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Column switching combined with hydrophilic interaction chromatography-tandem mass spectrometry for the analysis of saxitoxin analogues, and their biosynthetic intermediates in dinoflagellates

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ABSTRACT

Hydrophilic-interaction chromatography (HILIC) is reportedly useful for the analysis of saxitoxin (STX) analogues, collectively known as paralytic shellfish toxins. Column switching and two-step gradient elution using HILIC combined with mass spectrometry enabled the simultaneous analysis of the 15 primary STX analogues and their biosynthetic intermediates, arginine, Int-A', and Int-C'2, and the shunt product, Cyclic-C'. Crude extracts of toxin-producing dinoflagellates can be injected without any treatment except filtration. Enrichment of the compounds using this method was highly reproducible with respect to retention times (% RSD was under 1%) and highly sensitive (limits of detection (LODs) were in the range 0.9 (Int-C'2) - 116 (C3) μM) in terms of avoiding matrix effects associated with co-eluting substances. Validation studies demonstrated acceptable performance of this method for specificity, repeatability, linearity and recovery. A comparison of the quantitative results for STX analogues in *Alexandrium tamarense* using HPLC with post-column fluorescent derivatization and the column-switching HILIC–MS method revealed good agreement. The presence of Int-A', Int-C'2, and Cyclic-C' in toxic dinoflagellate species with different toxin profiles was confirmed using this method. Our data support the hypothesis that the early stages of the STX biosynthesis and shunt pathways are the same in dinoflagellates and cyanobacteria.

1. Introduction

Paralytic shellfish poisoning is one of the most common human poisonings and is caused by biotoxins of marine organisms. The causative substances are saxitoxin (STX) and its analogues, collectively known as paralytic shellfish toxins (PSTs) (Fig. 1). PSTs are strong and selective blockers of voltage-gated sodium ion channels [1]. Marine dinoflagellates of the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium* are the most common producers of STX and its analogues [2–5]. The toxins produced by these dinoflagellates accumulate in filter-feeding shellfish and are then transferred to higher animals through the food chain. Toxic dinoflagellates form blooms in many areas of the world, and their distribution is spreading [6]. As damage to fisheries by the resulting harmful algal blooms is significant, the need for techniques to predict and regulate these blooms in order to minimize algal toxicity is growing. Toward that end, it is necessary to obtain a more complete understanding of the biosynthesis and metabolism of STX and its analogues in dinoflagellates.

The chemical structures of the biosynthetic intermediates of STX were proposed based on the predicted functions of the putative biosynthetic genes found in cyanobacteria (Fig. 1) [7]. Subsequently, the putative biosynthetic intermediates Int-A', Int-C'2 in the early stages of STX biosynthesis, and the tricyclic bisguanidine compound, Cyclic-C', were chemically synthesized, and were identified in extracts of the toxic cyanobacterium *Anabaena circinalis* [8,9]. Moreover, among these compounds, only Cyclic-C' showed

Abbreviations: PSTs, paralytic shellfish toxins; GTX, gonyautoxin; STX, saxitoxin; dc, decarbamoyl; HILIC, hydrophilic interaction chromatography; ESI, electrospray ionization.

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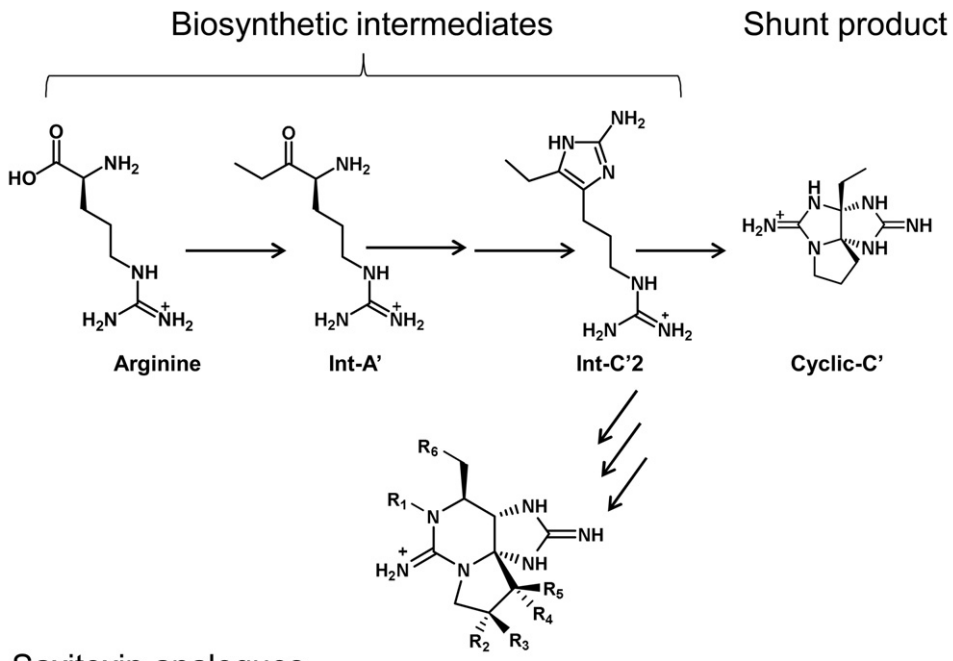

Saxitoxin analogues

R_1	R_2	R_3	R_4	R_5	R_6: $OCONH_2$	R_6: $OCONHSO_3^-$	R_6: OH
H	H	H	OH	OH	STX	GTX5	dcSTX
H	H	H	H	OH			12α-deoxy-dcSTX
H	H	H	OH	H			12β-deoxy-dcSTX
OH	H	H	OH	OH	neoSTX	GTX6	
OH	OSO_3^-	H	OH	OH	GTX1	C3	
H	OSO_3^-	H	OH	OH	GTX2	C1	
H	H	OSO_3^-	OH	OH	GTX3	C2	
OH	H	OSO_3^-	OH	OH	GTX4	C4	

Fig. 1. Putative biosynthetic pathway of STX and STX analogues and the shunt pathway. STX = saxitoxin; GTX = gonyautoxin; dc = decarbamoyl.

a weak inhibitory activity against voltage gated sodium channel by using a cell-based assay with mouse neuroblastoma cell line Neuro2A (its IC_{50} value was 36 μM, whereas that for dcSTX used as a reference was 99 nM) [9]. The feeding experiments of ^{15}N-labeled compounds to *A. circinalis* suggested that while Int-A' and Int-C'2 are genuine precursors of C2 (the major STX analogue in this strain), Cyclic-C' is a shunt product excreted from the cells [10]. The same route in the first several steps through which Int-C'2 is biosynthesized from arginine through Int-A', as well as the shunt pathway, could exist in the toxic dinoflagellate, since one toxic subclone, *A. tamarense* Axat-2, was shown to produce Int-A', Int-C'2, and Cyclic-C' [8,9]. However, less evidence had been obtained for the biosynthesis of STX and its analogues in dinoflagellates [11], due to the unique characteristics of dinoflagellates which made the genetic study very difficult [12–16]. It led us to consider a chemical approach.

The development of an analytical method to identify and quantitate the biosynthetic intermediates along with STX analogues in toxic organisms would accelerate the study of the fate of STX in dinoflagellates. Such an approach could lead to the identification of a novel biosynthetic and metabolic pathway that in turn could facilitate elucidation of the mechanisms of the toxicokinetics of PSTs in dinoflagellates. However, no methods that would enable simultaneous analysis of STX analogues and their biosynthetic intermediates have been reported. STX analogues exist naturally as a complex mixture of structurally related compounds classified into three groups: (1) carbamate toxins such as STXs and gonyautoxins (GTXs), (2) sulfo-carbamate toxins such as the C-toxins, and (3) decarbamoyl toxins such as decarbamoyl STX (dcSTX) [17]. In addition to these primary toxins, many analogues with modified functional groups on the common STX skeleton have been described, with more than 50 analogues thus far reported [18].

As differences in molecular profiles are the result of differences in the enzymatic activities expressed by each strain, it is necessary to identify each compound in order to fully characterize the biosynthesis and metabolism of dinoflagellate toxins. HPLC with post- or pre-column fluorescent derivatization has been widely used for the analysis of STX and its analogues [17,19]; however, the intermediates and some STX analogues, such as 12-deoxy-dcSTX, are difficult to detect using these methods [20]. Early in our study, some reports appeared describing the use of liquid chromatography-mass spectrometry (LC–MS) with hydrophilic-interaction chromatography (HILIC) columns for the identification of STX and its analogues. The use of TSK-gel amide-80 and SeQuant® ZIC®HILIC columns with linear gradient elution was reported [21–23]. There was also a report describing HILIC-mode LC–MS to analyze water-soluble cellular metabolites including arginine and some guanidino derivatives [24]. The application of these columns for the simultaneous analysis of STX analogues and their biosynthetic intermediates using LC–MS was thought to be effective. Because co-eluting substances affect the signal intensity and retention time of analytes, a clean-up step is typically necessary for LC–MS analyses of biological samples