Discovery of novel ovatoxin isomers in several Ostreopsis strains in Japan

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**Ostreopsis** spp.

- The dinoflagellate genus, *Ostreopsis*, has an increasingly global distribution

- Some *Ostreopsis* produces palytoxin analogues

- *O. ovata* is held responsible for respiratory illnesses due to inhalation of aerosols during blooms in the Mediterranean region

Dinoflagellate genus

*Ostreopsis* spp.

(*O. siamensis* *O. ovata* etc 9 species)
Ostreopsis found along the coastal area in Japan
Palytoxins

Ostreopsis

The Soft coral
Palythoa toxica
Human poisoning cases due to consumption of seafood suspected to be contaminated with palytoxins

- Human fatalities due to consumption of seafood suspected to be contaminated with palytoxins were reported in the Philippines, after consumption of the crab *Demania reynaudii* (1988), and in Madagascar following consumption of the sardine *Herklotsichthys quadrimaculatus* (clupeotoxism) (1999)

- Respiratory illness has also occurred when people were exposed to *Ostreopsis ovata* bloom aerosols during recreational or working activities, in Italy (2006)

![D. reynaudii](image1)

![H. quadrimaculatus](image2)

![Ostreopsis ovata](image3)
Blue humphead parrotfish (*Scarus ovifrons*) poisoning in Japan (*Palytoxin like poisoning*)

- Symptoms: Rhabdomyolysis, a syndrome injuring skeletal muscle, causing muscle breakdown, and leakage of large quantities of intracellular (myocyte) contents into blood plasma
- The symptoms are similar to palytoxin poisoning
- Palytoxins have not been confirmed yet from the blue humphead parrotfish which was identified as the causative food in the poisoning

![Diagram](image)

- *Ostreopsis*
- ?
- Palytoxin
- ?
- *Scarus ovifrons*
- The Soft coral *Palythoa* spp.
Blue humphead parrotfish (*Scarus ovifrons*) poisoning in Japan (*Palytoxin like poisoning*)

In 2000
1 patient

In 2001
2 patients

In 2003
3 patients

In 2004
2 patients
1 person died

In 2007
9 patients

In 2011
4 patients were reported in Tokyo in November
1 patient was reported in Miyazaki in March
Objectives

• LC-MS/MS analysis of palytoxin analogues in several Ostreopsis strains collected in Japan

• LC-MS/MS analysis of palytoxin analogues in the blue humphead parrotfish (Scarus ovifrons) which was identified as the causative food in human poisoning cases in Tokyo and Miyazaki in 2011
LC-MS/MS analysis of palytoxin analogues in several Ostreopsis strains collected in Japan
References

LC-MS/MS chromatogram of palytoxin

**LC-MS condition**

Column: Hypersil-BDS-C8  
(150 mm x 2 mm i.d)  
Flow rate: 0.2 mL/min  
Mobile phase: A water, B 95% MeCN  
both containing 2 mM HCOONH$_4$ and 50 mM HCOOH

Step 1: 5 % B  100%B for 15 min  
Step 2: 100 % B for 5 min

**Detection**  
[M+2H-H$_2$O]$^{2-}$ 1331$>$ 327

**LOD**: 10ng/ml (0.1ng)
LC-MS/MS chromatogram of palytoxin analogues in *Ostreopsis* collected in coastal waters in Japan

**Ostreopsis culture**

Cells were harvested by centrifugation at 3000 rpm

Toxins were extracted with MeOH

An aliquot of the MeOH extract was analyzed by LC-MS/MS

Peak #1: Ovatoxin-a
Peak #2: Ovatoxin-b
Peak #3: Ovatoxin-b isotope
Peak #4: Ovatoxin-d
Peak #5: Ovatoxin-a isotope
Peak #6: Ovatoxin-e
Peak #7: Palytoxin
LC–MS/MS chromatogram obtained from the mixture of Italian and Japanese *Ostreopsis* strain extracts

(A) Ovatoxin-a AC from Japanese culture

(D) Ovatoxin-e AC from Japanese culture

AC: Porf. Adachi Culture
**[M+2H-nH2O]^{2-}**

- **A**
  - 327.1935 (+4.5 ppm)
  - 327.1920 (345.2026)
- **B**
- **C**
  - 726.4040 (-3.5 ppm)
  - 786.4276 (-3.0 ppm)
  - 804.4382

Additional masses:
- 744.4170
- 377
- 419
- 406
- 726
- 744
- 786
- 804

**m/z**

- 300
- 400
- 500
- 600
- 700
- 800
- 900
- 1000
- 1100
- 1200
- 1300
- 1400
LC–MS/MS spectra of palytoxin analogues in *Ostreopsis*

**Palytoxin**

- A
- C
- \([\text{M+2H-nH}_{2}\text{O}]^{2-}\)

**Ostreocin-D**

- A-CH₃
- C
- \([\text{M+2H-nH}_{2}\text{O}]^{2-}\)

**Ovatoxin-a AC**

- A
- C
- \([\text{M+2H-nH}_{2}\text{O}]^{2-}\)

**Ovatoxin-b**

- A +2CH₂+O
- C
- \([\text{M+2H-nH}_{2}\text{O}]^{2-}\)
High-resolution LC-MS/MS product ion spectra obtained for authentic palytoxin standard by Qtof LC-MS/MS
High-resolution LC-MS/MS product ion spectra obtained for Ovatoxin-a AC by Qtof LC-MS/MS
Proposed fragmentation diagram of palytoxin
Elemental formulae of palytoxin analogues determined by QTOF LC-MS spectra on the positive mode

<table>
<thead>
<tr>
<th></th>
<th>m/z (measured value)</th>
<th>Formula</th>
<th>Tolerance (ppm)</th>
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</thead>
<tbody>
<tr>
<td>Ovatoxin-a AC</td>
<td>2647.5062</td>
<td>$C_{129}H_{224}N_3O_{52}$</td>
<td>3.2</td>
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<tr>
<td>Ovatoxin-d AC</td>
<td>2663.4918</td>
<td>$C_{129}H_{224}N_3O_{53}$</td>
<td>-0.3</td>
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<tr>
<td>Ovatoxin-e AC</td>
<td>2663.4841</td>
<td>$C_{129}H_{224}N_3O_{53}$</td>
<td>-3.2</td>
</tr>
</tbody>
</table>
Clades classified by the phylogenetic analysis reported in our previous study (Sato et al., 2011)
Toxin profiles of several *Ostreopsis* strains collected in Japan and Italy (*1) analyzed by MRM LC-MS/MS

*1

Ostreopsis strains

- ostreocin-d
- ovatoxin-e AC
- ovatoxin-d AC
- ovatoxin-c AC
- ovatoxin-b AC
- ovatoxin-a AC
- palytoxin
Total cellular toxin contents of several *Ostreopsis* strains collected in Japan and Italy (*1) analyzed by MRM LC-MS/MS.

*1*
Summary

• Novel isomers of ovatoxin-a, -b, -d, -e were found in Japanese Ostreopsis. The isomers of ovatoxin-a,-b,-d, and –e detected in Japanese Ostreopsis were tentatively named ovatoxin-a AC, -b AC,-d AC and –e AC.
• LC-MS analysis revealed that ovatoxin-a AC is a structure analogue of palytoxin reduced a hydroxyl group at both C16-C20 and C53-C73 moieties of palytoxin.
• Toxin profiles of Ostreopsis collected in middle to southern coastal area in Japan were clarified by LC-MS/MS
• The toxin contents of some strains collected in Japan were comparable to that obtained in a strain (KAC 85) collected in the Italian coast where human health problems occurred
LC-MS/MS analysis of palytoxin analogues in the blue humphead parrotfish (*Scarus ovifrons*) which was identified as the causative food in human poisoning cases in Tokyo and Miyazaki in 2011
Human poisoning cases by consumption of the blue humphead parrotfish in 2011

• In March 2011, the human poisoning case by consumption of the blue humphead parrotfish occurred in Miyazaki. One patient was diagnosed as the palytoxin like poisoning

• In November 2011, the human poisoning case by consumption of the blue humphead parrotfish occurred in Tokyo. Four patients were diagnosed as the palytoxin like poisoning

• The blue humphead parrotfish samples caused the human poisoning were provided to NRIFS to identify palytoxin by LC-MS/MS
meat, head, curry soup (20g) homogenize with 90% MeOH (180mL)

palytoxin for recovery check (30 ppb)

centrifugation at 3000 rpm for 2 min

20 mL supernatant

hexane (20 mL)

distilled water (8 mL), chloroform (30 mL)

aqueous MeOH hexane
distilled water (8 mL), chloroform (30 mL)

aqueous MeOH chloroform

evaporation

50% MeOH (1 mL) or 5% MeOH (1mL)

(head, curry soup) (meat)

20uL injection to LC-MS/MS
LC-MS/MS analysis of the blue humphead parrotfish

Fish meat causing of the human poisoning case (A)  
Fish meat (A) fortified with palytoxin at 30 ppb
Recovery (%) of palytoxin from the blue humphead parrotfish fortified with palytoxin at 30 ppb

<table>
<thead>
<tr>
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<th>Recovery (%)</th>
<th>LOQ (ppb)</th>
<th>LOD (ppb)</th>
</tr>
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<tbody>
<tr>
<td>Head</td>
<td>58.3</td>
<td>1.18</td>
<td>0.35</td>
</tr>
<tr>
<td>Curry soup</td>
<td>66.7</td>
<td>1.50</td>
<td>0.45</td>
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<tr>
<td>Meat</td>
<td>57.1</td>
<td>1.60</td>
<td>0.48</td>
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</table>
Summary

- LC-MS/MS method of palytoxin analogues in cooked seafood samples was developed.
- The LOD and LOQ are 0.5 and 2 ppb, which are lower than the EFSA regulatory level (30 ppb) of palytoxins in seafood.
- Palytoxins were not detected in the blue humphead parrotfish samples causing the human poisoning in 2011.
- Palytoxins are not the causative toxin in the blue humphead parrotfish poisoning in Japan.