Research of marine dinoflagellate bioactive compounds in Taiwan

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SUMMARY: Thirty-five clones belonging to twelve dinoflagellate species have been isolated from coastal waters of Taiwan and cultured since 1996. Crude extracts of them were screened for bioactive compounds by mouse and brine shrimp toxicity assays. Fifteen of the 35 tested clones (42%) showed potent mouse toxicity and 19 of the 34 tested clones (56%) showed brine shrimp toxicity. Clones belonging to *Amphidinium carterae*, *A. klebsii*, *Gambierdiscus toxicus*, *Gyrodinium instriatum*, *Ostreopsis lenticularis*, *Prorocentrum lima*, and an unknown *Prorocentrum* species were found toxic in one or both animal assays, but not all the clones of the same species were toxic. From *P. lima* PL01 and *P. sp1*. PM08, several derivatives of okadaic acid and prorocentrolide were isolated to be active components. Among them, spiro-prorocentrimine was purified in crystal and its relative configuration resolved as the first one in this group of dinoflagellate toxins. 14-O-acetyl-4-hydroxyprorocentrolide, 4-hydroxyprorocentrolide and prorocentrolide were also found scattered in different species of *Prorocentrum*, and not limited to *P. lima* only. From *P. lima*, OA-D9d, DTX-4b and DTX-6 were isolated as new okadaic acid diol esters, together with several known OA analogues and derivatives in this research.

KEY WORDS: dinoflagellate, *Prorocentrum lima*, macrolides, okadaic acid, dinophysistoxin

INTRODUCTION

Aquaculture for seafood supply has been more important than catch fisheries in Taiwan. Wastes and dissolved organic nutrients from feed in aquaculture ponds have been supporting the growth of various plankton species to form the abundant floating biomass in the surrounding waters. Some of these waters are also important in supporting the shellfish farming. Thus sporadic shellfish poisonings and ciguatera have inevitably occurred and have been reported in this island province. Search for the possible causative agents of these shellfish and fish poisonings has started from a mouse or a brine shrimp toxicity assay of the extract of cultured clones of planktonic and benthic dinoflagellates isolated from the surrounding waters. Various toxins of different categories have been known in the same or similar group of dinoflagellate around the world¹⁾. Our interest on dinoflagellates lies not only on their toxicities but also on their pharmaceutical potentials, especially the tropical benthic ones, that have been revealed rich in bioactive compounds^{2, 3)}. In this paper we summarize the result of a toxicity screening conducted on a batch of thirty-five clones, belonging to twelve different species of tropical and subtropical dinoflagellates that have been isolated from the waters

of aquaculture pond and the epiphytic wash-offs of seaweed collected in coral reef areas of Taiwan. Further descriptions are on the chemical nature of the toxins isolated from some of the toxic species.

MATERIALS AND METHODS

Field collections of the dinoflagellate were made at various brackish ponds and coral reefs along the coastal area of the main-island and offshore islets of Taiwan in different seasons. Single cell of the wanted dinoflagellate species was picked up using a glass capillary under a binocular microscope. The isolated cell was then washed several times in drops of sterilized seawater until it was free of visible contaminants before it was inoculated in capped tube with 15 ml sterilized K-medium-enriched seawater. Thirty-five clones of the following twelve species were isolated in 1997 and subjected to culture stock: *Amphidinium carterae*, *A. klebsii*, *Coolia monotis*, *Gyrodinium instriatum*, *Gambierdiscus toxicus*, *Gonyaulax* sp., *Gymnodinium* sp., *Ostreopsis lenticularis*, *Prorocentrum. lima*, *P. mexicanum*, *P. minimum*, and an unknown species of *Prorocentrum*.

Culture and Extraction

Cultures of different clones were scaled up stepwise from 15 ml in test tubes to 2 L in flasks. Growth conditions were maintained at 25°C under low illumination of 1500 lx for 16 hr daily. Algal cells were harvested at the end of the exponential phase by either filtration or centrifugation. The harvested cell

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masses of different clones were re-suspended in 30 ml methanol respectively and sonicated for extraction twice. The combined methanol extracts were then partitioned with 15 ml *n*-hexane twice to remove the non-polar pigments. After making up the extract to 60% of methanol with de-ionized water, it was further partitioned with 30 ml chloroform for two times. The aqueous methanol and chloroform fractions thus obtained were subjected for both mouse and brine shrimp toxicity assays.

Bioassay

Both methanol and chloroform extract of dinoflagellate cells were suspended in 1 ml of 1% Tween-60 saline solution. Concentrations of the different algal extracts for injection in each mouse ranged from 2 to 20 mg, depending on sample availability. Male, white mice of ICR strain, 20 g each, were injected intraperitoneally and then observed for 24 hr. If mice showed dyspnoea, gasping, diarrhoea, and/or hypersalivation before death, figures of mouse unit (MU) corresponding to ciguatera toxicity of the extracts were calculated on the duration from injection to death according to the Lewis formula⁴⁾.

The brine shrimp toxicity assay applied was similar to the method previously reported⁵⁾. In brief, preserved eggs of brine shrimp (*Artemia salina*) were hatched in a beaker of filtered seawater. The free-swimming nauplii were collected using a Pasteur pipette 36 to 48 hr after sowing. Attentions were paid on the collection of the nauplii to exclude the un-hatched eggs and debris. Ten to twenty *Artemia* nauplii in 100 μ l were transferred to each well of a multi-welled culture plate, and then a serial dilution of extracts in concentrations of 4000, 2000, 1000, 500, 100 ppm were added into each well. Counting of the survival started after 24 hr treatment of the algal extracts. The estimated LC₅₀ was calculated from the plot of percentage mortality against dosage.

Toxin Isolation⁶⁾

Okadaic acid (OA) 1, dinophysistoxin-1 (DTX-1) 2, methyl okadate 3, and two diol esters of okadaic acid, OA-D8 4 and OA-D9d 5 were isolated from chloroform extracts of *P. lima* by successive separations on columns of Baker's silica gel for flash column (CHCl₃-MeOH, stepwise), Sephadex LH-20 gel permeation (MeOH) and Cosmosil 5C₁₈-ARII RP-HPLC (CH₃CN-H₂O, 1:1). From clone PL01 of *P. lima* two sulfated okadaic acid derivatives (6 and 7) and two prorocentrolide derivatives (8 and 9) were identified in the butanol extract. They were purified from successive separations on columns of Sephadex

LH-20 (MeOH-H₂O 7:3), Baker's silica gel (EtOAc-MeOH-H₂O 7:3:1) and Cosmosil 5C₁₈-ARII RP-HPLC (CH₃CN-0.01M NH₄OAc, linear gradient of 35 to 40% in 10 minutes). From clone PM08 of an unknown *Prorocentrum* species prorocentrolide 10 and a microlide 11, with an unprecedented skeleton and a spiro-linked cyclic imine moiety were isolated from the butanol extracts. Isolation of compounds 10 and 11 was carried out in a similar manner with the separation of 8 and 9 in PL01.

¹H NMR and ¹³C NMR spectra of the isolated compounds were taken on a Bucker DMX-500 spectrometer, and mass spectra were taken on a Jeol SX-102A spectrometer. Data from 2D-NMR of COSY, HMBC, HMQC and NOESY, and mass spectrometry gave sufficient information for structure elucidation of the known and new compounds. Single crystal x-ray crystallography was also applied for the compound 11, spiro-prorocentrimine.

RESULTS

As shown in Table 1, all clones of these twelve dinoflagellate species were maintained in good growth shapes, although their cell concentrations varied a lot at the time of end of exponential phase. They were always kept in exponential phase by transferring the culture to a fresh medium once they reached the beginning of stationary phase. Most of the clones reached their stationary phase about a month after inoculation except *A. carterae*, which reached its maximum cell concentration two weeks after each inoculation into fresh medium.

Table 1. Clones of the dinoflagellate in culture.

Species	Cell concentration (10 ⁴ cells/ml)	Total cells (10 ⁸ cells)
<i>Amphidinium carterae</i> , 9 clones	10.5-19.0	2.95-5.79
<i>A. klebsii</i> , 1 clone	0.46	0.08
<i>Coolia monotis</i> , 1 clone	9.70	2.72
<i>Gonyaulax</i> sp., 1 clone	0.82	0.10
<i>Gyrodinium instriatum</i> , 7 clones	0.22-0.48	0.03-0.07
<i>Gymnodinium</i> sp., 1 clone	0.96	0.15
<i>Gambierdiscus toxicus</i> , 1 clone	0.16	0.02
<i>Ostreopsis lenticularis</i> , 1 clone	4.90	1.37
<i>Prorocentrum lima</i> , 1 clone	1.42	0.4
<i>P. mexicanum</i> , 1 clone	0.88	0.32
<i>P. minimum</i> , 1 clone	5.65	1.30
<i>Prorocentrum</i> sp1., 10 clones	1.26-4.85	0.38-1.4

Mouse and brine shrimp toxicity assays revealed that a total of 15 clones among 35 showed toxicity to mice (Table 2). Five of the 9 clones of *A. carterae*, 6 of the 10 clones of *Prorocentrum* sp1., and clones of *A. klebsii*, *P. lima*, *G. toxicus*, and *O. lenticularis* are found toxic to mice. It was obvious that different

clones of dinoflagellate might show different toxicity from their various toxin composition and different content. This was the case found in the various clones of *A. carterae* and *Prorocentrum* sp1. (Table 2). It was also noted that most of the toxic dinoflagellate clones took hours to kill the mice after injection of the extract except some clones of *Prorocentrum* sp1. that killed the mice in a very short time. It was speculated that fast-acting toxins might exist in some of the toxic clones of this unknown *Prorocentrum*. The general symptoms of the intoxicated mice were weakness, weak paralysis in limb, gasping, and dyspnoea.

Brine shrimp larvae responded to the methanol extract of dinoflagellate clones in variable extents (Table 2). Among them, clones 1, 2, 3, 4, 5, and 9 of *A. carterae* and the tested clone of *A. klebsii* were the most toxic ones in addition to *P. lima*. Chloroform extract of *P. lima* also showed toxicity to brine shrimp, like the chloroform extract of *Gyrodinium instriatum*. Various clones of *G. instriatum* were found the most toxic group among the tested clones in their chloroform extract. However their methanol extracts showed less toxicity or nontoxic to brine shrimp.

Table 2. Mouse and brine shrimp larvae toxicity assays for the cloned dinoflagellates: *Amphidinium carterae* (AC01-AC09), *A. klebsii* (AK01), *Prorocentrum lima* (PL01), *P. mexicanum* (PM03), *P. minimum* (PM12), *P. sp1.* (PM01-PM02 & PM04-PM11), *Coolia monotis* (CM10), *Gambierdiscus toxicus* (GT03), *Ostreopsis lenticularis* (OL01), *Gymnodinium* sp. (GS01), *Gyrodinium instriatum* (GI01-GI07), *Gonyaulax* sp. (GD01). *MU represents the mouse unit, the LD₅₀ dose on a 20 g mouse; ** ND, not detected.

Clone	Mouse toxicity MU*		Estimated LC ₅₀ (ppm) on brine shrimp at 24hr	
	MeOH fraction	CHCl ₃ fraction	MeOH fraction	CHCl ₃ fraction
AC01	1	<1	186	>4000
AC02	<1	<1	109	>4000
AC03	35	<1	164	>4000
AC04	2	1	103	>4000
AC05	<1	<1	191	>4000
AC06	3	<1	962	>4000
AC07	<1	<1	2105	>4000
AC08	<1	<1	2140	>4000
AC09	3	<1	114	>4000
AK01	315	48	100	>4000
CM10	<1	<1	>4000	>4000
GD01	<1	<1	593	2791
GI01	<1	<1	>4000	100
GI02	<1	<1	>4000	167
GI03	<1	<1	>4000	189
GI04	<1	<1	2960	366
GI05	<1	<1	1339	295
GI06	<1	<1	>4000	184
GI07	<1	<1	1777	141
GS01	<1	<1	>4000	777
GT03	25667	<1	ND**	ND
OL01	<1	4	>4000	2746
PL01	8	3846	187	357
PM01	<1	<1	>4000	2000
PM02	<1	<1	>4000	2500
PM03	<1	<1	>4000	4230
PM04	<1	<1	>4000	2546
PM05	551	<1	1540	2070
PM06	<1	<1	2523	2150
PM07	173	1065	2051	1267
PM08	1138	748	835	1754
PM09	1142	<1	2660	2340
PM10	99	<1	1412	>4000
PM11	132	<1	2848	3200
PM12	<1	<1	2840	>4000

Following the guide of mouse and brine shrimp toxicity assays, okadaic acid 1, and its derivatives, DTX-1 2, methyl okadate 3, and OA diol esters, OA-D8 4 and OA-D9d 5 were isolated from chloroform extract of *P. lima*, in addition to two sulfated OA diol esters, DTX-6 6 and DTX-4b 7. from *n*-butanol extract. Other than the above mentioned polyether toxins, 4-hydroxyprorocentrolide 8 and 14-*O*-acetyl-4-hydroxyprorocentrolide 9, were isolated from the same clone, PL01 of *Prorocentrum*, and the prorocentrolide 10 from PM08 clone of the unknown *Prorocentrum*. From PM08 a new macrolide spiro-prorocentrimine 11 was also isolated and its structure confirmed by x-ray crystallography⁸). Spiro-prorocentrimine was the first one in this group of dinoflagellate toxins with its relative configuration resolved.

- 1 okadaic acid (OA) $R_1 = H$ $R_2 = H$
 2 dinophysistoxin 1 (DTX-1) $R_1 = H$ $R_2 = CH_3$
 3 OA methyl ester $R_1 = CH_3$ $R_2 = H$
 4 OA-D8 $R_1 = -CH_2-CH=CH-CH=CH-CH_2OH$ $R_2 = H$
 5 OA-D9d $R_1 = -CH_2-CH=CH-CH=CH-CH_2OH$ $R_2 = H$

Okadaic acid and DTX-1 were further analyzed in different clones of *Prorocentrum* by an HPLC. The result revealed that many *Prorocentrum* species other than *P. lima* may contain both OA and DTX-1, and all of the clones of *P. lima* contain OA or DTX-1 in varied contents. It was also noted that OA was released into medium by the active-growing cells and an aged PL01 culture contained more OA in medium than in the cells. Clones of *P. lima* were also found to contain prorocentrolide or its derivatives in different composition. However, clones of some unknown *Prorocentrum*, that contain prorocentrolide or its derivatives were found in lack of OA and DTX-1.

DISCUSSION

The search for the toxic benthic dinoflagellates has revealed that clones of *A. carterae*, *A. klebsii*, *G. toxicus*, *G. instriatum*, *O. lenticularis*, *P. lima*, and an unknown *Prorocentrum* species were toxic in one or both animal assays in this report. This fact led to the speculation that these epiphytic dinoflagellates may be the toxin producers that cause the seafood poisonings from ingestion of contaminated coral reef fishes or gastropods. These types of poisoning may be regarded as ciguatera outbreaks in a broad view on its definition. However direct evidence has never been obtained to show the toxins involved in the poisonings are from dinoflagellates. Besides not all the clones of toxic species are toxic.

Prorocentrum is a rather common dinoflagellate group in Taiwan. Various species of known or unknown belonging to this genus have been found in different collection sites. All our clones of *P. lima* produce okadaic acid and DTX-1 that have been known the toxins to be involved in diarrhetic shellfish poisoning. Several derivatives of okadaic acid were also found in the PL01 of *P. lima*. Among them OA-D9d, DTX-6 and DTX-4b are new in this group of toxins. PL01 is also known capable to produce 4-hydroxyprorocentrolide instead of prorocentrolide which is a known toxin isolated from Okinawan *P. lima*⁷⁾ and from our PM08 clone of an unknown *Prorocentrum* species.

Spiro-prorocentrimine, a new compound which contains a spiro-linked cyclic imine group with the *ortho*, *para*-disubstituted 3'-cyclohexene, rather than the hexahydro-isoquinoline moiety of prorocentrolide was also isolated from PM08⁸⁾. However both spiro-prorocentrimine and prorocentrolide share some common structure in part. Similar spiro-linked imine moiety was also found in some dinoflagellate toxins produced by *Gymnodinium*⁹⁾ and *Alexandrium*¹⁰⁾. It

might imply that spiro-imine toxins are widely distributed among marine dinoflagellates.

ACKNOWLEDGMENTS

The authors are grateful to Shouou-ling Huang and Shu-yun Sun for spectral measurements in Instrument Center, National Taiwan University. This work was supported in part by grants (NSC88-2312-B-002-039 and NSC89-2323-B-002-013) from National Science Council, Taiwan.

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