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Toxic *Alexandrium minutum* (Dinophyceae) from Vietnam with new gonyautoxin analogue

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Abstract

Clonal cultures of *Alexandrium* species collected from a shrimp pond on the northern coast of Vietnam were established and morphologically identified as *Alexandrium minutum*. Nucleotide sequences of domains 1 and 2 of the large subunit ribosomal (LSU) rRNA gene showed high sequence similarity to *A. minutum* isolates from Malaysia. Paralytic shellfish toxin profile of the clones was characterized by the dominance of GTX4, GTX1, and NEO. GTX3, GTX2, and dcSTX were also present in trace amount. Toxin content varied among the strains and growth stages, ranged from 3.0 to 12.5 fmol cell^{-1}. In addition to these known toxin components, a new gonyautoxin derivative was detected by HPLC, eluting between GTX4 and GTX1. The peak of this compound disappeared under non-oxidizing HPLC condition but unchanged either after treated with 0.05 M ammonium phosphate/10% mercaptoethanol or 0.1N HCl hydrolysis. LCMS ion scanning showed a parental ion of $[M + H]^+$ at *m/z* 396, $[M - SO_3]^+$ at *m/z* 316, and $[M - SO_4]^+$ at *m/z* 298. Based on these results, the derivative was identified as deoxy-GTX4-12ol, and this represents the first report of this toxin analogue.
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Keywords: *Alexandrium minutum*; Large subunit ribosomal RNA; Morphology paralytic shellfish toxins; Vietnam

1. Introduction

In the Asia Pacific region, the toxic *Alexandrium minutum* Halim was first reported in Taiwan (Su and Chiang, 1991; Hwang and Lu, 2000), followed by Australia (Cannon, 1990), Japan (Yuki, 1994), Thailand (Matsuoka et al., 1997), New Zealand (Chang et al., 1995; Mackenzie and Berkett, 1997), Vietnam (Yoshida et al., 2000), Malaysia (Usup et al., 2002), and Philippine (Bajarias et al., 2003). *A. minutum* was commonly occurred in semi-enclosed water such as harbor and coastal lagoon (Delgado et al., 1990; Giacobbe et al., 1996), estuary (Lim et al., 2004), and shrimp ponds (Matsuoka et al., 1997; Yoshida et al., 2000). Strong freshwater influence coupled with a stratified water column has been suggested as an essential prerequisite for blooms of this species (Delgado et al., 1990; Giacobbe et al., 1996; Lim and Ogata, 2005).

Most *A. minutum* isolates studied to date produced paralytic shellfish toxins (PSTs) which were dominated

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by gonyautoxins GTX1, GTX2, GTX3 and GTX4 (Hallegraeff et al., 1991; Mackenzie and Berkett, 1997; Hwang and Lu, 2000; Lim et al., 2004). However, different toxin profiles have been reported for some isolates. For example, isolates from New Zealand (Chang et al., 1997) and Denmark (Hansen et al., 2003) produced neosaxitoxin (NEO) and sulfocarbamoyl toxins (C-toxins) as the principal toxin congeners.

The presence of toxic and potential toxic *Alexandrium* species in coastal waters of Vietnam have been well documented (Yoshida et al., 2000; Nguyen-Ngoc, 2004). The occurrence of *A. minutum* was first reported from plankton net haul samples (Yoshida et al., 2000). However, more detailed toxicity and molecular studies was hampered by unavailability of culture materials.

The present study was aim to investigate the morphological, genetic, toxin composition and toxicity of tropical *A. minutum* established from coastal water in northern Vietnam in relation to *A. minutum* reported in other regions. Recently, several clonal cultures of *A. minutum* from northern Vietnam were established. Here we report the toxicity of these clones and also their molecular phylogenetic affiliation based on sequence analysis of the partial LSU rRNA gene. Presence of a new GTX analogue in these strains was also characterized and documented in this report.

2. Materials and methods

2.1. Cultures

A. minutum cells used in this study were isolated from shrimp ponds in Do Son, Hai Phong, Vietnam in October 2004 (Fig. 1). The cultures were initially established in Daigo's IMK medium for Marine Microalgae (Daigo, Tokyo, Japan). The cultures were later transferred to Laboratory of Aquatic Microbiology, Kitasato University and maintained in ES medium (Kokinos and Anderson, 1995), at 25 °C under a 14:10 h light:dark cycle at 140 μmol photons m^{-2} s^{-1}. Seawater of 33 PSU from Okkirai Bay was used as the medium base. Salinity of the medium was adjusted to 15.0 ± 1.0 PSU by addition of deionized distilled water.

For species identification, mid exponential phase cultures were harvested by centrifugation at 2000 × *g* for 5 min and preserved in 4% formaldehyde solution. Cells were then stained with 1% Calcofluor White M2R (Sigma Aldrich Co. Ltd., Gillingham, UK) (Fritz and Triemer, 1985) and observed under an Olympus BX51 epi-fluorescence microscope (Olympus, Tokyo, Japan). Digital images under UV excitation were captured using a Pixera Penguin 600XL cooled CCD camera (Pixera Corporation, Los Gatos, CA, USA). A total of 50 randomly selected cells were measured with a mean determined.

2.2. DNA extraction, amplication and sequencing

Mid-exponential batch cultures were harvested by centrifugation at 3000 × *g* for 5 min. Total DNA was extracted using ISOGEN (Nippongene, Tokyo, Japan) according to the manufacturer instruction. Approximately 700 bp of domains 1 and 2 (D1–D2) of LSU rRNA gene was amplified by polymerase chain reaction (PCR) using primers D1R and D2C (Scholin et al., 1994) from Sigma Genosys (Sigma, The Woodland, TX, USA). PCR was carried out on an ASTEC PC707 thermacycler (ASETC, Kanagawa, Japan). Purified products were stored at −20 °C. DNA sequencing was performed using dideoxy terminator (DYEnamic ET terminator cycle sequencing kit, Amersham Bioscience, Sweden) on an ABI 377 automated DNA sequencer (Applied Biosystem, CA, USA).

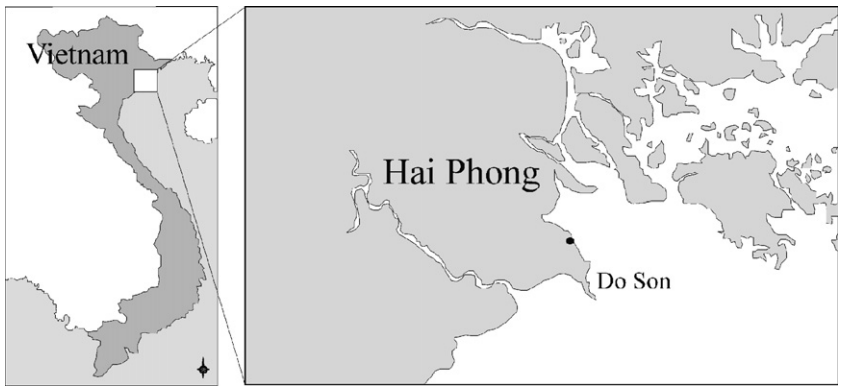

Fig. 1. Map showing location of Do Son, Hai Phong in Vietnam from where samples were collected.

2.3. Molecular phylogenetic analysis

Sequences obtained were aligned using the Clustal-X program (Thompson et al., 1997). These and previously published (Table 1) sequences were used in the phylogenetic analysis. Phylogenetic analyses were carried out using PAUP* Ver. 4.0b10 (Swofford, 1998) with maximum parsimony and likelihood algorithms. Maximum parsimony was performed by heuristic search of 1000 random additions and TBR branch swapping. The MODELTEST Ver. 3.06 program (Posada and Crandall, 1998) was used to determine the best model of evolution. The best fit evolutionary model selected for the sequence data set was the general time reversible model with gamma distribution (GTR + Γ) and estimated base frequencies of $A = 0.2693$, $C = 0.1526$, $G = 0.2580$, $T = 0.3201$; base substitution rates of $[G - T] = 1.0000$, $[A - G] = 2.3324$, $[A - T] = 1.0000$, $[C - G] = 1.0000$, $[C - T] = 6.8275$ and $[G - T] = 1.0000$, a Γ distribution shape of 0.5178, and zero proportion of invariable sites. The GTR + Γ model and maximum likelihood parameters were then used in the maximum likelihood analysis with the previous parsimony tree as the starting tree.

2.4. Toxin extraction and analysis

Fifteen millilitres of clonal cultures (AmSp01, 03, 04, 05 and 07) at early and late exponential phases were harvested by centrifugation at 2000 × *g* for 15 min. One millilitre of duplicate subsamples were taken for cell counts. The cells were preserved in Lugol's solution. Cell pellets for toxin extraction were then sonicated with ultrasonic homogenizer UH-50 (SMT Co. Ltd., Japan) in 0.5 M acetic acid (AcOH) for 2 min on ice. Cell debris was removed by centrifugation at 12,000 × *g* for 10 min. The supernatant was collected and kept in −20 °C until further analysis.

Toxins analysis was carried out by HPLC using the isocratic, post-column derivatization method of Oshima (1995b) on a JASCO HPLC system (JASCO, Japan) fitted with post-column system and fluorescence detector. The samples were run through a Wakosil C18 column (Ø 4.6 mm × 150 mm; Wako, Osaka, Japan). The post-column temperature was kept at 70 °C for all runs. Detection wavelengths were set at 330 nm excitation and 390 nm emissions. Authentic toxins provided by Dr. Y. Oshima, Tohoku University, Japan were used as toxin references. Further toxin verification was carried out in non-oxidizing post-column condition by replacing the oxidizing reagent with distilled water and the reaction coil was kept in the ice bath during the analysis. Hydrolysis of sample was carried out by boiling the extract in 0.1N of HCl for 10 min (Hall and Reichardt, 1984).

2.5. Isolation and fractionation of potentially new toxin analogue

Twenty litres of *A. minutum* clonal culture at late exponential phase was harvested by sieving at 10 μm mesh size sieve and followed by centrifugation. Toxins were extracted according to the procedure as described above. The extract was then proceeded to lyophilization. The freeze-dried sample was treated with 0.05 M ammonium phosphate/10% (v/v) mercaptoethanol (ME) and boiled for 10 min to remove the sulformoyl moiety of GTXs (Sakamoto et al., 2000; Sato and

Table 1
Strains of *Alexandrium* species used in the phylogenetic analysis, with origin of isolate, GenBank accession number, and citation

Strain	Species	Origin	GenBank accession number	Reference
AmKB01, AmKB06	*A. minutum*	Malaysia, Tumpat	AY566185, AY566187	Leaw et al. (2005)
AL3T	*A. minutum*	Italy, Gulf of Trieste	AJ535353	John et al. (2003)
AMAD06	*A. minutum*	Australia, Port River	U44936	Scholin et al. (1994)
X20	*A. minutum*	France, the Rance	AF318232	Guillou et al. (2002)
3.9h	*A. minutum*	England, Fleet Lagoon	AY705869	Nascimento et al. (2005)
95/4	*A. minutum*	France, Bay of Concarneau	AF318264	Guillou et al. (2002)
CAWD13	*A. minutum*	New Zealand, Malborough Sounds	AY338751	Direct submission
–	*A. minutum*	New Zealand, Anakoha Bay	AF033532	Walsh et al. (1998)
GHmin04	*A. minutum*	Denmark, Korsor Nor	AY294613	Hansen et al. (2003)
Al1V	*A. minutum*	Spain, Galicia	L38626	Zardoya et al. (1995)
SZN29	*Alexandrium tamutum*	Italy	AJ535372	John et al. (2003)
AI104	*Alexandrium insuetum*	Japan	AB088248	Direct submission
K0287	*Alexandrium ostenfeldii*	Denmark, Limfjordan	AJ535356	John et al. (2003)
AlMS02	*Alexandrium leei*	Malaysia, Malacca	AY566183	Leaw et al. (2005)
X12	*Alexandrium margalefi*	France, Bay of Concarneau	AF318230	Guillou et al. (2002)

Kodama, 2003). Conversion of GTX1–4 to STXs (STX and NEO) was confirmed by HPLC analysis. The sample was then purified using a Bio-Gel P-2 column (fine; 15 mm × 450 mm) (BioRad, Hercules, CA, USA) equilibrated with deionized distilled water. The sample was loaded to the column and eluted with 0.2 M AcOH at a flow rate of 0.5 mL min^{-1}. Five-milliliter fractions were collected using Redifrac fraction collector (Pharmacia Biotech, New Jersey, USA). Individual fractions were further analysed by HPLC. Fractions containing compound of interest were combined, lyophilized and then dissolved in 0.05 M AcOH. Samples were kept frozen at −20 °C until further analysis.

2.6. Liquid chromatography–mass spectrophotometry (LCMS/MS) analysis

An Agilent 1100 LC system (Agilent Technologies, CA, USA) and a API 2000 quadru-pole MS/MS system (Applied Biosystems, CA, USA) were used to analyse the purified compound. Chromatographic separation was performed using a column of Wakosil Navi 5C-18 (2 mm × 150 mm; Wako, Japan) with a linear gradient system that was run from 0.2% heptafluorobutyric acid (HFBA) to 30% acetonitrile containing 0.2% HFBA in 12 min at a flow rate of 0.2 mL min^{-1}. The electrospray ionization interface (ESI) was operated in positive mode. The mass spectrometer was operated in both Q1 scan and product ion scan mode in which N_2 was used as desoluvation, cone and collision gas (curtain gas 50 psi; ion spray voltage 5500 V; ion-source gas 1, 40 psi; ion-source gas 2, 60 psi; declustering potential 10 V; collision energy 30 V; collision cell exit potential 15 V).

3. Results

3.1. Morphology and molecular phylogenetic analysis

Cells from Vietnam were oval in shape, small with transdiameter between 20 and 28 μm (Fig. 2A). Gamete was approximately half the size of vegetative cell (Fig. 2B). The first apical plate (1′) was rhomboidal with ventral pore (vp) located on the anterior right margin of the plate (Fig. 2B and C). Some cells showed long and narrow 1′, with almost parallel right and left margins (Fig. 2A). Apical pore complex (APC) was comma in shape without anterior attachment pore (Fig. 2D). Sixth precingular plate (6″) was longer than wide (length width ratio = 1.5–1.8). Posterior sulcal plate (s.p.) was wider than long (Fig. 2E and F).

Nucleotide sequences of domain 1 and 2 of LSU ribosomal RNA gene were obtained for four strains (AmSp01, AmSp03, AmSp05, and AmSp17) with sequence length of 657 bp. The aligned sequences contained 626 characters (including gaps) for 18 taxa. Of these, 393 were constant, 154 were variable but parsimony uninformative, and 79 were parsimony

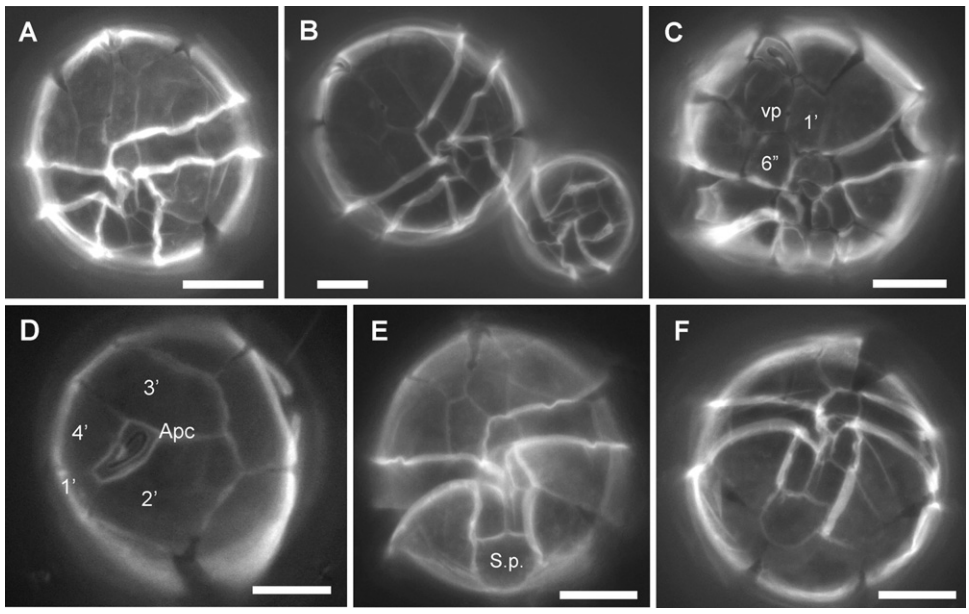

Fig. 2. Epi-fluorescent micrographs of *Alexandrium minutum* from Vietnam. (A) An oval vegetative cell and gamete (B); (C) ventral view showing the first apical plate (1′), a long sixth precingular plate (6″) and the position of ventral pore (vp); (D) apical view showing the shape of apical pore complex (Apc); (E and F) wide posterior sulcal plate (s.p.). Scale bar: 10 μm.

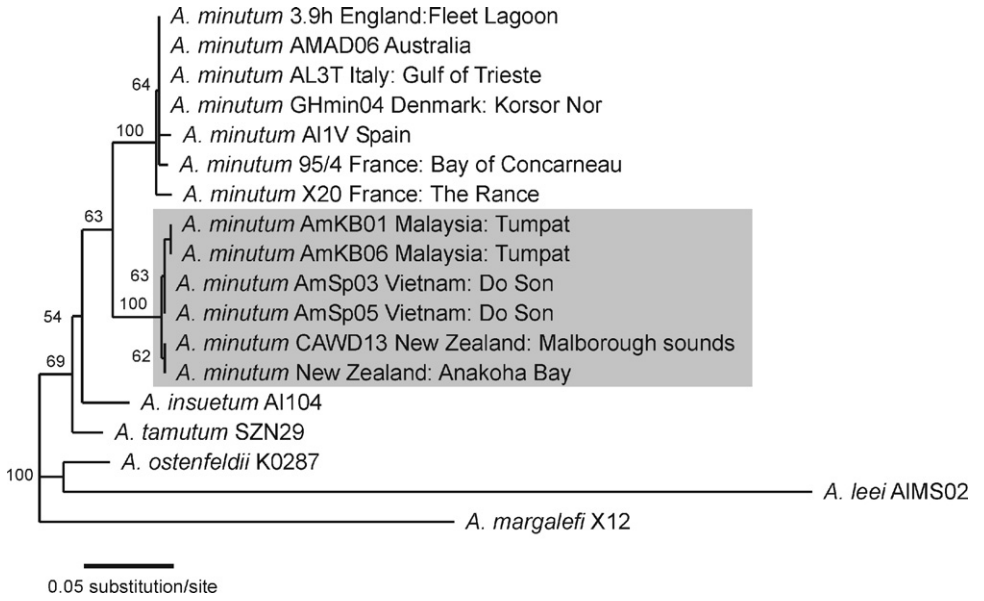

Fig. 3. Tree topology based on maximum likelihood analysis. Log likelihood = −2257.34. Values at nodes indicate branch frequency from 1000 bootstrap samples of two most parsimonious trees.

informative. Parsimony analysis resulted in two most parsimonious trees, each with a length of 318 steps, consistency index (CI) of 0.7315, retention index (RI) of 0.8413, and homoplasy index (HI) of 0.1258. The most likely tree obtained from the maximum likelihood analysis is shown in Fig. 3. Two clades were resolved with bootstrap support of 63%. Isolates from Vietnam, Malaysia and New Zealand formed a monophyletic group, with pair-wise sequence divergence of 0.33–0.65% (2–4 base differences) while the second group comprised *A. minutum* mainly from Europe and Australia. Both clades were strongly supported. The intrageneric divergences between the two clades ranged from 4.72 to 5.71% (29–35 base differences). The sequences of Vietnamese strains were 100% identical to each others.

3.2. Toxin profile and contents

All isolates of *A. minutum* from Vietnam examined produced PSTs. The main toxin component was GTX4, with GTX1 and NEO as minor components (Table 2). In addition, trace amount of GTX3, GTX2 and dcSTX were also detected in some strains. The contribution of GTX4 to total toxin content varied from 91–94 mol% at

Table 2
Toxicity of Vietnamese strains of *Alexandrium minutum*

Toxins (fmol cell^{-1})	Strains									
	AmSp01		AmSp03		AmSp04		AmSp05		AmSp17	
	Day 5	Day 10	Day5	Day 10	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10
GTX 4	11.523	8.185	11.139	7.643	4.588	6.878	2.687	6.181	5.038	4.489
GTX 1	0.468	1.126	0.392	0.915	0.08	0.782	0.057	0.881	0.036	0.232
GTX 3	n.d.	0.003	n.d.	n.d.	0.005	n.d.	n.d.	0.005	n.d.	0.011
GTX 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.013	n.d.	n.d.
dcSTX	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.072	n.d.	0.035
neoSTX	0.463	0.280	0.324	0.613	0.205	0.327	0.239	0.304	0.403	0.177
New derivative[a]	(0.366)	(1.701)	(0.287)	(4.639)	(0.239)	(3.292)	(0.087)	(2.062)	(0.185)	(1.449)
Cellular toxin quota (fmol cell^{-1})	12.454 (12.820)	9.593 (11.294)	11.856 (12.143)	9.171 (13.810)	4.878 (5.117)	7.987 (11.279)	2.983 (3.070)	7.458 (9.520)	5.476 (5.661)	4.944 (6.393)

n.d., not detected.
[a] Concentration of the new GTX derivative was estimated based on GTX4 fluorescence response. Value in bracket represent cellular toxin quota with inclusion of the new analogue.

early exponential phase to 83–91 mol% at late exponential phase. Cellular toxin quota varied among strains and growth stages with the range of 3.0–12.5 fmol PST cell^{-1}.

3.3. Existence of a new toxin analogue

In HPLC analysis, a fluorescence peak consistently appeared between GTX4 and GTX1 with retention time of 11–12 min (Fig. 4a). The presence of this peak was confirmed in all clonal cultures. No PST derivative has been reported at this position previously. The peak, together with other GTXs, disappeared under non-oxidizing conditions (Fig. 4b). However, the peak remained after HCl hydrolysis (Fig. 4c). Further treatment of the extract using phosphate buffer and 2-mercaptoethanol successfully removed the *o*-sulfate moiety in GTX1–4 and converted them into STXs, yet

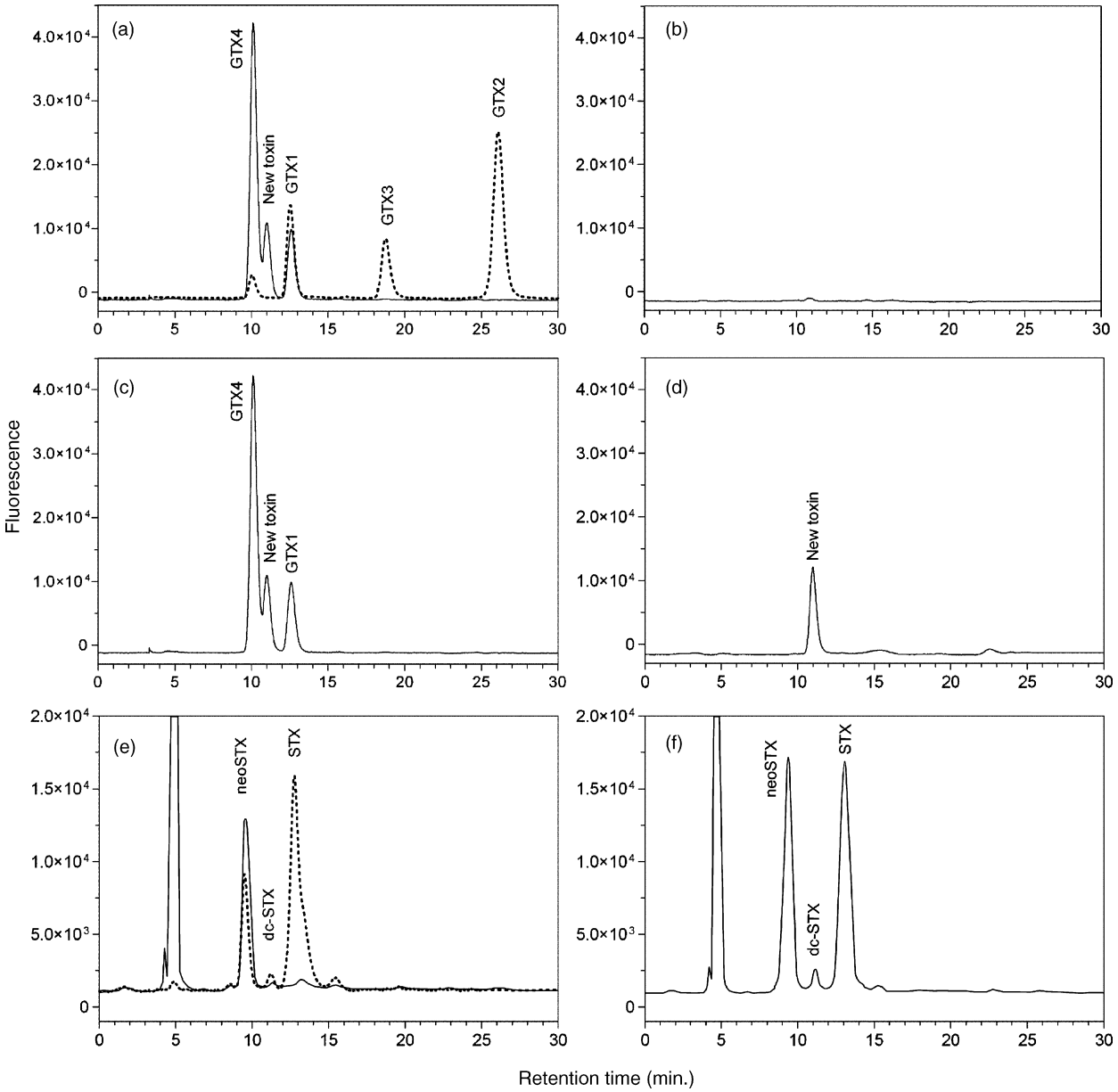

Fig. 4. HPLC chromatographs of *A. minutum* strain AmSp03 extract, GTXs calibration standard was shown as dash line (a). The same extract run through non-oxidizing condition (b) and after HCl hydrolysis (c). The extract after mercaptoethanol treatment, note the disappearance of GTX4 and GTX1 peaks (d). (e and f) STXs run before (e) and after (f) mercaptoethanol treatment showing the increment in STX, dcSTX and neoSTX after mecaptoethanol treatment, STXs calibration standard was shown as dash line.

the peak was unaffected (Fig. 4D–F). The compound also displayed significant changes over the growth stages (Table 2). The concentration of this compound was estimated based on the fluorescence response of GTX4 with the assumption that it gave similar response as GTX4 in the HPLC analysis. The concentration was low (2.4–4.7 mol%) at the early exponential phase but increased significantly to 15–33.6 mol% in all the strains at the late exponential phase (day 10).

LCMS/MS analysis carried out on the unknown toxin analogue showed that in total ion scanning Q1 mode, two major peaks were observed at 3.55 and 7.10 min (Fig. 5a). Mass spectra obtained by full scan mass chromatogram at retention time of 7.07–7.29 min showed predominant $[M + H]^+$ ion at *m/z* 396 (Fig. 5b). The peak was also observed in selected ion scanning mode at *m/z* 396. Further analysis of the peak using product ion scan from 150 to 450 Da successfully found

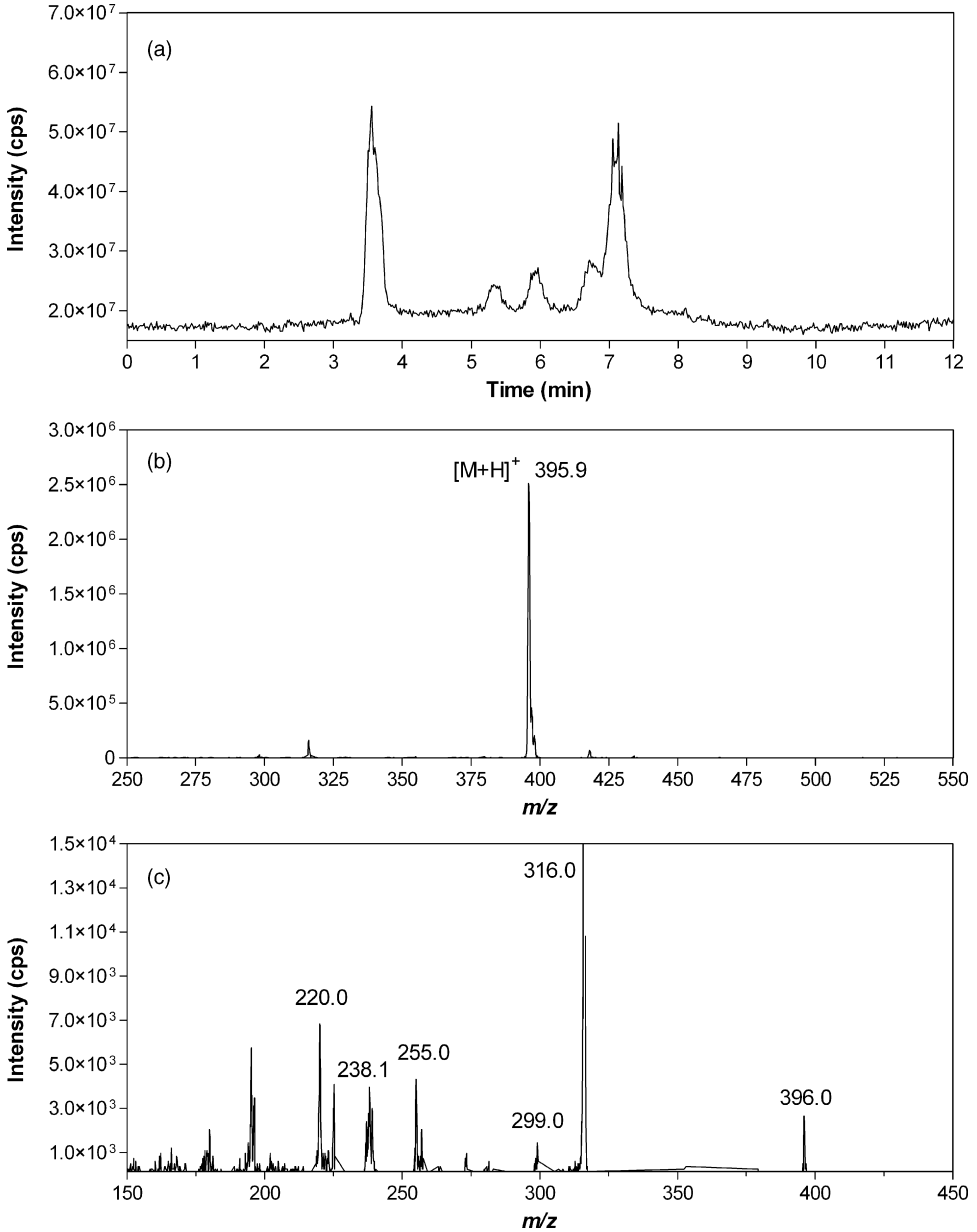

Fig. 5. (a) Total ion scanning of the compound. (b) Mass spectra obtained by full scans mass at retention time of 7.1 min showed a parental ion at *m/z* 396. (c) Fragmentation pattern of the peak. A parental ion was observed at *m/z* 396 with daughter ions at *m/z* 316.0 $[M + H - SO_3]^+$, *m/z* 299.0, 255.0, 238.1 and 220.0.

similar GTXs fragmentation (Fig. 5c). In the mass spectrum of the peak, ion at *m/z* 316 corresponding to $[M + H - SO_3]^+$, a structure that eliminated SO_3 moiety from the site chain, was predominant. The results clearly showed the presence of *o*-sulfate side chain in the compound. Other fragment ions at *m/z* 255, 238 and 220 were also observed.

4. Discussion

Identification of *A. minutum* based on morphological thecal plates tabulation was a straight forward task. The size and shape of the cells, position of ventral pore (vp), the shape of posterior sulcal plate (s.p.) and other sulcal plates observed in all the isolates from Vietnam fitted well with Balech's description (Balech, 1989, 1995). However, some morphological differences have been reported from the European strains. Some of the European strains were reported to have reticulation on hypotheca (Montresor et al., 1990) and some do not have vp (Hansen et al., 2003). In contrast, most of the Asia Pacific strains reported thus far possessed smooth cell surface with the presence of vp (Usup et al., 2002).

In this study, *A. minutum* was genetically separated into two main groups based on partial LSU rRNA gene analysis. The Vietnamese isolates were clustered together with the Malaysian and New Zealand isolates, forming a monophyletic group with large sequence divergences compared to the European and southern/werstern Australian isolates. Several previous studies have also shown the biogeographical separation of *A. minutum* populations, i.e. the Asia Pacific clade and European clade (including southern/western Australia) (Hansen et al., 2003; Lilly et al., 2005). Two discrete ribotypes were also reported among the Australian *A. minutum* (De Salas et al., 2001). In the present study, high sequence homogeneity (>99.5%) was observed between the Vietnamese and Malaysian strains of *A. minutum*, in relative to other strains reported in the region. The similarity between both populations suggested the presence of gene flow. This could be explained by the monsoon currents pattern occurred annually in the South China Sea. During the winter monsoon, northeast current flows through the coastal waters of Vietnam and eventually ended in the Gulf of Thai and northeastern of Peninsula Malaysia.

The cellular toxin quota (Qt) of Vietnamese *A. minutum* isolates ranged from 3.0 to 13.8 fmol PST $cell^{-1}$ (Table 1), which was at comparable level with the strains from Malaysia (4.0–12.0 fmol $cell^{-1}$; Lim and Ogata, 2005), New Zealand (3.4–10.1 fmol $cell^{-1}$; Mackenzie and Berkett, 1997), and Spain (1.0–18.0 fmol $cell^{-1}$; Franco et al., 1994). GTX4 and GTX1 were reported as predominant toxin congeners in most of the strains from Asia Pacific regions, including strains from Taiwan (Hwang and Lu, 2000), New Zealand (Mackenzie and Berkett, 1997), Thailand (Piumsomboon et al., 2001) and Malaysia (Lim et al., 2004). Similar toxin composition was also observed for the Vietnamese isolates. However, some of the New Zealand strains were reported with NEO as principle toxin congener (Chang et al., 1997). Toxin profile of *A. minutum* from Europe was far more diverse compared to the Asia Pacific strains. Some European strains such as those from France (Belin, 1993) and UK (Percy et al., 2002) possessed different toxin composition with GTX3 and/or GTX2 as the predominant toxin congeners. In contrast, GTX4 and GTX1 were the main toxin congeners for Portugal (Cembella et al., 1987) and Spain strains (Franco et al., 1994; Carreto et al., 2001). The Danish strains, on the other hand, were reported with predominant C1 and C2 (Hansen et al., 2003). Recently, a strain with GTX3 and STX as the major toxin congeners has been reported from Fleet Lagoon, UK (Nascimento et al., 2005). Nonetheless, geographical divergence of the toxin profiles has been reported in many other *Alexandrium* species (Cembella et al., 1987; Anderson et al., 1994; Cembella and Destombe, 1996; Yoshida et al., 2001) and *Gymnodinium catenatum* (Oshima et al., 1993).

In this study, a new GTX analogue was found to occur naturally in the Vietnamese strains of *A. minutum*. In our HPLC analysis, a fluorescent peak appeared consistently between the peaks of GTX4 and GTX1. As a matter of fact, some non-PST compounds also gave false fluorescence signals in the post-column reaction system (Gulavita et al., 1988; Onodera et al., 1996; Sato and Shimizu, 1998) and remained under non-oxidizing condition. In our analysis, however, the peak disappeared under the non-oxidizing condition. The results showed that the compound reacted similarly to oxidizing reagent as other GTXs. In addition, the retention time of the peak showed that it might possess similar polarity and ion state as other GTXs. Furthermore, the peak remained when hydrolyzed with HCl. This indicated that *N*-sulfocarbamoyl moiety is absent from this compound.

Results of LCMS/MS showed a parental ion $[M + H]^+$ at *m/z* 396 for the compound. The molecular weight was differed from GTX4/GTX1 ($[M + H]^+ = 412$) by 16, but identical to the *m/z* values of GTX3 and GTX2. This results indicated the absence of one oxygen atom in this compound compared to GTX1 or GTX4. In this study, the compound did not react with ME and this indicated that the atom oxygen was most probably absent at the C12

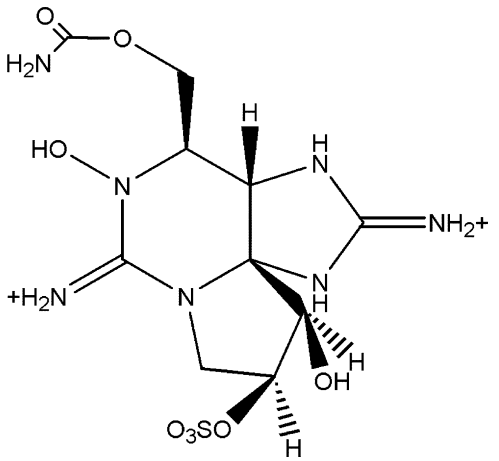

Fig. 6. Chemical structure of deoxy-GTX4-12ol.

position. The characteristic of keto-geminal-diol structure at the position of C12 was essential for the ME reduction (Sato and Kodama, 2003). On the other hand, fragmentation pattern at *m/z* 316 was due to the loss of SO_3 from the $[M+H]^+$ ion, clearly evident in the presence of *o*-sulfate side chain at R2 or R3 (C11) position. These result strongly suggested that the compound is a new GTX analogue, deoxy-GTX4-12ol (Fig. 6).

Recently increasing number of new saxitoxin derivatives have been reported. Six new saxitoxin analogues were found in the freshwater filamentous cyanobacterium, *Lyngbya wollei*, three congeners showed the reduction at C12 position (Onodera et al., 1997). Three new saxitoxin analogues were also identified from the dinoflagellate *G. catenatum*, which possessed a hydroxyl benzonate moiety rather than carbamoyl side chain in the saxitoxin molecules (Llewellyn et al., 2004). Two of the analogues, GC1 and GC2 also possessed sulfate at the C11 position and existed as epimeric mixture (Llewellyn et al., 2004). Reduced form of saxitoxins (also referred as saxitoxinol) and it epimers have also been produced chemically (Shimizu et al., 1981; Kao et al., 1985; Mahar et al., 1991). Nonetheless, the derivative reported here was distinctively different from those reported earlier. Recently, a peak labeled as NT was also reported in the Australian strains of *A. minutum* (Parker et al., 2002). The position of the peak in the HPLC analysis was almost identical to the peak observed in this study. If the peak is identical to the one reported here, it might indicate another strain that possessed this unique derivative.

In our *A. minutum* cultures, the new GTX analogue was also found to be variable among the strains as well as different growth stages. Interestingly, the derivative was found to be negatively correlated to GTX4 (Table 2). Close structural similarity between the two derivatives and relative proportional changes may reveal some enzymatic conversion between the two. Enzymatic transformation of toxin congeners has been widely reported in PST-producing dinoflagellates. For example, an oxidase that converted GTX3/2 to GTX4/1 has been reported in *A. tamarense* (Oshima, 1995a). Another enzyme, *N*-sulfotransferase has also been found in *G. catenatum* that converted carbamate toxins to C-toxins (Oshima, 1995a).

Final structural information of this derivative could only be provided with NMR analysis. Nonetheless, results of HPLC and LCMS/MS in this study have clearly showed the presence of a new GTX analogue in the Vietnamese strains of *A. minutum*. Further study on enzymatic transformation of this derivative might give valuable clues in understanding the metabolism and biosynthesis of PSTs.

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