGTX1/4, respectively.

moyl analogues in addition to the newly discovered GC toxin analogues (Vale, 2008a, 2008b).

Potential applications from this reaction were not envisaged at that time. The aim of the present research was to look into possible usefulness of the readiness with which GC analogues loose its benzoate moiety. One application could be simplification of food safety analysis and the other could be simplification of production of decarbamoyl analogues standard's for use in chemical assays.

2. Experimental

2.1. Reagents

Reagents were of analytical grade, except for acetonitrile that was LC grade. Water was purified using a Milli-Q 185 Plus system (Millipore, Bedford, MA, USA). Standard solutions from STX, NEO, GTX2 + 3, GTX1 + 4, dcSTX, dcNEO, dcGTX2 + 3, B1, and C1 + 2 were purchased from the Certified Reference Materials Program (CRMP) of the Institute for Marine Biosciences, National Research Council (Halifax, Canada).

2.2. Microalgae cultures

The strain of *G. catenatum* originated from the algal library of Instituto de Oceanografia, Lisbon University (strain n° IO.13.04). It was cultured in seawater adjusted to 30 psu and enriched with f/2 nutrients without silica. Culture media was autoclaved in 2-l flasks used for cultivation. Continuous illumination was achieved with fluorescent lamps in a culture room kept at 18 °C.

Cell chains and isolated cells were collected by passing successively in 60 μm and 20-μm pore size plankton meshes. The cell concentrates were centrifuged at 2300*g* for 10 min, seawater discarded and suspended in 1% acetic acid. Extraction was performed by a freeze/thaw cycle followed by sonication in an ultrasonic bath for 10 min. The supernatant was obtained by centrifugation at 2500*g* for 10 min, and filtered by disposable 0.2 μm regenerated cellulose syringe filters.

2.3. Toxin fractionation

A 500 mg Supelclean LC18 SPE cartridge (No. 57012; Supelco, Bellefonte, PA, USA) was conditioned with 3 ml methanol and 3 ml water. An aliquot of 1 ml of *G. catenatum* supernatant was applied and eluted with 3 ml of water (these combined fractions contain the hydrophilic toxins).

The hydrophobic toxins were eluted in the next consecutive fractions by passing different methanolic solutions, obtaining different types of enriched mixtures, as outlined below:

2.3.1. Full suite of GCs

Elute with 3 ml 9 + 1 methanol/water containing 0.1 M acetic acid.

2.3.2. Hydroxybenxoate GCs

Wash first with 3 ml of 10% aqueous methanol and elute with 3 ml 9 + 1 methanol/water containing 0.1 M acetic acid.

2.3.3. Detailed GCs

Elute with 3 ml of 10% aqueous methanol, then 9 + 1 methanol/water containing 0.001% acetic acid and by last elute with 9 + 1 methanol/water containing 0.1 M acetic acid. This aims at separating the benzoate analogues lightly bound (sulphated-benzoate GCs or GC1b-6b) from the gonyautoxin-type hydroxybenzoate analogues (GC-GTXs) plus the saxitoxin-type hydroxybenzoate analogues (GC-STXs).

The SPE methanolic fractions were vacuum dried at 55 °C (RapidVap, Labconco, Kansas City, MO, USA), resuspended in 500 μl of 0.1% acetic acid.

2.4. Preliminary hydrolysis assessment with modified periodate oxidation

Extracts selectively enriched in GC-GTXs plus GC-STXs (Section 2.3.2) were hydrolysed in a vial by mixing 100 μl of semi-purified extract with 200 μl of base solution, and heated in a BT1 Block Thermostat (Grant Instruments (Cambridge) Ltd., Royston, UK). Periodate oxidation was carried out after the selected hydrolysis time by further adding 200 μl of 0.03 M periodic acid, reacting for 1 min. and acidification with 10 μl acetic acid:water (50:50 v/v). 12.5 μl were injected on column. Preliminary experiments started with a base solution made from 1 ml of 0.3 M Na_2HPO_4 plus 100 μl of 1 M NaOH, and reacting at 50 °C for 25 min intervals up to 75 min. As these conditions were not enough for complete hydrolysis, further modifications to the NaOH concentration, temperature, and time were researched, as reported in Section 3.

2.5. Toxin hydrolysis for production of standards

For maximising recovery of dcGTXs, extracts enriched either in the sulphated-benzoate analogues (eluted in SPE with 10% metha-