Pahutoxin: A Fish Poison

Structure, activity, and synthesis of a metabolite isolated from the Hawaiian boxfish is described.

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The widespread occurrence of alarming and repellent substances among marine organisms is well known to biologists (*1, 2*), but the chemical nature of these substances has received little attention. Brock (*3*) had observed that the boxfish, *Ostracion lentiginosus*, a member of the trunkfish family (Ostraciontidae), when placed in a container, secreted a substance that rapidly killed other fish in its vicinity. Thomson (*4*) confirmed this observation and isolated a crude preparation that, on the basis of similarities in detergent and hemolytic activities, he thought was structurally related to the steroidal saponins isolated from echinoderms (*5, 6*). Thomson (*7*) named this material ostracitoxin, while we had in the meantime isolated a pure crystalline substance and had named it pahutoxin (*8, 9*). We now report the isolation of pahutoxin, the determination of its molecular structure, its synthesis, certain biological activities, and a comparison of these activities with those of several synthetic homologs (*10*).

Brock's (*3*) relatively recent observation that the boxfish secretes a toxin that is lethal to other fishes was preceded in the literature by a number of reports which indicate that the boxfish and other members of the trunkfish family have been used as food. Malo in his treatise on ancient Hawaiian culture (*11*) mentions that the *pahu* was *kapu* to Hawaiian women and eaten only by the men. The emphasis in this connection should be on the use of the boxfish as a food. Its restricted use for men only, we might add, was rather common in ancient Hawaiian society, where men and women ate apart from one another. The best-known example of such a sexually restricted food is the banana. Herald (*12*) reports that the trunkfishes are considered a delicate food in some parts of the Pacific and are roasted in their carapaces in the same way in which chestnuts are roasted. According to Buddle (*13*), boxfishes and cowfishes are eaten and even esteemed by the inhabitants of Singapore. He further states, however, that human consumption should be prohibited since many of these fishes are poisonous. This reference to their toxic nature is probably quite unrelated to the boxfishes' toxic secretion, but may stem from the not uncommon confusion of the boxfishes with the puffers which belong to the same order (Plectognathi), have similar shapes, but produce the poison tetrodotoxin (*14*). Buddle's (*13*) statement regarding the toxicity of trunkfishes may also be reconciled on the basis of Halstead's (*15*) observation that members of the trunkfish family may contain ciguatoxin, a fish toxin of known geographical and chronological variability (*16*). We have not attempted to test this idea experimentally, but we believe that secretion of a toxin is doubtless compatible with the use of a fish as food after it is cooked.

Secretion of a toxin capable of killing fish seems to be a characteristic property of the trunkfishes (*4*). A single report (*17*) that a New Guinea toadfish (family Batrachoididae) also possesses this ability has not been confirmed.

Isolation and Purification

The boxfish for our study (Fig. 1) were netted along the reefs in Kaneohe Bay and at Waikiki, Oahu. Specimens collected at the islands of Maui and Kauai in the Hawaiian chain and at Tahiti in the Society Islands also produced the toxin. The fish were immediately placed in containers of distilled water, where they released copious amounts of a mucous secretion. The boxfish were then quickly returned to the sea since prolonged contact with the toxic solution would kill them. Initially, the purification was monitored by Thomson's (*4*) bioassay with brackish-water mollies as the test animal. The bioassay was replaced by the Dragendorff test for tertiary or quaternary amines in all routine isolations when we noted that the toxin responded positively to this test, producing a light orange turbidity characteristic of a quaternary nitrogen function.

We soon discovered that the toxic aqueous solutions gradually became less toxic (bioassay) when allowed to remain at room temperature. Immediate heating of this solution followed by cold storage inhibited this decrease, but furnished a crude preparation which again became progressively less toxic when removed from cold storage. Adsorption of the aqueous solution on a column of powdered polyethylene and elution of the toxin with aqueous methanol yielded a more stable product. However, best results were obtained when the toxin was immediately extracted from the aqueous solution into 1-butanol. This step achieved a 20-fold purification as shown by bioassay. The toxic butanol solution was then chromatographed on a column of silicic acid as described by Wren (*18*). Elution with a mixture of chloroform and methanol (88:12 by volume) yielded the toxic fraction. Removal of the solvent furnished a toxic white amorphous solid. This solid could not be crystallized from a hot acetone solution or after repeated chromatography. However, a single passage through an anion-exchange column (Dowex 1-X4) treated with picric acid yielded a product which crystallized from acetone in the form of long colorless needles, which had toxic properties and which we call pahutoxin. Identity of crystalline and amorphous pahutoxin was demonstrated spectrally. This isolation procedure consistently furnished up to 60 mg of pahutoxin from one adult boxfish.

Crystalline pahutoxin melts at 74° to 75°C and has a specific rotation of + 3.05° (22°; 2.30 g per 100 ml of methanol). It is soluable in water, ethanol, chloroform, hot acetone, and hot ethyl acetate. In addition to giving a positive Dragendorff test it gives an immediate reaction when tested for ionic

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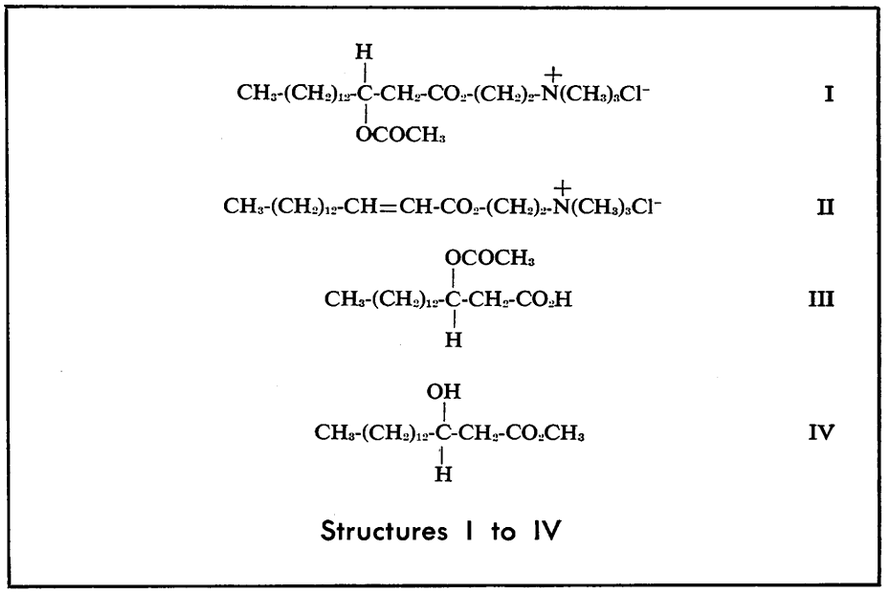
halide. Combustion data (*19*) were inconsistent and left the molecular formula initially in doubt. Spectroscopic data, hydrolytic degradations, and synthesis established unambiguously $C_{23}H_{46}NO_4Cl$ as the formula of pahutoxin and I as its structure.

Spectral data. The infrared spectrum of pahutoxin in chloroform solution (Fig. 2) showed the presence of quaternary nitrogen (3320 cm^{-1}), saturated hydrocarbon (3000 cm^{-1}), and ester (1730, 1250 cm^{-1}) functions. The nuclear magnetic resonance (NMR) spectrum in deuteriochloroform (Fig. 3) confirmed the presence of a large aliphatic portion by a strong signal centered at 1.38 δ and indicated a choline moiety by signals at 3.68, 4.28, and 4.68 δ.

Hydrolytic degradations. Treatment of pahutoxin with an excess of 1N sodium bicarbonate at 50°C for 7 hours, followed by acidification and extraction with butanol, furnished an aqueous phase from which choline chloride could be recovered and crystallized from a mixture of acetone and 2-propanol. Identity was proven by direct comparison of infrared and NMR spectra with those of authentic choline chloride. The butanol solution, after concentration and chromatography on silicic acid yielded three components. The first product appeared to be an olefinic fatty acid since its NMR spectrum resembled that of crotonic acid (*20*). Catalytic hydrogenation of this acid over a platinum catalyst yielded palmitic acid, identical in all respects with an authentic sample. The hydrolysis product was therefore assigned the structure of 2-hexadecenoic acid.

The second product of the butanol portion gave a positive Dragendorff test and yielded a crystalline picrate, m.p. 100° to 102°C, $C_{27}H_{44}N_4O_9$. The picrate was converted to the chloride by anion exchange. This chloride had an infrared spectrum not unlike that of intact pahutoxin, but occurrence of a sharp band at 1650 cm^{-1} suggested the presence of an olefinic function. Further hydrolysis of the chloride led to isolation of choline chloride and of 2-hexadecenoic acid, both of which were identified by direct comparison with authentic samples. The initial hydrolysis product must therefore have structure II.

A third product of the butanol extract could be crystallized after sublimation in a vacuum of the chromatographic fraction, m.p. 44° to 45°C, $C_{18}H_{34}O_4$. Its infrared spectrum ex-



Structures I to IV

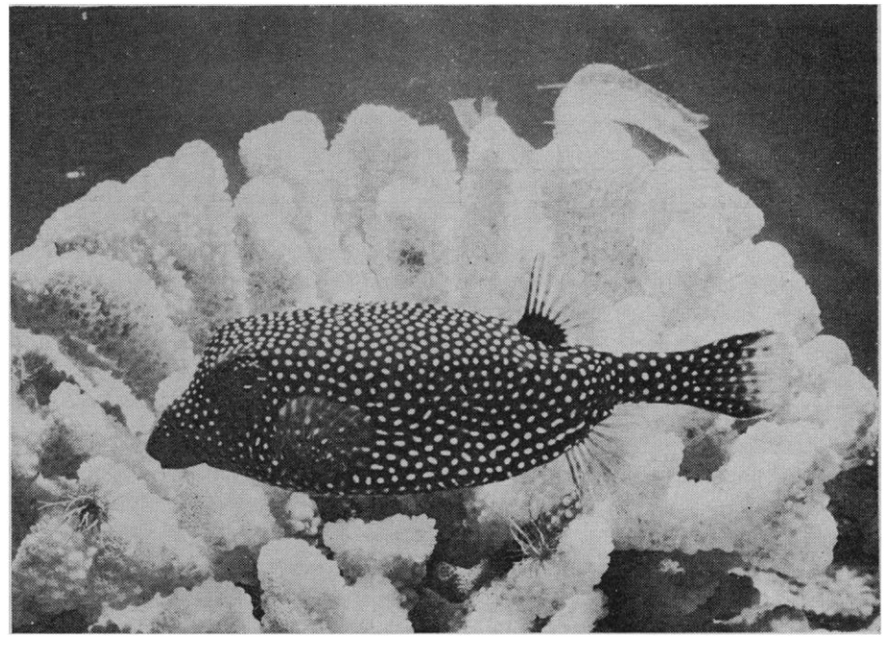


Fig. 1. Female boxfish, *Ostracion lentiginosus.* Length, 13.3 cm; width, 3.8 cm; eye diameter, 1.2 cm. Photo by Ralph Bowers, Hawaii Institute of Marine Biology.

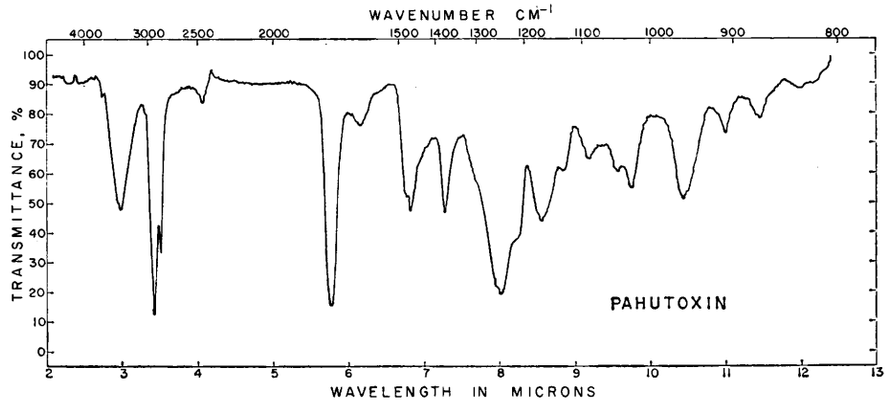
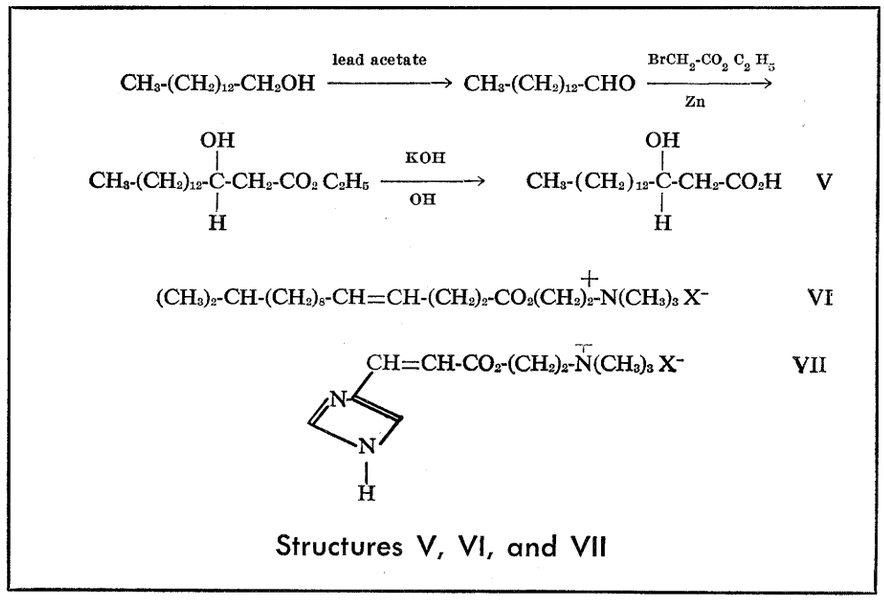


Fig. 2. Infrared spectrum of pahutoxin in chloroform.



Structures V, VI, and VII

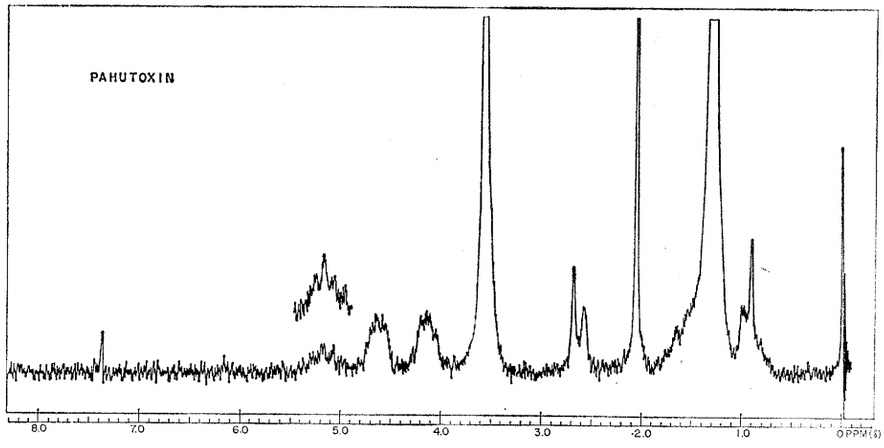


Fig. 3. Nuclear magnetic resonance spectrum of pahutoxin in deuteriochloroform.

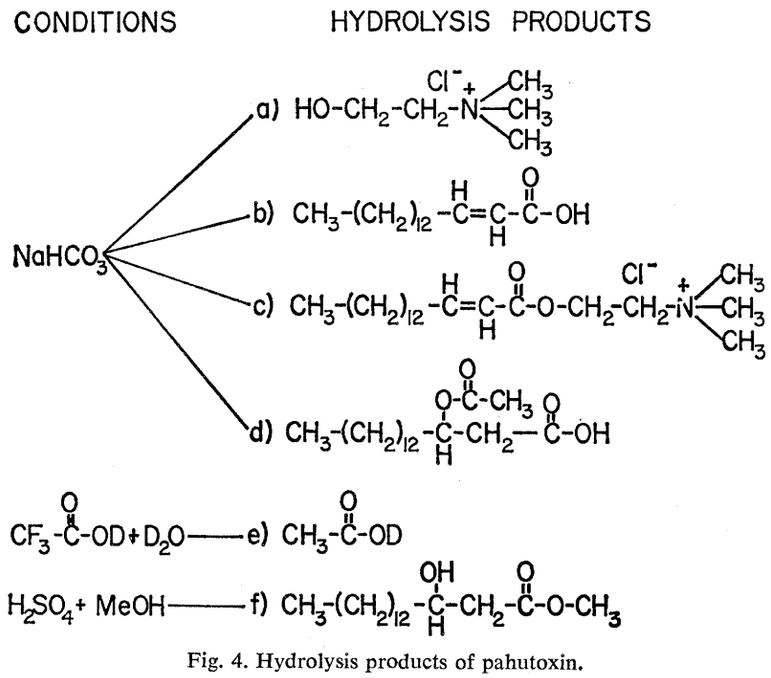


Fig. 4. Hydrolysis products of pahutoxin.

hibited two carbonyl peaks, at 1740 and 1720 cm^{-1}, and a characteristic ester band at 1235 cm^{-1}. Its NMR spectrum closely resembled that of methyl-3-acetoxybutyrate (*21*). The compound was therefore assigned structure III, 3-acetoxyhexadecanoic acid.

In contrast to the multiplicity of products obtained from mild alkaline hydrolysis of pahutoxin, acid hydrolysis conducted in 2*N* methanolic sulfuric acid at 60°C for 3 hours furnished only a single crystalline compound, m.p. 46° to 48°C. It proved to be identical with a synthetic sample of methyl-3-hydroxyhexadecanoate (IV).

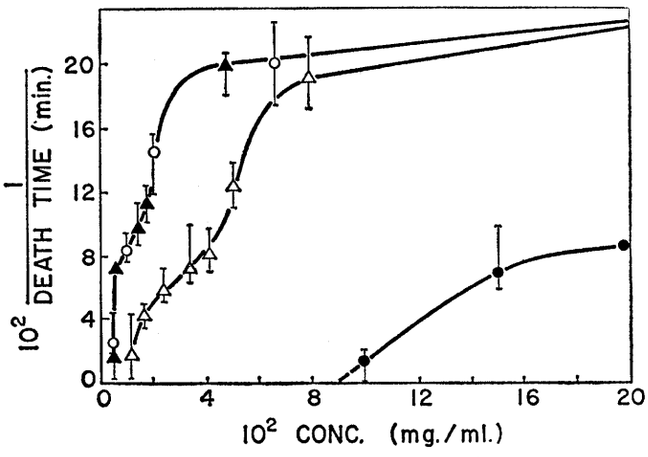
Finally, in a hydrolysis experiment designed to detect volatile fragments only acetic acid was found. Pahutoxin was treated with trifluoroacetic acid in deuterium oxide at 60°C for 12 hours in a moisture-proof system. Analysis of the distillate by NMR indicated a singlet at 2.18 δ, the signal growing in intensity upon the addition of acetic acid. The hydrolysis experiments are summarized in Fig. 4.

As a result of the hydrolyses of pahutoxin, structure I was considered the most likely. Because the combustion data of the intact toxin was inconsistent, we sought additional proof by synthesis.

Synthesis. The key intermediate V, 3-hydroxydecanoic acid, was prepared by (i) oxidizing tetradecanol with lead tetraacetate (*22*), (ii) treating the resulting aldehyde with ethyl bromoacetate in the presence of zinc (Reformatsky reaction), and (iii) hydrolyzing the resulting ethyl-3-hydroxyhexadecanoate with 10 percent alcoholic KOH. Crystallization of the hydrolysis product from carbon tetrachloride furnished the white crystalline compound V, m.p. 183.5°C, identical with the free acid of IV obtained by degradation. Compound V treated with acetic anhydride yielded a product identical with III. Formation of the acid chloride of III, and subsequent esterification with choline chloride yielded I, identical with natural pahutoxin in all respects except optical activity and melting point. Combustion analysis of this synthetic pahutoxin also gave ambiguous results.

Biological Activity

Since the synthesis of pahutoxin presented no difficulties, we synthesized pahutoxin homologs of the corresponding C_{14} and C_{12} fatty acids (choline



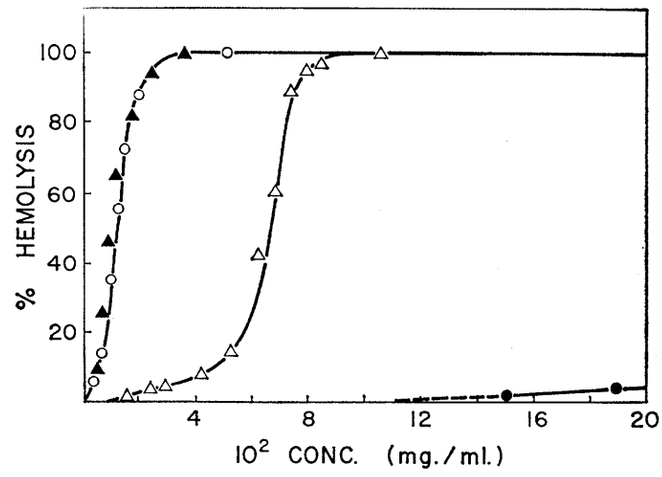


Fig. 5 (left). Toxicity of pahutoxin and homologs. ▲ Natural pahutoxin; ○ synthetic pahutoxin; △ C_{14} homolog; ● C_{12} homolog.
Fig. 6 (right). Hemolytic activity of pahutoxin and homologs. ▲ Natural pahutoxin; ○ synthetic pahutoxin; △ C_{14} homolog; ● C_{12} homolog.

chloride esters of 3-acetoxytetradecanoate and 3-acetoxydodecanoate) from the appropriate starting materials by the route outlined above. The two new compounds were characterized in the usual fashion.

Lethality of the four compounds, natural and synthetic pahutoxin (C_{16}) and the C_{14} and C_{12} homologs, were compared by exposing four adult brackish-water mollies (*Mollienesia lallipinna*) in the preadult stage to known concentrations of the four substances in 5 ml solutions. In Fig. 5 reciprocal time-to-death in minutes is plotted against concentration. The vertical extensions of the respective symbols indicate the variability of individual test fish in a group of four.

The values for natural and synthetic pahutoxin fall on the same curve although the synthetic compound is a racemate. Apparently the lethality is not dependent on the configuration of the optically active center at carbon atom 3. The shape of the curve for the C_{14} homolog is the same as that for pahutoxin, but the compound is markedly less toxic. A dramatic decrease in lethality occurs when one proceeds from the C_{14} to the C_{12} compound.

The capacity of boxfish toxin to hemolyze red blood cells was noted by Thomson (*4*) in his work with crude extracts. We determined the hemolytic activity of crystalline pahutoxin quantitatively and compared it with the activity of the two lower (C_{14} and C_{12}) homologs.

Blood cells from the bigeye tuna were washed and diluted with modified Alsever's buffer (*23*). A standard solution of cells was prepared so that upon dilution with a sevenfold volume of hemolyzing agent it had an optical density of 0.68 at 541 mμ (*24*). One milliliter of the standard blood cell suspension was added to 7 ml of the solution to be tested. The mixture was allowed to stand at room temperature for 10 minutes and was then centrifuged for 3 minutes. The optical density of the supernatant was measured at 541 mμ and compared with the optical density of a totally hemolyzed identical blood sample. The ratio of the two densities was considered the extent of hemolysis and was reported as percent hemolysis (Fig. 6).

Again, as with the data on lethality, those on hemolysis for optically active and racemic pahutoxin fall on the same curve; again the shape of the C_{14} curve is the same but shows diminished hemolytic activity; and once more, a sharp break occurs between the C_{14} and C_{12} activities. Furthermore, the similarity between lethality and hemolysis plots is striking. This would suggest a relation between lethality and hemolysis although previous studies on antimycin as a fish poison (*25*) lend no *a priori* support for this hypothesis.

Our finding that the chain length of the acid portion of the choline ester bears a relation to the degree of lethality and hemolysis is in accord with related studies. The lethality of alkylbenzene sulfonates (detergents) increased progressively with the lengths of the aliphatic chain up to C_{14} (*26*). Studies with sodium salts of fatty acids of from 10 to 20 carbons (soaps) showed that hemolytic activity reaches a maximum between the C_{14} and C_{18} homologs (*27*). This study further showed that structural variations (introduction of hydroxyl groups or double bonds) had no influence on the hemolytic activity. Lastly, among a series of choline esters of fatty acids those of palmitic (C_{16}) and stearic (C_{18}) acids had the highest hemolytic activity (*28*).

Direct comparison of the hemolytic activity of pahutoxin and that of other zootoxins such as the holothurins was not made. Comparison of our data with those of others (*29*) is not feasible because of widely divergent experimental conditions. An approximate comparison of the lethality of pahutoxin with a fish toxin such as antimycin can be made. We found that the minimum lethal concentration of pahutoxin required to kill all fish within 1 hour in 50 ml solutions was 0.176 μg/ml, or 176 ppb (parts per billion). Lethal doses of antimycin occur at dilutions of 1.0 ppb (*25*). However, these measurements were made in 2.5 liter containers; time-to-death was not noted, and the test fish (goldfish) were different. It may tentatively be concluded that pahutoxin may be as toxic to fish as antimycin is, but direct comparison under identical experimental conditions is clearly indicated.

Among those of known fish toxins the structure of pahutoxin is unique. It resembles the fatty acid moiety of a glycolipid (VI) which has been isolated from a Japanese oyster (*30*). On the basis of our work one would expect compound VI to have similar biological activity, but this has not been verified. A less obvious structural congener is the compound murexine (VII), which was isolated from *Murex trunculus* and related species (*31*). The biological ac-

tivity of murexine is reported to resemble that of nicotine and curare, activities which are not shared by pahutoxin (*32*). The biological activity of pahutoxin is, however, analogous to the activities of the steroidal saponins isolated from echinoderms (*5, 6*).

To our knowledge this is the first chemical identification of an alarming or repellent substance of a marine organism. Marine biologists have postulated that such substances are of common occurrence in that they constitute a logical defense of slow-moving unprotected species against predators. Our finding that crude solutions of pahutoxin lose their toxicity rapidly may well be the result of an unknown biological (enzymatic?) mechanism which is designed to protect the boxfish from its own toxin. Since pahutoxin is a compound of simple chemical structure and is readily synthesized, it will be an excellent vehicle for further biological research of the mechanism of fish repellents.

Pahutoxin, the toxic secretion of the boxfish, *Ostracion lentiginosus*, has been isolated in crystalline form and identified as the choline chloride ester of 3-acetoxyhexadecanoic acid. It and its C_{14} and C_{12} homologs have been synthesized as the racemates. Their lethalities toward fish and their hemolytic activities have been compared. Of the compounds studied, pahutoxin, the C_{16} compound, has the highest activity. This work constitutes the first chemical identification of an alarming substance secreted by a marine organism.

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NEWS AND COMMENT

World Food Supply: Problems and Prospects

The critical problems of food production and population control were discussed, provocatively at times, by several panels at the AAAS's annual meeting in Washington last week. Although in most cases the two problems were treated separately, few people these days, when many are predicting a doubling of the present world population of 3.2 billion by the year 2000, overlook the Malthusian prophecy that ultimately population growth will outstrip food supplies, with apocalyptic results.

Provided the widely held concept of a "population explosion" is valid, which not everyone concedes, the outlook seems gloomy indeed. One AAAS panelist, Lester R. Brown, administrator of the U.S. Department of Agriculture's International Agricultural Development Service, held out little hope that increases in food supplies through the expansion and improvement of conventional farming will be sufficient. The prospects, he said, for significantly increasing cultivated acreage in the hungry and potentially hungry nations are not bright unless there should be technological breakthroughs, such as the development of a desalination process cheap enough to permit the irrigation of vast desert areas.

As for increasing the yield of existing farm land through more extensive use of improved seed varieties, fertilizers, modern farm equipment, and the like, Brown spoke more encouragingly. He made it clear, however, that widespread use of advanced agricultural practices in the developing nations will not come easily, despite the new emphasis on "self-help" in U.S. aid programs (*Science*, 6 May 1966). Thoroughgoing social and economic changes will be necessary, Brown indicated. As others have noted, for example, the subsistence farmers of traditional societies are largely outside the cash economy, and, even when shown the advantages of modern farming methods, they have no money to invest in them.

At best, adoption of such methods will not, if the experience of the advanced nations is indicative, lead to an indefinitely increasing food supply. When first used, Brown observed, such things as hybrid seeds and weed control agents lead to major gains in crop yields, but ultimately the yields tend to level off. "A one-time phenomenon—this simple phrase has ominous overtones for the long-run food production capacity of conventional agriculture," Brown said.

However, if the outlook for an ade-