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TOXICITY STUDIES ON PUFFERS FROM TROPICAL WATERS(南方産フグの毒性に関する研究)

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論 文 内 容 要 旨

TOXICITY STUDIES ON PUFFERS FROM TROPICAL WATERS

INTRODUCTION

Tetrodotoxin (TTX) has attracted the interests of so many researchers for a long time as have the only few natural products. It is one of the most toxic nonprotein substances having an unique chemical structure and specific physiological properties. The toxin is quite important for public health, as it causes one of the most violent forms of intoxication. From economic stand points, the toxin is also important in Japan, where puffers are highly esteemed and widely consumed.

Puffers are also eaten, though implicitly, in many southern seas, and occasional toxicity incidences have been reported without sufficient proof on the presence of tetrodotoxin. It is urgently needed, therefore, to conduct toxicity studies on southern species for prevention of intoxication. Because some of the tropical species dwell in southern part of Japan but remain unelucidated with regard to their toxicity, determining the toxicity level of these fish and dissemination of the information to the public are necessary.

In the present investigation, we tested, first, toxicity levels of the flesh to provide the criteria for the acceptability of the fish as foods. Distribution of toxins among other tissues and organs was also examined, because contamination of flesh by other toxic parts or mistakenly ingested liver or gonads occasionally cause intoxication. Toxin distribution in the body is also interesting to infer the biological role of the toxin in fish. Secondly, toxin composition was investigated by comparing HPLC analyses with mouse bioassays. Close and careful examination of HPLC chromatograms enable us to detect novel components. They were later isolated and structures determined. Further efforts to elucidate the discrepancy observed between HPLC data and bioassay date led to detection of saxitoxin and other unidentified toxins.

I. TOXICITY STUDIES OF PUFFERS

1) Toxicity of Okinawan puffers

A total of 66 specimens comprising 9 species listed in Table 1 were collected in 1988-1989. The following four species have not been reported previously with regard to the toxicity: *Arothron mappa*, *A. manillensis*; *A. nigropunctatus*, and *A. stellatus*. Lethal potencies of the muscle, skin, liver, gonad and intestine were determined by an official mouse bioassay method and expressed in mouse units (MU).

Toxicities of the flesh (Fig. 1) indicate that six species should be rejected from markets. Extremely high toxicity of the flesh of *Chelonodon patoca* is worth of special mentioning. Three species, *A. mappa*, *A. stellatus*, and *Fugu niphobles* were weakly toxic but await further examination on increased number of specimens. The *Diodon* was nontoxic in agreement with its wide consumption as foods.

The results on the skin (Fig. 2) and visceral part (Fig. 3) indicate that they should not be eaten. The high toxicity of the skin was the characteristic feature of Okinawan specimens.

2) Toxicity of puffers from tropical countries

A total of 67 specimens comprising 9 species of puffers (Table 2) collected at Negros Island of the Philippine, Saipan of the Federation of North Mariana, Pingelap Island of Federated States of Micronesia, Tahiti Island of French Polynesia, Bay of Bengal of India and from Mexico were investigated.

Irrespective of locality, tissues of *A. nigropunctatus* was highly toxic followed by *Canthigaster margaritatus* from Saipan. *A. meleagris* from Saipan was moderately toxic which was nontoxic from Tahiti provides typical example of regional variation (Table 3) *C. patoca*, *A. stellatus*, and *Lagocephalus lunaris* showed weak toxicity and *A. mappa* was nontoxic (Fig. 4-6). Further studies are necessary to draw a conclusion, as the tests were run on one specimen each except *L. lunaris*. *Diodon* showed no

significant toxicity, as was observed with Okinawan specimens. Musculature of *Sphaeroides annulatus* was also not toxic (Fig. 4) but remarks on safety relies on place of catch. In most of specimens the highest toxicity was detected in skin (Fig. 5) rather than gonad (Fig. 6).

3) Toxicity of puffers from Tohoku

To find the exogenous source of toxins and toxicosis in the visceral organs of puffers, fresh specimens from Tohoku area were examined (Table 4). Interestingly in one of the *L. wheeleri* specimen only gut-content was 40 MU/g, while other tested tissues were nontoxic in all other specimens. The toxicity of intestine were higher than stomach but liver toxicity was the highest in most of toxic species.

II. CHEMICAL ANALYSIS OF PUFFER TOXINS

By the HPLC analyzer extracts used for bioassay were analyzed first, and then the same extracts treated with Amberlite CG 50 (NH_4^+ form), were analyzed at the improved condition of the analyzer. The discrepancies of toxicity between bioassay and HPLC both on crude and semipure sample (Fig. 7) were remarkable.

1) Toxins profiles in different tissues

The toxin profiles of different tissues for all tested species were recorded. Typical result is shown in Table 5. Presence of large amount of 11-deoxyTTX and 6-*epi*TTX and substantial amount of 11-oxoTTX in tissues of tropical puffers was remarkable. Profile mainly varies from species to species but not among tissues.

2) Toxins recovery on different columns

Mainly tissues like skin, liver and muscles which showed

large discrepancies by TTX analyzer were charged on different column conditions (Table 6). The recovery of toxins were recorded and compared with HPLC data. Poor recoveries by bioassay from tropical samples mainly on charcoal column and increase of HPLC toxicity indicate loss of toxin other than TTX. The toxicity recovery from the samples of Tohoku was relatively good. This supports the prediction about the presence of other toxins along with TTX in tropical puffers.

III. ISOLATION, PURIFICATION AND STRUCTURE DETERMINATION

1) Identification and isolation of 6-*epi*TTX and 11-deoxyTTX

From liver of *Fugu niphobles* (4.2 kg) from Tohoku, isolations of 6-*epi*TTX and 11-deoxyTTX were successful (Fig. 8). These were confirmed by HPLC, TLC and ^{1}H NMR spectra with those of reference toxins isolated from newts. These were the first reports from puffers after newts.

2) Isolation and structure determination of 11-*nor*TTX-6(*R*)-ol

From the same extract stated above isolation, purification of novel toxin (1 mg) eluted in between 6-*epi*TTX and 11-deoxyTTX was successful (Fig. 8). The SIMS suggested $C_{10}H_{15}N_3O_7$ ($[M+H]^+$ *m/z* 290) as the probable formula. ^{1}H NMR, ^{1}H-^{1}H COSY, NOESY (Fig. 9) and NOE spectra confirmed the novel toxin as 11-*nor*TTX-6(*R*)-ol. It is 1/7th toxic of TTX (70 μg/kg).

3) Isolation and structure determination of 11-oxoTTX

Micronesian puffer *A. nigropunctatus* (1.2 kg) which showed the highest discrepancies between bioassay and HPLC toxicity was used for separation, and successfully a new toxin (3 mg) eluted before TTX was isolated (Fig. 10). High resolution FAB-MS suggested $C_{11}H_{17}N_3O_9$ ($[M+H]^+$ *m/z* 336) as the probable formula. The structure was established basing on the ^{1}H NMR, ^{1}H-^{1}H COSY and

NOE (Fig. 11) spectra and named as 11-oxoTTX. This was further confirmed by reduction with $NaBH_3CN$ (Fig. 12). Its toxicity is 1/10th of TTX (120 μg/kg). From the original extract other three unknown compounds were isolated and measured the ^1H NMR spectra but further studies on their structures were impossible due to paucity of the samples.

4) Isolation and purification of saxitoxin

The ovary of *A. mappa* from Okinawa showed 50% discrepancies in between HPLC and Bioassay toxicity. Attempt was made to isolate the toxin which does not give any peak by TTX analyzer (Fig. 13). In the strongly basic fraction from an ion exchange column, saxitoxin (STX) was detected by paralytic shellfish toxin analyzer (Fig. 14). Evaluation of STX in the original extract explained only 33.3% of its toxicity, indicating the presence of other toxic substance(s).

SUMMARY

1. This work covers about 40% of tropical and 35% of Japanese puffers comprising first report for 4 species of Japan. The virulence varies with species, specimens and tissues.

2. Much significant extent of toxicity was observed in skin and muscle from tropical species than that of temperate species.

3. Diodon and Gulf-puffer may be considered for consumption from the localities covered in this work.

4. Toxicity discrepancies on the tissues of tropical puffers between bioassay and HPLC revealed the presence of large amount of toxins other than tetrodotoxin. Part of the discrepancies (33.3%) was explained by saxitoxin and its analogs.

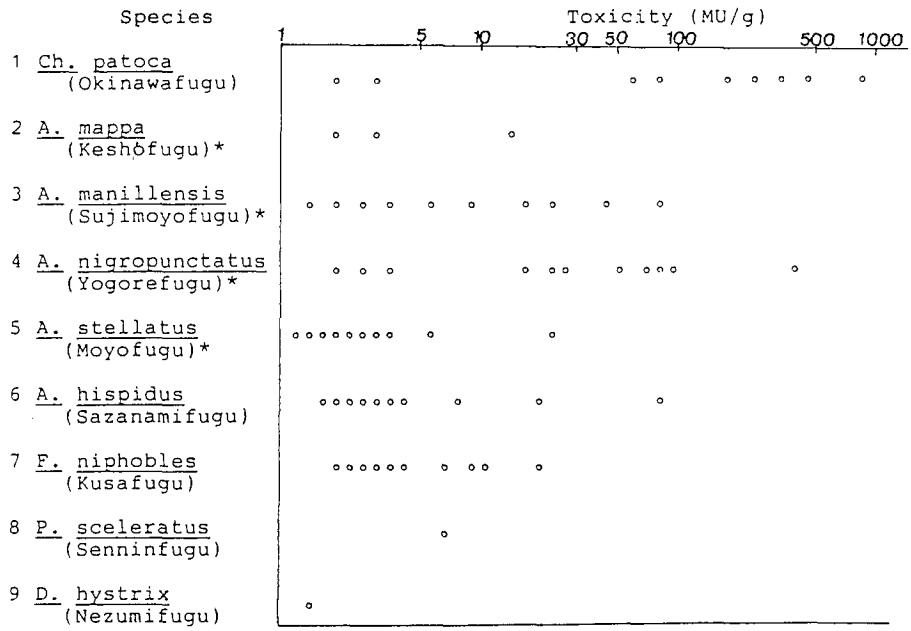
5. Toxins profiles among tissues of same species do not vary significantly but do vary among the species.

6. Findings of 11-*nor*-tetrodotoxin-6(R)-ol, 11-oxotetrodotoxin and identification of 11-deoxytetrodotoxin and 6-*epi*-tetrodotoxin explain probable biosynthetic and metabolic pathway of tetrodotoxin in puffers (Fig. 15).

Table 1. LIST OF PUFFERS EXAMINED FROM OKINAWA

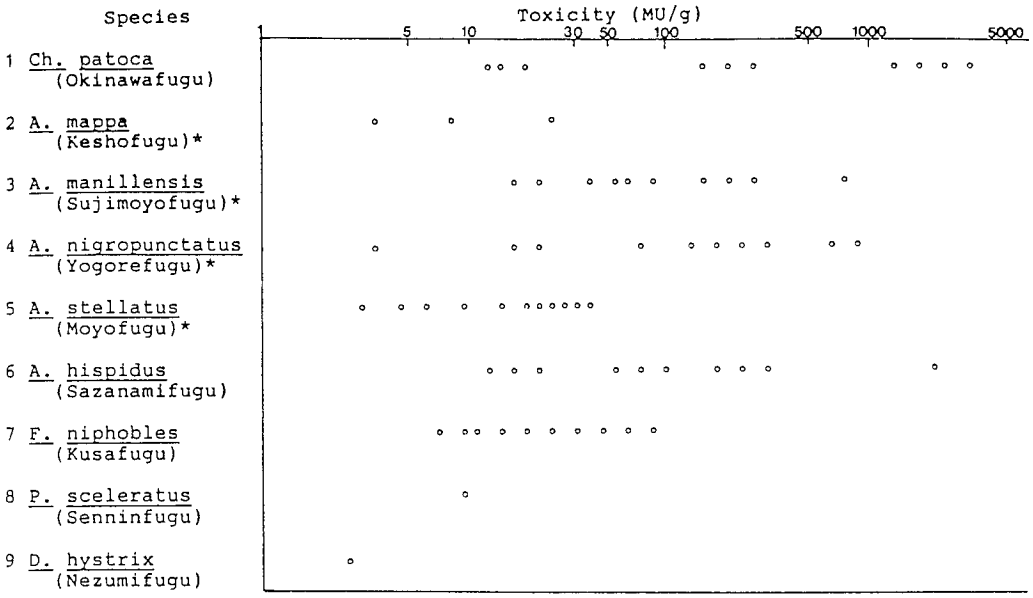
Name	Japanese Name	No. Specimen
Chelonodon patoca	Okinawafugu	10
Arothron mappa	Keshofugu*	3
A. manillensis	Sujimoyofugu*	10
A. nigropunctatus	Yogorefugu*	10
A. stellatus	Moyofugu*	10
A. hispidus	Sazanamifugu	10
Fugu niphobles	Kusafugu	10
Pleuranacanthus sceleratus	Senninfugu	1
Diodon hystrix	Nezumifugu	1

* First report.



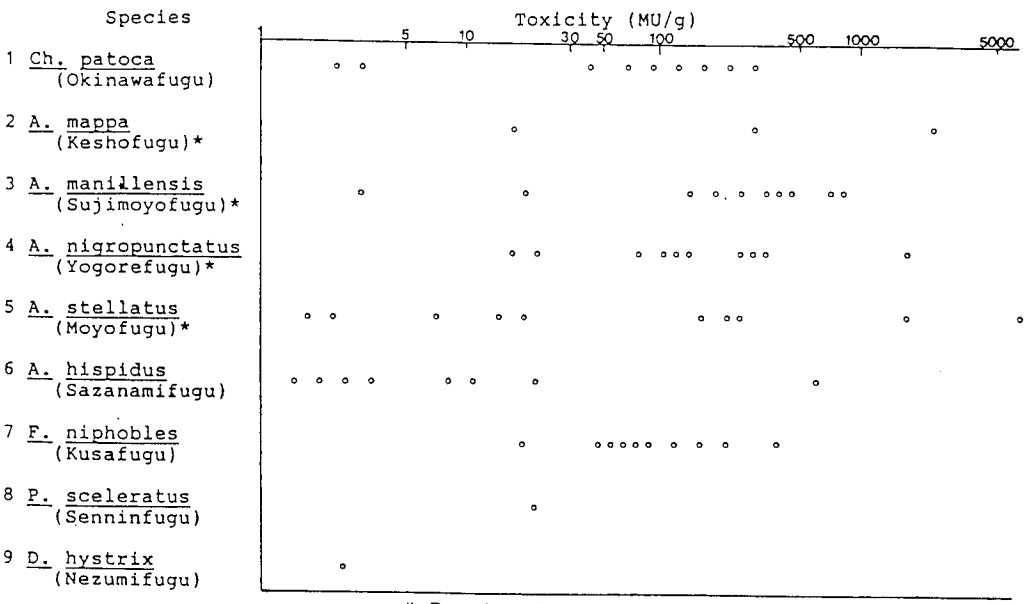
*Previously unreported species.

Fig. 1. TOXICITY IN MUSCLE OF OKINAWAN PUFFERS



* Previously unreported species

Fig. 2. TOXICITY IN SKIN OF OKINAWAN PUFFERS



* Previously unreported species.

Fig. 3. TOXICITY IN GONAD OF OKINAWAN PUFFERS

Table 2. LIST OF PUFFERS EXAMINED FROM TROPICAL COUNTRIES

Name	Japanese Name	Locality**
Chelonodon patoca	Okinawafugu	Philippine (1)
Canthigaster margaritatus	-	Saipan (12)
Arothron mappa	Keshofugu*	Pingelap (1)
A. meleagris	Mizorefugu*	Saipan (7)
〃	〃	Tahiti (4)
A. nigropunctatus	Yogorefugu*	Philippine (2)
〃	〃	Saipan (6)
〃	〃	Pingelap (1)
A. stellatus	Moyofugu*	India (1)
Lagocephalus lunaris	Dokusabafugu	India (12)
Sphaeroides annulatus	-	Mexico (13)
Diodon hystrix	Nezumifugu	Saipan (1)
〃	〃	Tahiti (6)

* First report. ** ( ): Number of specimens examined.

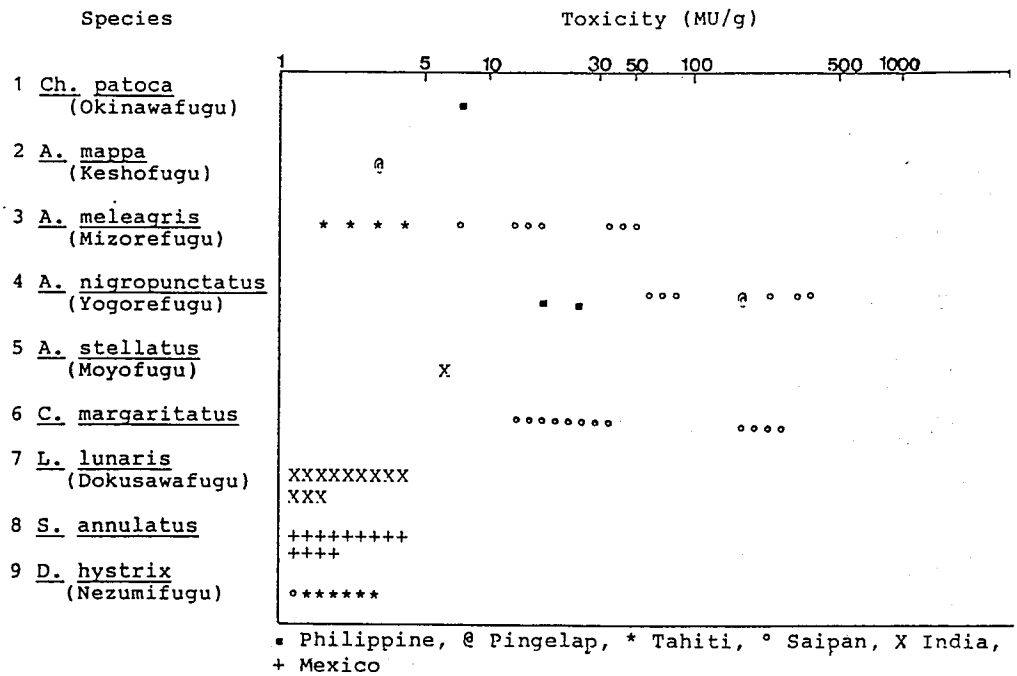


Fig. 4. TOXICITY IN MUSCLES OF PUFFERS FROM TROPICAL COUNTRIES

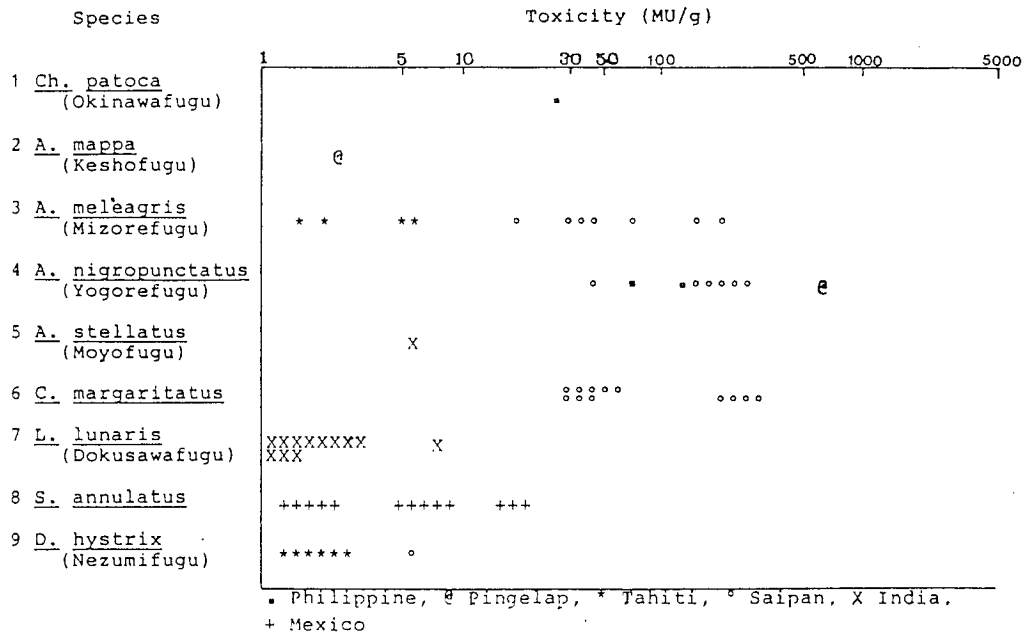


Fig. 5. TOXICITY IN SKINS OF PUFFERS FROM TROPICAL COUNTRIES

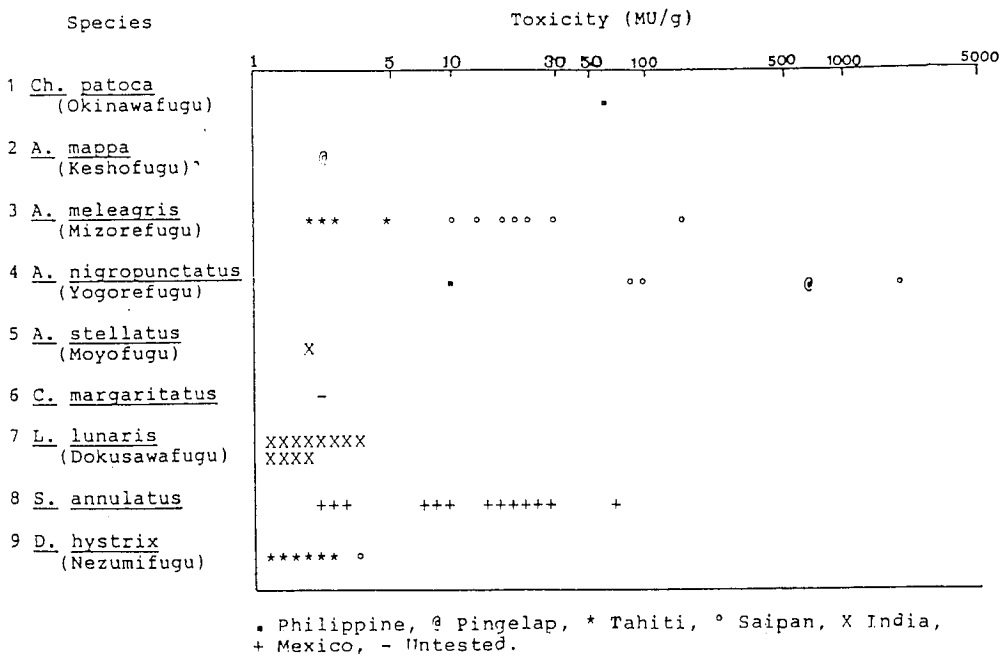


Fig. 6. TOXICITY IN GONAD OF PUFFERS FROM TROPICAL COUNTRIES

Table 3. REGIONAL VARIATIONS OF TOXICITY*

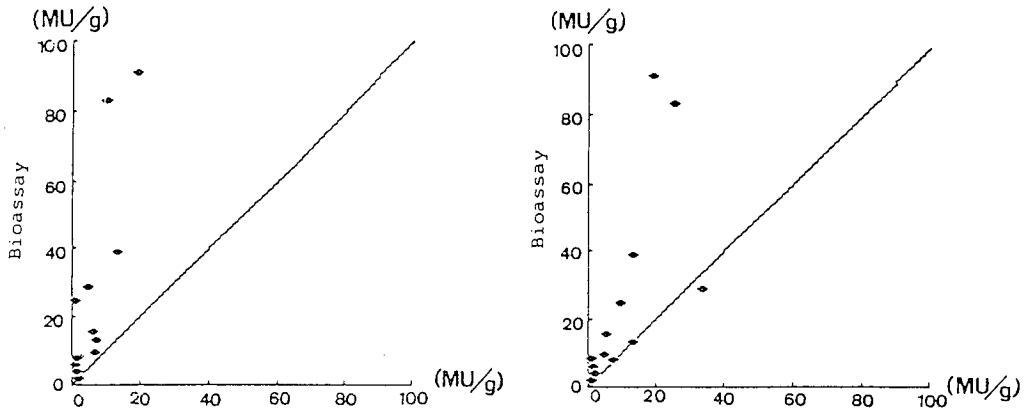
Tissues	Average Toxicity (MU/g)	
	Saipan(7)**	Tahiiti(4)**
Muscle	24.8	ND
Skin	63.0	2.7
Liver	20.4	ND
Gonad	38.4	ND
Intestine	24.7	ND

* Bioassay toxicity of *Arothron meleagris*
**Number of specimens tested. ND: not detected.

Table 4. TOXICITY TESTS ON FRESH VISCERA OF PUFFERS FROM TOHOKU

No.	Sex	T.Wt. (g)	T.L. (cm)	Toxicity (MU/g) Liver	Stomach	Intestine	Gut-content
Fugu pardalis							
1	M	54.0	14.5	1920	700	1435	70
2	M	43.4	13.0	90	230	ND	8
3	F	33.0	12.0	210	70	750	20
Lagocephalus wheeleri							
1	F	73.0	16.0	ND	ND	ND	ND
2	M	52.1	14.0	ND	ND	ND	ND
3	F	46.2	14.0	ND	ND	ND	40
Fugu poecilonotus							
1	F	228.9	22.5	5900	2610	3910	
2	F	200.7	20.6	330	160	16*	
3	F	184.8	20.0	6930	920	230*	
4	F	167.7	18.5	4595	290	630*	
5	M	151.0	21.5	140	40	6*	
6	M	148.1	20.0	5100	355	2100*	
7	F	167.0	20.0	380	-	200*	

T.Wt.: total weight, T.L.: total length,
F: female, M: male, (-): not tested,
*Including gut-content



HPLC on crude sample

HPLC on semipure sample

Fig. 7. HPLC ANALYSES OF TROPICAL PUFFERS

Table 5. TOXINS PROFILES ON TISSUES OF TROPICAL PUFFER*

	Muscle	Skin	Liver	Gonad	Intestine
TTX	62.5	60.9	58.4	40.4	61.7
11-oxoTTX	3.7	4.3	2.6	1.7	2.6
6-epiTTX	12.8	15.8	11.6	11.1	10.8
4-epiTTX	2.4	2.5	2.0	3.6	2.0
4,9-anhydroTTX	9.6	10.9	10.4	15.9	11.3
11-deoxyTTX	8.8	5.3	14.7	27.0	11.4
HPLC (MU/g)	25.2	121.8	28.5	44.1	30.7
Bioassay (MU/g)	48.0	445.0	77.0	195.0	66.0

* Compositions in mole %

Sample: *Arothron nigropunctatus* from Okinawa

Table 6. TOXICITY RECOVERY ON DIFFERENT COLUMNS

Species/ Locality/ (Tissue)	Column	Initial Bioassay(MU)	Initial HPLC(MU)	Recovery Bioassay(MU)	(%)	Recovery HPLC(MU)	(%)
A. nigropunctatus Micronesian							
(Liver)	Charcoal	430	178	275	(64)	225	(126)
"	"	330	139	225	(68)	140	(100)
(Muscle)	"	1680	530	1089	(65)	512	(96)
(Whole body)	"	140,600	21009	91070	(65)	19924	(94)
A. nigropunctatus Okinawa							
(Skin)	"	1232	250	800	(65)	375	(150)
"	"	8327	4089	6700	(80)	6181	(151)
"	Biogel P-2	195	31	75	(38)	25	(80)
"	"	75	42	46	(61)	42	(100)
A. hispidus Okinawa							
(Skin)	Charcoal	480	375	425	(88)	350	(93)
A.manillensis Okinawa							
(Skin)	Charcoal	1367	530	1347	98	630	118
	Biogel P-2	1585	600	1372	86	960	160
Fugu niphobles Tohoku							
(Liver)	BioRex 70	145674	89245	135558	93	82477	92

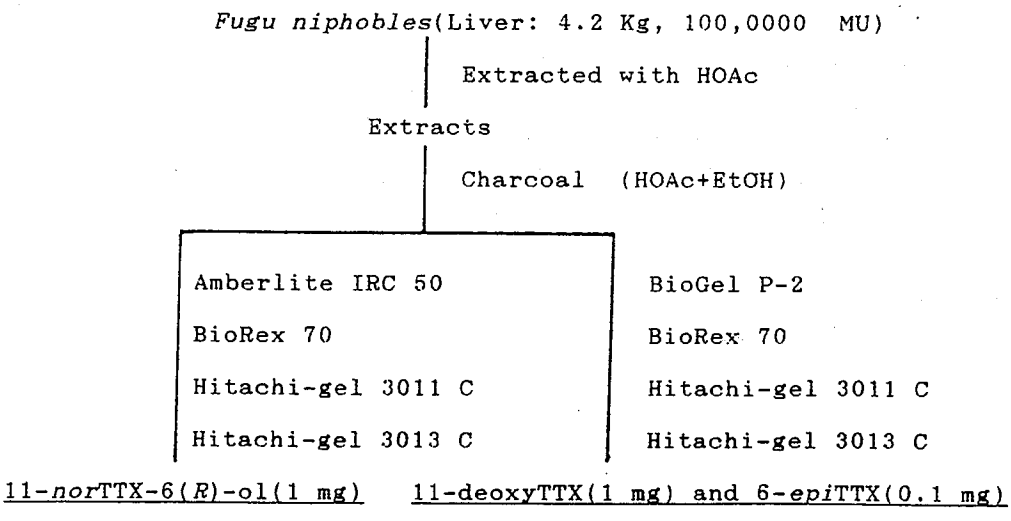


Fig. 8. ISOLATION OF 11-*nor*TTX-6(*R*)-ol, 11-deoxyTTX and 6-*epi*TTX

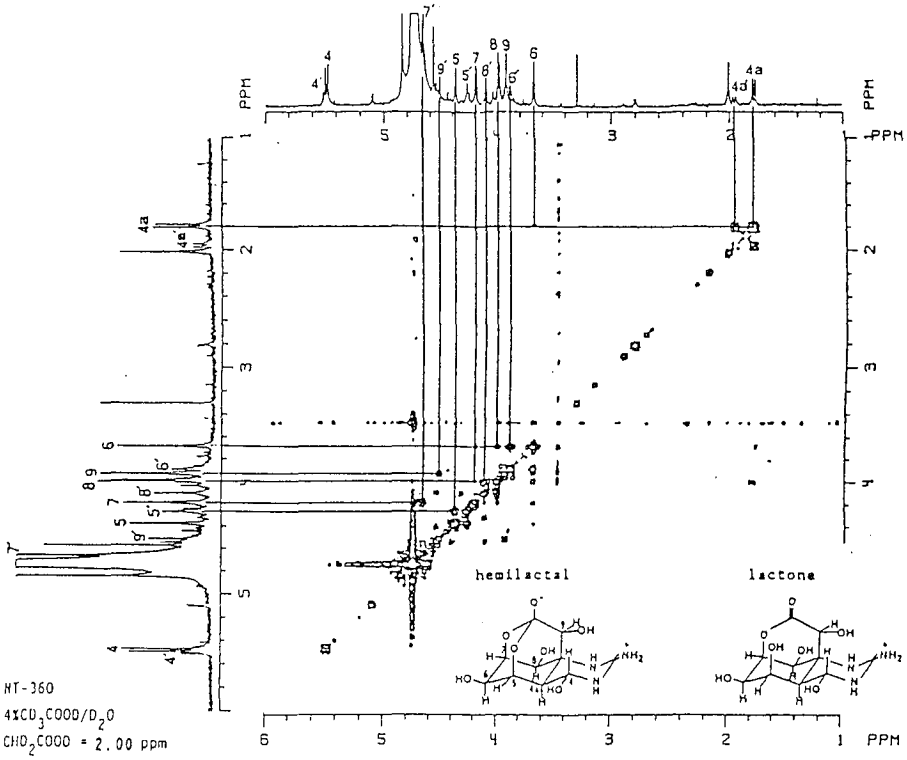


Fig. 9. NOESY SPECTRUM OF 11-*NOR*TETRODOTOXIN-6-(*R*)-OL

Arothron nigropunctatus (1.2 Kg, 23,9600 MU)

Extracted with HOAc

Extracts

Charcoal (HOAc+EtOH)

BioGel P-2

BioRex 70

Hitachi-gel 3011 C	Hitachi-gel 3011 C
TSK-Gel 1000	Hitachi-gel 3013 C
Unknown 1-3*	TSK Gel 1000

11-oxoTTX (3 mg)

Fig.10. SCHEMATIC DIAGRAMS ON ISOLATION OF 11-oxoTTX AND OTHERS

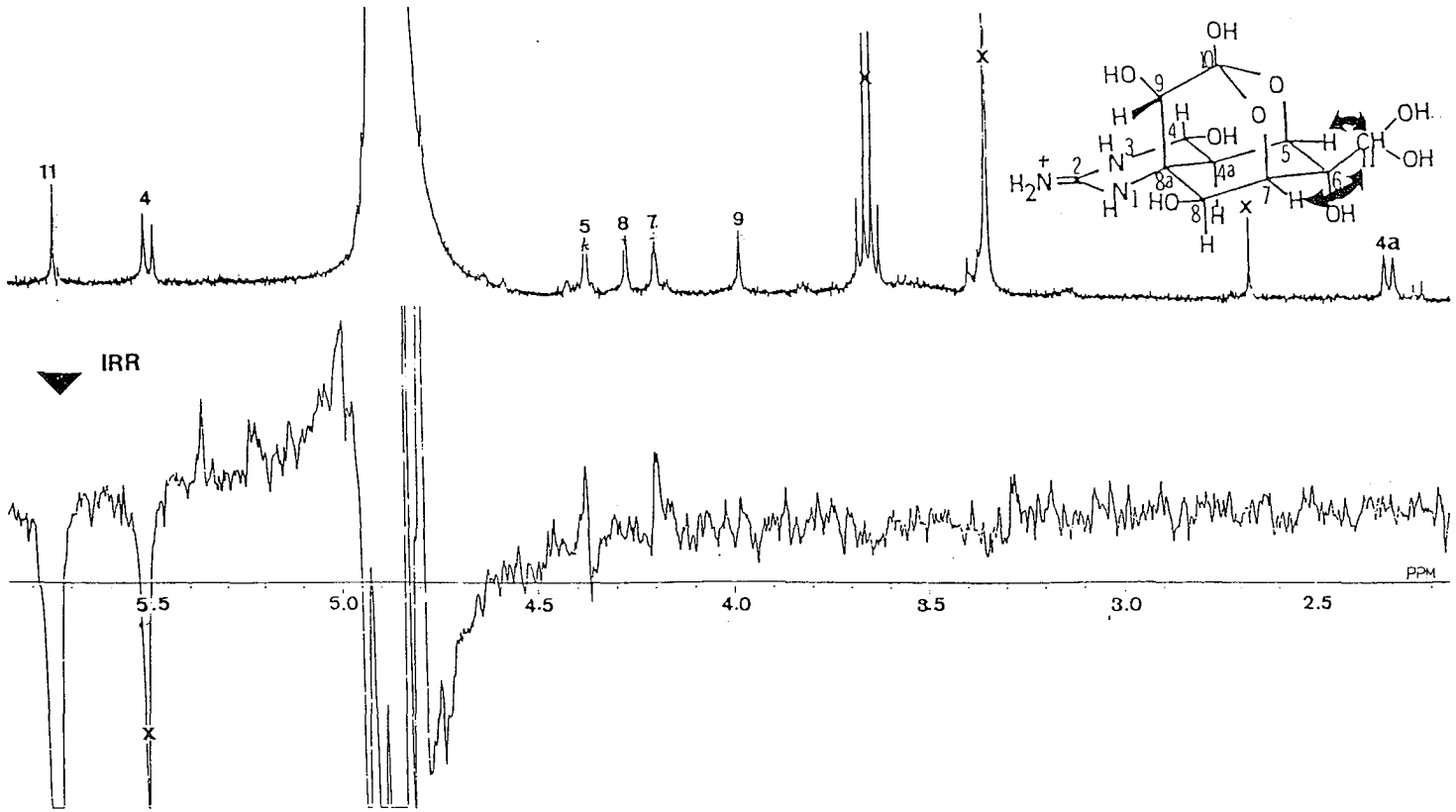


Fig. 11. NOE DIFFERNCE SPECTRUM OF 11-oxoTTX
(400 MHz, 4% CD_3COOD/D_2O, CHD_2COOD as 2.06 ppm)

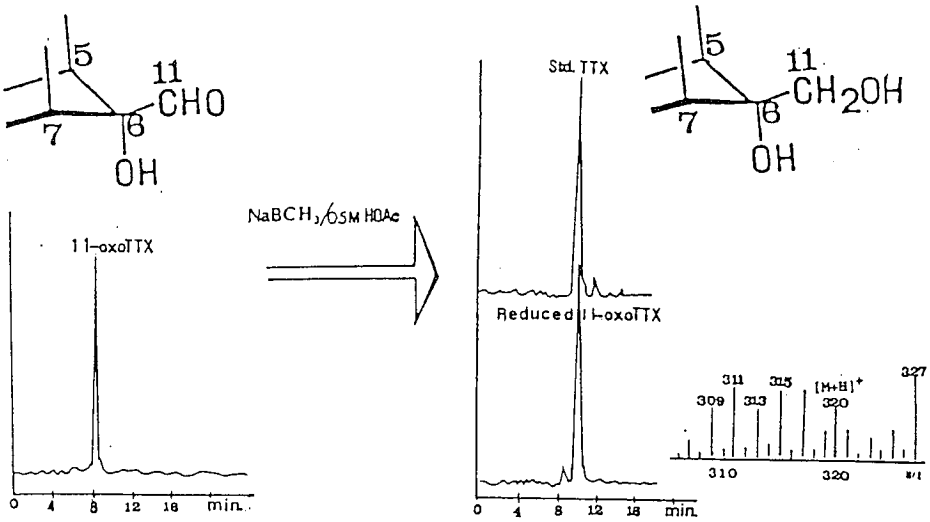


Fig. 12 . REDUCTION OF 11-oxoTTX

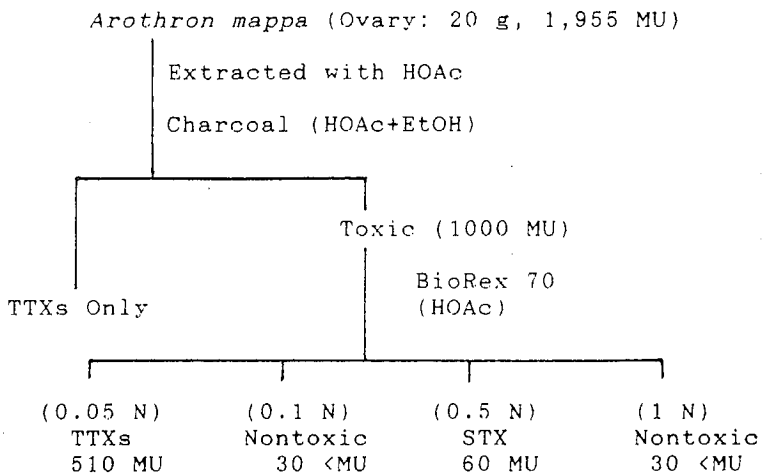


Fig. 13. ISOLATION SCHEME OF STX FROM TROPICAL PUFFER

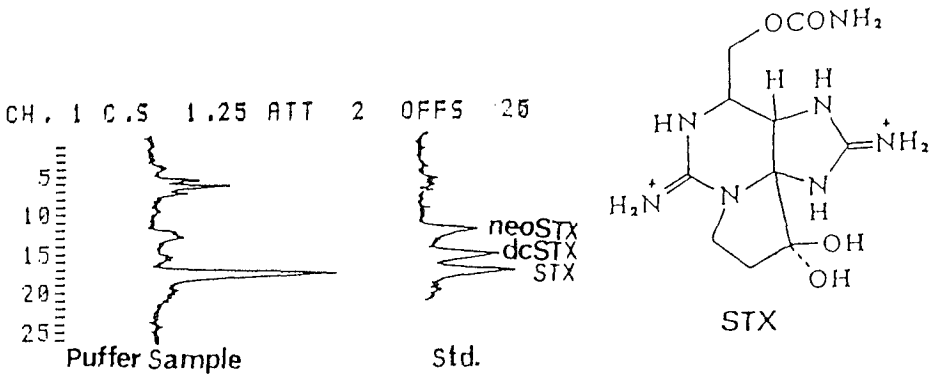


Fig. 14. CHROMATOGRAMS OF SAXITOXIN FROM PUFFER

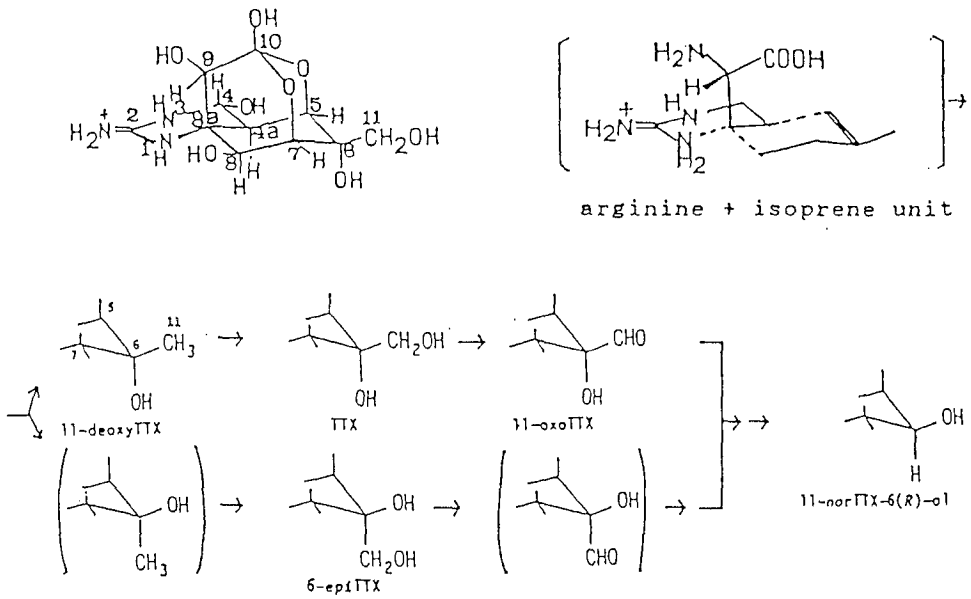


Fig. 15. PROBABLE BIOSYNTHETIC AND METABOLIC PATHWAY OF TTX
(Structure in parentheses are not found)

審査結果の要旨

わが国ではフグは美味な高級魚として広く賞味されている。しかしながら種類や組織によっては猛毒テトロドトキシン（TTX）が高濃度で存在するため，高死亡率の食中毒の原因となる。わが国で発生する食中毒死亡率事例の大半はフグ中毒が原因であり，フグの毒性監視は食品衛生上極めて重要な課題である。近年，フグの需要が著しく拡大したため，従来は食用の対象とされなかった種類や，外国産の種類が消費の対象とされつつある。このような新たな食用対象種についての毒性調査は十分になされていず，特に，南日本沿岸種には毒性不明種が多く，早急な調査が急務とされていた。

本研究では，まず，沖縄県及び太平洋熱帯域の６カ国から入手した９種65検体のフグの筋肉，皮，肝臓，生殖巣，腸管の毒性を測定し，食用種として適否について資料を作製した。また，本州沿岸種と南方種では毒の組織別分布に顕著な差があり，前者では通常肝臓が高い毒性を示すのに対して後者では大部分の種類で皮，もしくは，卵巣が最高の毒性を有し，肝臓の毒性は極めて低いことを明らかにした。これらの知見は調査・加工の際に高毒部位による汚染や混入の排除に役立つ。

次に，南方産の毒組成を精査し，TTX以外に６－エピTTX，11－デオキシTTX，11－オキソTTX，11－ノルTTX－６(R)－オールを単離し，各種スペクトル測定で構造を決定した。前２成分をフグから同定したのは初めてであり，後２成分は天然物としては初めて構造が決定された成分である。これらの成分の構造からTTXの生合成，及び，代謝経路について重要な情報が得られた。

高速液体クロマトグラフィーを用いて，TTX関連成分の微量迅速分析条件を設定し，得られた値とマウス毒性とを対比したところ，顕著な差異が認められた。このことよりTTX関連化合物以外の有毒成分の存在を推定し，細心な分析によりサキシトキシンが全毒量の20％も占める事を発見した。プランクトン由来の貝毒成分として知られる本成分がフグ中に高含量で存在することは極めて特異である。

以上のように本論文は本邦南岸種，及び，南方産フグの食用種としての適否を明らかにしたばかりでなく，毒の特異な分布と組成の解明，新成分の構造決定を行っており，その成果は農学博士の授与に値すると判断された。