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Similarities of Scaritoxin to Ciguatoxin on the Chromatographic Behaviours

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Scaritoxin과 Ciguatoxin의 크로마토그라피상에서의 몇가지 유사성

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Abstract

In studying the structural work on ciguatoxin, parrot fish collected were identified as *Scarus sordidus, S. frenatus, S. scaber* and *S. pectarlis*, in which only *S. sordidus* contained toxic materials. Crude toxins obtained by silicic acid column chromatography, could be separated on a DEAE-cellulose column into two fractions, ST-1(less polar) and ST-2 (polar) eluted with chloroform and chloroform-methanol(1 : 1).Furthermore ST-1 could be changed into ST-2 by repeated chromatography on DEAE-cellulose. Rf values of ST-1 and ST-2 were 0.60-0.75 and 0.30-0.54 on TLC coated with silica gel 60F-254 developed by chloroform-methanol-water-acetic acid (90 : 9.5 : 0.2 : 0.3) mixture. The peaks of ST-1 and ST-2 were not observed on each HPLC chromatogram at low sensitivity(2X), but by bioassay they were detected in the fraction of 24-27*ml*(less polar toxin, 120*ng*) and 22-27 *ml* (polar toxin, 150 *ng*). Less polar ciguatoxin from morey eel viscera also showed its peak in the same elution volume(25*ml*). Being subjected to chromatography on basic aluminum oxide (activity grade I) or to alkaline treatment, followed by basic aluminum oxide (activity grade I) chromatography ST-1 toxin was remarkably converted into the polar toxic component supposed to be polar ciguatoxin in both cases. In the latter case, approximately 74% of the residual toxicity was changed into the polar component, accompanied by about 50% loss of the initial toxicity. More than 26% of ST-2 toxicity was transformed into the less polar toxic component supposed to be less polar ciguatoxin on a deactivated aluminum oxide (activity grade V) column.

Introduction

According to Bagnis *et al.*[1], the patients intoxicated by parrot fish, *Scarus gibbus*, exhibited two types of symptoms, ciguatera-like syndrome and subsequent symptom caused by the effect in the cerebellum. The first symptoms appearing in a few hours after consumption of the fish, were very close to those induced by ciguatoxin. However, after 5-10 days, the patient progressed into the second phase characterized by failure of static and dynamic equilibrium with marked locomotor ataxia. These disorders remained for a few weeks, and it took usually more than 1 month to recover.

The "scaritoxin" which was said to be responsible for the second phase of symptoms, was isolated from parrot fish by Chunge *et al.*[2,3], and Yasumoto[7] also described that two toxins, of which chromatographic behaviours resembled those of ciguatoxin and "scaritoxin", were present in the viscera of a turban shell.

In a recent paper Yasumoto[9] reported that partially purified toxin (1.1 *mg*; MLD 2.2 *g/kg*) isolated from about 1,000*kg* of toxic fish was separated into two toxic fractions, a "neutral" fraction eluted with chloroform-methanol (9 : 1) and an "acidic" fraction, eluted with methanol-water (1 : 1), by chromatography on basic alumina. The acidic fraction was further separated into two components by HPLC (high performance liquid chromatography) on Bondapak C_{18}, thereby suggesting that ciguatoxin may not be a

single entity, but may in fact comprise multiple toxins.

Nukina[4] revealed that crude ciguatoxin, freshly prepared from morey eel viscera, could be separated into two fractions, less polar ciguatoxin (LPCTX) and polar ciguatoxin (PCTX) by basic aluminum oxide column chromatography, and both fractions were interchangeable with each other.

In studying the structural work on ciguatoxin, it was observed that crude toxins, freshly isolated from flesh and viscera of a parrot fish, *S.sordidus*, could be separated on a DEAE-cellulose (acetate form) column into two fractions, a less polar toxin (ST-1, tentatively) eluted with chloroform and a polar toxin (ST-2, tentatively) eluted with chloroform- methanol (1 : 1), and that ST-1 could be changed into ST-2 by repeated chromatography on DEAE-cellulose. Further study of the chromatographic behaviour of the toxins on aluminum oxide revealed that each of the toxins ST-1 and ST-2 was fractionated into polar (MeOH : H_2O, 1 : 1) and less polar fraction ($CHCl_3$: MeOH, 9:: 1), respectively.

Materials and Methods

Parrot fish collected at Bikenibeu in Kiribati and morey eel from the Johnstone Islands, U.S.A. territory in the Pacific, were kept frozen before use. The parrot fish collected were identified as *Scarus sordidus, S.frenatus, S.scaber, S.pectoralis*, but all species except *S.sordidus* did not contain toxic materials. All solvents were distilled and triple distilled solvents were used for HPLC.

Extraction of Toxins from Fish

The frozen fish were thawed and dressed, and then were cut into flesh and viscera. The flesh was blended to a mush with a little acetone in a Waring Commercial Blendor. It was then transferred to a 4 L erlenmeyer and extracted for 2 days with acetone. The first extract was filtered off and fresh acetone added and the flesh then reextracted for 4 days.

The 1st and 2nd acetone extracts were evaporated to an aqueous suspension and then washed 2 times with equal portions of hexane. The hexane layer was backwashed 3 times with 100*ml* portions of methanol-water (8 : 2,v/v). The aqueous methanol layer was then combined with the aqueous suspension and evaporated. The new aqueous suspension was then extracted 3 times with equal amounts of ethyl acetate.

The viscera of parrot fish (in case of morey eel the viscera were chopped into small pieces) were processed in the same manner as the flesh without blending.

Determination of Toxicity

The toxicities of each extract and fraction in the process of isolation and purification were evaluated by intraperitoneal (i.p.) injection into mice. Each sample was diluted to a known volume with methanol, and portions calculated from the resultant solution were dried under a stream of nitrogen. The 0.5*ml* or 0.1 *ml* of 1% or 5% Tween 80 was added and subjected to a Vortex mixer,followed by i.p.injection into mice (male or female, Swiss Webster, 16-22 *g*). A dilution giving death time less than 3 hours was used for an estimation of the amount of toxin according to a dose-death time relationship curve proposed by Tachibana[6].

Silicic Acid Chromatography

The crude toxic material was dissolved in a small volume of chloroform and applied to a silicic acid column packed with silicic acid (SilicAR, 200-425 mesh, Mallinckrodt Inc., Paris, ky., U.S.A.) (about 28 *g* per 1 *g* sample). The column was eluted successively with chloroform (10 *ml* to 1 *g* silicic acid), chloroform-methanol (9 : 1) mixture (14 *ml* to 1 *g* silicic acid), chloroform-methanol (1:1)mixture (10 *ml* to 1 *g* silicic acid), and methanol (10 *ml* to 1 *g* silicic acid). All the solvents were removed immediately after elution.

DEAE-Cellulose Chromatography

The chloroform-methanol (9 : 1) fraction from silicic acid column was subjected to chromatography on a DEAE-cellulose column packed with Cellex D (acetate form, 0.79 *meq/g*, Bio Rad laboratories, Richmond, CA., U.S.A.) (9.3 *g* to 1 *g* sample) prepared according to the method of Rouser *et al*[5]. The

column with sample was eluted successively with chloroform (60 *ml* to 1 *g* adsorbent), chloroform-methanol (1 : 1) mixture (90 *ml* to 1 *g* adsorbent), and methanol (60 *ml* to 1 *g* adsorbent).

Non-polar coloured guaiazulene was run through the newly prepared column to determine the bed volume and to detect imperfections in the packing. For the test of changeability of ST-1 toxin on DEAE-cellulose (acetate form), the toxin was subjected to rechromatography.

Aluminum Oxide Chromatography

A column of aluminum oxide (activity grade 1, Alumina Woelm, Woelm Pharma, Eschwege, Germany) was prepared by suspending aluminum oxide (100 *g* to 1 *g* sample). Then the toxic fractions eluted from DEAE-cellulose chromatography were applied as a solution of chloroform and eluted with the following solvent systems; chloroform, chloroform-methanol (9 : 1), chloroform-methanol (1 : 1), methanol, and methanol-water (1:1). In this process, 3.75 *ml* of the eluants to 1 *g* aluminum oxide was recommended to be used. Each eluate was concentrated to dryness immediately after elution.

For the study of the interchangeability of the toxic extracts on aluminum oxide, activity grade V column in addition to activity grade 1 column, was prepared by adding water according to the Brockmann scale. Each grade aluminum oxide (10 *g*) was packed in a column (18.5 x 0.7*cm*) and each sample was eluted with the following solvents; chloroform (40 *ml*), chloroform-methanol (36 *ml* + 4*ml*), chloroform-methanol (20*ml*+20 *ml*), methanol (40 *ml*), and ethanol-water (20*ml*+20 *ml*).
The eluates were concentrated soon after elution and then kept in methanol solution.

Sephadex LH-20 Chromatography

To a column (103×1.4 *cm* or 115×2.5 *cm*) of Sephadex LH-20 (Pharmacia, Piscataway, New Jersey, U.S.A.), each toxic fraction from an aluminum oxide column (less polar toxin, 810 *ng* as pure form; polar, 450 *ng* as pure form) was applied and eluted with 600*ml* of chloroform-methanol (2 : 1) solvent. The eluates were monitored at 254 *nm* using an ISCO model UA-5 and were collected 4.9 *ml* every ten minutes in a 10 *ml* test tube.

Thin-Layer Chromatography

Aluminum plates coated with silica gel 60F-254 (thickness, 0.2 *mm*) and glass plates spread with silica gel H (thickness, 1 *mm*) were used for analytical and preparative separations. The plates with sample were developed with chloroform-methanol-water-acetic acid (90 : 9.5 : 0.3 : 0.2). For lethality tests, spots or bands visualized by iodine vapor were scraped off from the plates and extracted with chloroform-methanol (4 : 1) and chloroform-methanol (1 : 1) mixture. Sometimes the scraped-off fractions, including silica gel were homogenized with 1% Tween 80 and then injected into mice.

High Performance Liquid Chromatography

A packed column of LiChrosorb RP-18 (250×4.6 *mm*, partcle size 10 μm, Unimetrics/Knauer, Anaheim, CA., U.S.A.) was used with an HPLC instrument (Waters 6000A, Milford, Mass., U.S.A.) equipped with a Waters model R 401 differential refractometer. The solvent systems used for routine operation were; a) methanol-water (17 : 3) containing ammonium carbonate (0.5 *g/l*) and b) methanol-water (4 : 1). The operational procedure was carried out in the room air-conditioned at 23℃.

Each of the less polar toxin (200 *ng*) and polar toxin (280 *ng*) purified from Sephadex LH-20 columns was forcibly passed by a microsyringe through a reverse-phase Bond Elut C_{18} cartridge (Analytichem, International, Harbor city, CA., U.S.A.) with methanol-water (2 : 1) and methanol, respectively.

The concentrated eluates (less polar toxin, 200 *ng*; polar toxin, 280 *ng*) were applied to a LiChrosorb RP-18 column and eluted with the solvent a) (flow rate, 2.5 *ml*/min) for 75 minutes, and was further purified on the same column using solvent system b) (flow rate, 1.0 *ml*/min.) for 75 minutes. Eluent was collected in a 10 *ml* vial every minute, and was then dried under a stream of nitrogen.

Results

Isolation of Toxic Components by Silicic Acid

Chromatography

The toxicity and yield of each step in the process of isolating the toxic materials from parrot fish, are given in Table 1. Like ciguatoxin, the toxins in the flesh and viscera of parrot fish were eluted with 10% methanol in chloroform from silicic acid column, the toxicity in the viscera (8,769 M.U.) was more stronger than that in flesh (5,839 M.U.). No toxicity was detected in the other fractions.

Purification of Toxins on DEAE- Cellulose Column

The toxic eluates from the flesh and viscera by silicic acid column chromatography were separated into the chloroform (ST-1) and chloroform-methanol (1 : 1) (ST-2) fractions as depicted in Table 1, and the toxin in the viscera also could be fractionated into two toxic fractions by DEAE-cellulose chromatography as shown in Table 1. Yasumoto[8] reported that scaritoxin could be converted from a precursor in the parrot fish diet,because of lack of "scaritoxin" in the liver and gut contents of parrot fish.

The ST-1 toxin from parrot fish viscera was converted into ST-2 toxin by repeated DEAE-cellulose column (Fig.1). Similarly, the toxic extracts of morey eel viscera from silicic column were eluted in the chloroform and chloroform-methanol (1:1) fractions on a DEAE-cellulose column. and the toxins in chloroform could be further separated into the chlo-

Table 1. Toxicity of each fraction from various purification stages of parrot fish toxins

Stage of Purification	Yield(*g*)	LD50(*mg/kg*)	Total toxicity(M. U.)
Flesh(5.337 *kg*)			
Ethyl acetate	8.99		
Silicic acid chromatography(250*g*)			
$CHCl_3$	3.52	non-toxic	
$CHCl_3$—CH_3OH (9:1)	1.53	23.0	3,319
$CHCl_3$—CH_3OH (1:1) — $CHCl_3$—CH_3OH (9:1)	0.63	12.5	2,520
$CHCl_3$—CH_3OH (1:1) — $CHCl_3$—CH_3OH (1:1)	1.13	non-toxic	
CH_3OH	2.20	non-toxic	
DEAE-cellulose chromatography(20*g*)			
$CHCl_3$	2.74	29.0	556
$CHCl_3$—CH_3OH (1:1)	0.52	22.5	1,160
CH_3OH	0.01	non-toxic	
Viscera(0.876 *kg*)			
Ethyl acetate	13.81		
Silicic acid chromatography(385*g*)			
$CHCl_3$	4.46	non-toxic	
$CHCl_3$—CH_3OH (9:1)	5.05	29	8,763
$CHCl_3$—CH_3OH (1:1)	1.48	non-toxic	
CH_3OH	1.58	non-toxic	
DEAE—cellulose chromatography(47*g*)			
$CHCl_3$	2.94	29.2	5,034
$CHCl_3$—CH_3OH (1:1)	1.00	27.8	1,799
CH_3OH	0.08	non-toxic	

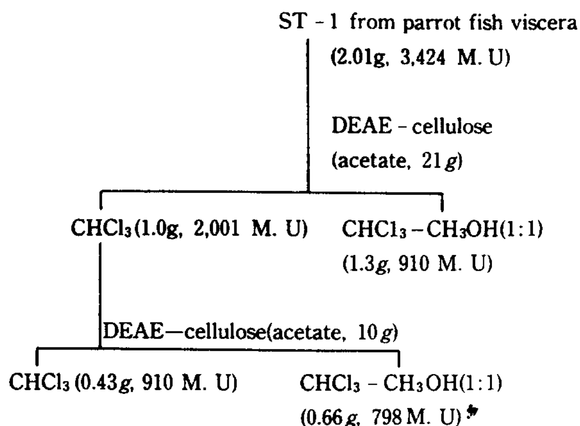

Fig. 1. Chromatographic behaviour of ST-1 toxin on DEAE-cellulose column

roform and chloroform-methanol (1 : 1) fractions by repeated chromatography on DEAE-cellulose (acetate form) (Fig.2).

Chromatographic Behaviour of ST-1 and ST-2 on Aluminum Oxide

When ST-1 fraction of parrot fish flesh was subjected to chromatography on basic aluminum oxide (activity grade 1), or to alkaline treatment (boiled with 1 *N* NaOH in aqueous methanol for 40 minutes), followed by basic aluminum oxide (activity grade 1) chromatography, ST-1 toxin was remarkably converted into the polar toxic comkonent supposed to be PCTX[4] in both cases. In the latter case, approximately 74% of the residual toxicity was converted into the polar component, accompanied by about 50% loss of the initial toxicity (Fig.3).

On the other hand, when ST-2 fraction was subjected to chromatography on a deactivated aluminum oxide (activity grade V) column, more than 26% of ST-2 toxicity was fransformed into the less polar toxic component supposed to be LPCTX.[4]

Sephadex LH-20 Chromatography

The less polar and polar toxins separated from the ST-1 toxin of parrot fish flesh by chromatography on basic aluminum oxide (activity grade 1), were applied to Sephadex LH-20 columns for the further purification. They were eluted in the 88-110 *ml* (200 *ng*) and in the 86-108 *ml* (280 *ng*) fractions, respectively.

Thin-Layer Chromatography of ST-1 and ST-2

ST-1 and ST-2 toxins from parrot fish flesh, and PCTX, gave Rf values on silica gel as shown in Table 2. ST-1 was developed much faster than ST-2, and

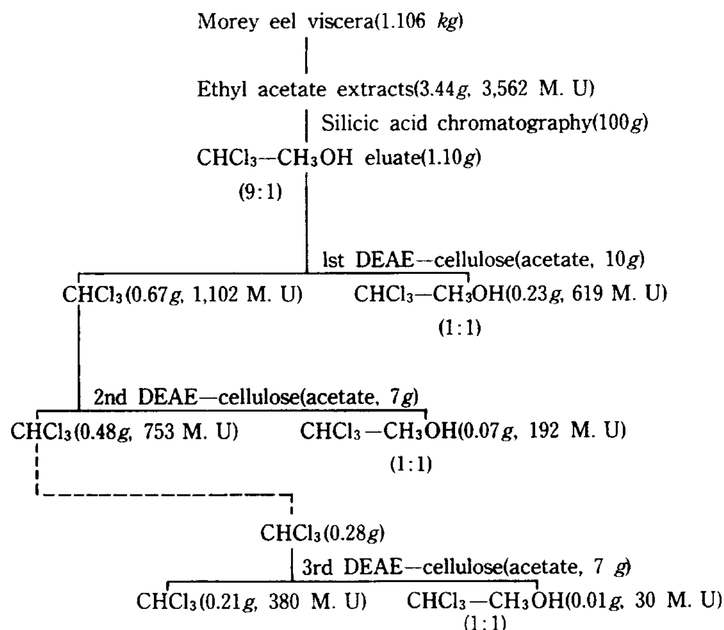

Fig. 2 Chromatographic behaviours of toxins from morey eel viscera on repeated DEAE-cellulose columns

Table 2. Rf values of parrot fish toxins(ST-1, ST-2) and PCTX

Solvent	ST-1	ST-2	PCTX	Scaritoxin[2,3]
$CHCl_3$—CH_3OH—H_2O—AcOH (90 : 9.5 : 0.2 : 0.3)	0.60~0.75	0.30~0.54	0.28~0.54	0.78~0.92

Column charge (10*g* for each colum)	ST-1 (1.10μg)					ST-1* (1.13μg)					ST-2 (1.20μg)				
Activity	I					I					V				
Eluates	A	B**	C	D	E	A	B	C	D	E	A	B	C	D	E
Amounts of toxin Recovered		0.2 μg	0.15 μg		0.55 μg		0.12 μg			0.34 μg		0.21 μg			0.80 μg

Fig. 3. Transformation of ST—1 and ST—2 toxins isolated from parrot fish flesh by aluminum oxide chromatography

*treated with 1 *N* NaOH in aqueous methanol

**A; Chloroform, B; Chloroform-methanol(9:1), C; Chloroform-methanol (1:1), D; Methanol, E; Methanol-water(1:1)

showed Rf value slightly different from that of "scaritoxin"[2] as shown in Table 2, but it can be explained by difference in the crude toxic materials applied to and procedures employed for thin-layer chromatography.

High Performance Liquid Chromatography

The peaks of the less polar and polar toxins could not be observed on each chromatogram at the low sensitivity (2X), but by bioassay they were checked to be present in the fraction of 24-27 *ml* (less polar toxin, 120 *ng*) and 22-27 *ml* (polar toxin, 150 *ng*).

LPCTX from morey eel viscera also showed its peak in the same elution volume (25*ml*)(Fig.4)

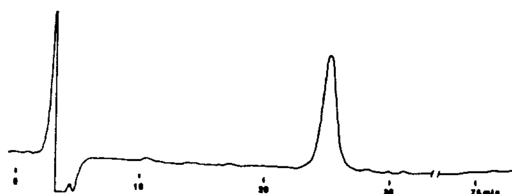

Fig. 4. HPLC chromatogram of LPCTX from morey eel viscera

Column: LiChrosorb RP-18(250×4.6*mm*, particle size 10 μ*m*, Unimetrics/Knauer), conditioned at 23℃
Sample amounts injected: 12.7μg of relatively pure LPCTX dissoved in 10 μ*l* methanol.
Solvent: Methanol/Water(4/1, v/v), flow rate: 1*ml*/min.
Detector: Differential refractometer(Waters model R-401), attenuation: 2X.

Discussion

ST-1 toxin isolated from a parrot fish, *S.sordidus*, on DEAE-cellulose column which was introduced for separation of "scaritoxin"[2,3,8] can be changed into ST-2 toxin by repeated DEAE-cellulose chromatography. This result also had been observed in the process of purification of "scaritoxin" from a parrot fish, *S. gibbus*.[2,3] It is further worth nothing that ciguatoxin freshly isolated from morey eel, *Gymonothorax javanicus*, in which "scaritoxin" has not been detected, also can be separated into two fractions, i.e. chloroform and chloroform-methanol (1:1) on a DEAE-cellulose column.

The purified ST-1 toxin can be further separated into two fractions, chloroform-methanol (9:1) and methanol-water (1:1), on a basic aluminum oxide column, and interestingly, the toxins in both fractions are interchangeable with each other on a basic aluminum oxide column. This finding is reminiscent to that of Nukina[4] who had isolated two interchangeable forms of ciguatoxin freshly prepared from morey eel, which were eluted in chloroform-methanol

(9 : 1) and methanol-water (1 : 1), by using a basic aluminum oxide column.

Attempts to get more toxic materials during this period were not made, because of the difficulty in obtaining parrot fish which had accumulated much toxin in their tissues.

Judging from its chromatographic behaviours, ST-1 toxin corresponding to "scaritoxin" seems to be one type of ciguatoxin.

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요 약

Ciguatoxin 구조 연구의 일환으로 파랑비늘돔 중에서 독성분을 추출하였는데 *Scarus sordidus*만 근육과 내장에 독이 있었으나, *S.frenatus*, *S.scaber*, *S.pectoralis*에는 모두 독이 없었다. 실리스산컬럼에서 얻은 조독을 DEAE-셀루로즈 컬럼상에서 클로로포름, 클로로포름-메타놀 (1 : 1) 및 메타놀로 전개하였더니 클로로포름 유출액 (ST-1) 과 클로로포름-메타놀 (1 : 1) 유출액에서 독성분이 발견되었고, 메타놀 유출액에서는 독성분이 검출되지 않았다. ST-1과 ST-2을 silica gel 60 F-254 에 spot하여 클로로포름-메타놀-물-식초산 (90 : 9.5 : 0.2 : 0.3)으로 전개하였더니, 그Rf 값이 각각 0.60-0.75, 0.30-0.54 였다. DEAE-셀루로즈 컬럼에서 얻은 ST-1을 재재크로마토그라피 하여도 역시 ST-1과 ST-2로 나누어졌다. 이렇게 얻은 ST-1과 ST-2는 세파덱스 LH-20에 흡착시켜, 클로로포름-메타놀 (1 : 1) 용매로 전개할 때, 머무름시간이 모두 같았고, 역상 LiChrosorb RP-18를 이용한 HPLC상에서도 머무름시간이 동일하였다. 또 순수한 ST-1 (1.10 μg) 그리고 이것을 1N NaOH로 처리한 것 (1.13 μg) 을 활성도V인 알루미나 컬럼에 흡착시켜 클로로포름-메타놀 혼합액으로 전개하였더니 알카리처리를 하지 않는 경우는 0.35 μg가 클로로포름-메타놀의 9 : 1, 1 : 1 유출액에서, 0.55 μg가 메타놀-물 (1 : 1) 유출액에서 검출되었으나, 알카리처리를 한 경우는 처음 독성의 50%가 소실되어, 클로로포름-메타놀 (9 : 1) 유출액에서 0.12 μg, 메타놀-물 (1 : 1) 유출액에서 0.34 μg 검출되었다. 또 ST-2 (1.2 μg) 을 활성도V인 알루미나 컬럼에서 전개하였더니 클로로포름-메타놀 (9 : 1) 유출액에서 0.21 μg, 메타놀-물 (1 : 1) 유출액에서 0.80 μg 검출되었다. 하면 morey eel내장에서 얻은 독물질도 DEAE-셀루로즈에서 ST-1과 ST-2로 나누어지며, 이 ST-1의 TLC, HPLC 및 알루미나 컬럼상의 거동이 파랑비늘돔에서 얻은 ST-1의 그것과 같으므로 scaritoxin으로 보고한 ST-1은 ciguatoxin의 형태인 less polar ciguatoxin (LPCTX) 으로 생각된다.

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