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CIGUATERA AND ITS OFF-SHOOTS— CHANCE ENCOUNTERS EN ROUTE TO A MOLECULAR STRUCTURE

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Abstract—The history of ciguatera research at the University of Hawaii during the past 35 years revolves around the search for a molecular structure and strays upon some unexpected paths, far removed from the original goal.

I first learned of ciguatera in 1957. My colleague, the late A. H. (Hank) Banner,[1] a University of Hawaii Professor of Zoology, invited me to join a group of biologists and chemists to study ciguatera. And what on earth, one might ask (as I did then), is ciguatera? The same question is the title of a 1986 article in *The Australian Medical Journal*,[2] which the author answers: "Ciguatera is an impressive type of fish poisoning which, once experienced, is never forgotten by either the victim or their doctors." In this Perspective I should like to recount some of the high (and low) points of ciguatera research during the past 35 years and report on its current status with emphasis on some important unanswered questions. I will conclude with a brief account of the remarkable impact which ciguatera research made on marine natural product research.

HISTORICAL BACKGROUND

A detailed and profusely illustrated history of ciguatera may be found in Halstead's treatise[3] and will not be repeated here. A synopsis should suffice for those readers who are unfamiliar with ciguatera. The term is of Spanish origin and first appears in a book published in Havana. Cuba, in 1787 by a Portuguese biologist, Don Antonio Para. It refers to a "disease contracted by persons who eat fish that is affected with disease or jaundice".[4] The word itself is derived from *cigua,* the Spanish trivial name of a univalve mollusk, *Turbo pica,* reputed to cause indigestion. The term ciguatera somehow was transferred to an intoxication caused by the ingestion of coral reef fishes. Authentic descriptions of ciguatera incidents are much older than the term itself. The first account from the Americas by Peter Martyr of Anghera published in 1511 refers to diverse strange maladies caused by eating fish. The fish in turn were believed to have acquired the toxin from fruits of a tree in the West Indies, which grows on nearby shores.[5] The first report from the Pacific is ascribed to the Portuguese explorer Pedro Fernandez de Queiros, whose sailors became sick from eating fish, probably red snappers, in the New Hebrides (now Vanuatu) in 1606.[6] Again, the assumption was expressed that the fish must have acquired toxicity from feeding on poisonous plants. A case history of a ciguatera outbreak aboard the H.M.S. *Resolution,* when she was anchored in July of 1774 off Malicolo island, New Hebrides, is due to William F. Anderson, Captain Cook's surgeon's mate aboard the vessel.[7] Of three fish, probably red snapper, one was particularly toxic and all five sailors who shared that fish became ill for about a week. One dog and two pigs, which ate the viscera of the fish, died.

Over the years, a wide range of symptoms has been recorded from many case histories. In addition

to gastro-intestinal malaise, vomiting and diarrhea, which are common in food poisoning, there is a neurological component. Dizziness, tingling of the extremities, and the sensation of temperature reversal are among the salient characteristics of ciguatera poisoning. Virtually all victims recover, albeit painfully and slowly. The low fatality rate is due solely to the minute concentration of the toxin in fish flesh.

World War II, specifically the Pacific Theatre, gave birth to modern ciguatera research. A Japanese survey of "Poisonous Fishes of the South Seas"[8] was conducted between July and December, 1941. Yoshio Hiyama, a noted ichthyologist, was in charge of this investigation. His estimate of fewer than a hundred species of poisonous fishes appears to be realistic.

Two Americans, who found themselves in the Pacific during WW II and learned of potentially toxic coral reef fishes, profoundly influenced ciguatera research in the United States. Halstead, a Navy physician, was sufficiently impressed to initiate ciguatera research[9] and between 1965 and 1970 published his monumental three-volume treatise.[3,4,9] Banner, a marine biologist serving in the U.S. Air Force, was told by his Commanding Officer to inspect all fish which the personnel in his command had caught and reject all toxic ones. Banner, a crustacean specialist rather than an ichthyologist, knew nothing of ciguatera and pragmatically decided to reject all reef (i.e. brightly colored) fishes and allow all drab (i.e. pelagic) fishes to be eaten. This spur-of-the-moment judgment not only worked, but it inspired Banner to initiate a scientific study of ciguatera in Hawaii in the mid-1950's. The four objectives of the planned research were deceptively simple, yet broad in scope and penetrating in substance:

1. What is its molecular structure?
2. What is the origin of the toxin?
3. Can a diagnostic test be devised that will distinguish toxic from non-toxic fish?
4. Can an effective human therapy be found?

Of these four goals, determination of the molecular structure seemed central. Hence it was given high priority.

Thirty-five years have elapsed, and an appraisal of the results should prove worthwhile. The ensuing discussion will follow the sequence of the above four questions.

RETROSPECTS AND PROSPECTS

Molecular structure

The wide range of clinical symptoms (gastrointestinal, cardiovascular, neurological) in ciguatera patients, which was documented by 350 case studies,[10] provided strong circumstantial evidence that more than one toxic entity can cause ciguatera poisoning. Primitive chemical evidence supported this notion by isolation of a lipid-soluble ciguatoxin[11] and the water-soluble maitotoxin.[12] Early ecological[13] studies had shown that large carnivores tended to have the highest concentration of toxin and hence provided the most desirable source for toxin isolation and structural elucidation. Because of the uncertainty, that more than one lipid-soluble toxin might be involved, we concentrated our studies on a single species of fish from one location—as long as that source was viable. We first isolated ciguatoxin from red snappers *(Lutjanus bohar)* from the Line islands (Palmyra atoll, 5°53'N, 162°5'W) until toxicity declined; we then turned to moray eels *(Gymnothorax javanicus),* initially from Johnston Island (16°44'N, 169°17'W), later from Tarawa atoll (1°30'N, 173°0'E), Republic of Kiribati.

Our goal, the molecular structure of the toxin(s), had to be approached from the greatest distance imaginable—the known symptomology of intoxicated humans. Hiyama[8] used kittens, who will eat raw fish and exhibit ciguatera symptoms (progressive weakness of limbs, paralysis of hind legs, death) as bioassay animals. This choice was not available to us. We turned to the mongoose (*Herpestes mungo*), an unfriendly animal that was introduced to Hawaii to prey on rats and exists as

a wild population. With the aid of traps that we distributed to Boy Scout troops ($1 per mongoose) we were able to screen raw fish in order to avoid the lengthy and expensive extraction of marginally toxic fish. While this was an acceptable first step, it was difficult even then to hire students to tend to the mongooses. Decidedly, nobody would be willing to inject or force-feed toxic extracts—not to mention the large quantities of toxin that would be consumed by these animals. Hence every step of isolation had to be monitored by an as yet undiscovered reliable bioassay, an effort that consumed the early years of the research.[14] We developed a bioassay based on intraperitoneal injection into mice.[15] An ethanolic extract of dried fish flesh was freed from nontoxic lipids and the toxin was extracted into ether. Since pure ciguatoxin lacks UV absorption,[16] the mouse bioassay has remained the principal toxin monitor. Of the wide-ranging attempts to develop an equally reliable bioassay that would require smaller amounts of toxin (chicks, fresh-water crustaceans, mosquitoes etc) only giant mosquitoes *(Aedes aegypti)* found limited use.[17]

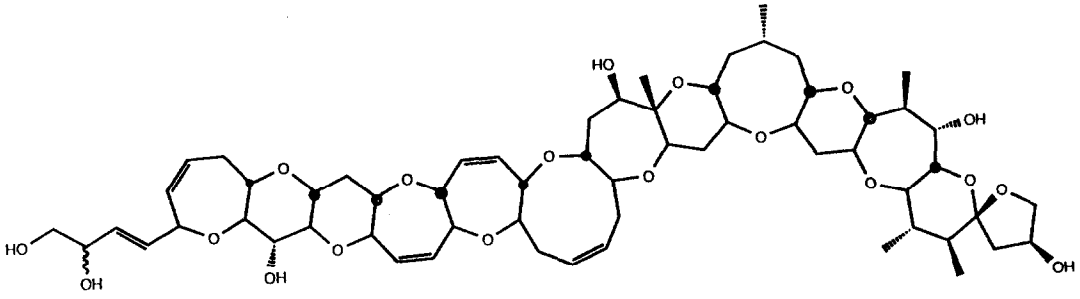
Even if we had known then that one terminus of ciguatoxin (1) was a handy 1,2-diol, the tools of affinity chromatography had not yet been invented and we were relegated to mouse-watching, a rather time-consuming task, since ciguatoxin is a slow-acting poison.

The crude toxin which served to establish the vital mouse bioassay[15] was crude, indeed. Had we realized in 1961 that pure ciguatoxin has an LD_{50} of 0.45 μg/kg,[16] we might well have abandoned the daunting task of purifying an extract with an MLD of 2×10^5 μg/kg, our 1961 "toxin." These figures are even more formidable when they are equated with fish. Despite frequent occurrences of ciguatera poisoning in many parts of the world, ciguatera poisoning is rarely fatal because of the low concentration of the toxin in fish flesh. We had shown[18] that fish viscera, particularly livers, are consistently more toxic than fish flesh. Yet even by restricting our starting material to moray eel viscera, it took 75 kg of toxic viscera, representing approximately 1,100 kg of eels (Fig. 1) to produce 1.3 mg (1.7×10^{-6}%) of HPLC-pure ciguatoxin.[16,19] It is worth noting that moray eels (also unfriendly animals) have to be speared or trapped (Fig. 2) one at a time and have an average weight of 5.5 kg. But the results were spectacular: by 1980 a mass spectral molecular weight (1,111.7 da) and a 600 MHz ^1H NMR spectrum of ciguatoxin.[16,20] By using the newly introduced Cf-252 Plasma Desorption technique the correct molecular ion at m/z 1111.7 was observed,[16] but only in 1989 an unambiguous molecular formula of the toxin, $C_{60}H_{86}O_{19}$, was realized with HRFAB instrumentation.[21] State-of-the-art NMR instrumentation at the time preceded FT techniques and did not allow measurement of a carbon spectrum. After an unsuccessful attempt to do so we received an unexpected reward. During the return flight from an NMR lab in New Jersey the sample (in MeOH-d_4) crystallized in the NMR tube; this provided welcome confirmation that our sample was indeed pure, even if the crystals proved to be unsuitable for single crystal x-ray diffraction. Our next attempt to obtain sophisticated NMR data, again in an east coast laboratory, ended in disaster. Our entire sample (1.3 mg) of ciguatoxin was destroyed when a pyridine solution of the toxin was transferred from a glass to a plastic tube. Attempted recovery of the toxin from a matrix of depolymerized plastic proved fruitless. Once again, a molecular structure of ciguatoxin became an elusive goal.

Two events, meanwhile, were approaching each other asymptotically and came to the rescue of the ciguatoxin structure. During 1975-76, Takeshi Yasumoto was retained by the World Health Organization to investigate "biotoxins in marine food fish" in French Polynesia. Collaboration with the resident French public health workers not only led to the discovery of *Gambierdiscus toxicus (vide infra),* but initiated a lasting collaboration that provided the Japanese investigators with partially purified toxin from moray eels. Simultaneously, NMR and computer technology were making rapid advances that greatly enhanced our knowledge of the molecular structure. With the same 350 μg of toxin that produced the correct molecular formula[21] Yasumoto and coworkers[22] deduced the two-dimensional structure of twenty-two carbons—including the vital piece of structural information—presence of a primary hydroxyl that might become useful for preparation of a

hemisuccinate, a prerequisite for conjugation with a protein and antibody formation. Finally, by pooling all available supplies (1.1 mg) the total planar structure was achieved.[23–25] There is little doubt that without the incredible advances in NMR technology, eventual structure elucidation of large complex molecules, available only on the micro- to milligram scale, would not have been possible.

Circumstantial evidence, predominantly chromatographic behavior reminiscent of okadaic acid (**8**, *vide infra*), had indicated that ciguatoxin (**1**) belonged to the class of polyethers, long-chain fatty acids—sometimes reduced to aldehydes or alcohols—characterized by a number of monoxa cycles. The actual structure, however, proved to be much more spectacular: a C_{55} fatty acid coiled into one terminal spiro and twelve contiguous *trans*-fused ether rings ranging in size from oxolane to oxonane. The remaining structural features were unremarkable—five olefins, five methyls, and six hydroxyls, two as a terminal vicinal diol. The closest structural analogs of ciguatoxin (**1**) are the brevetoxins, produced by a dinoflagellate *Gymnodinium breve* (syn. *Ptychodiscus brevis*) prevalent in the Gulf of Mexico and exemplified by brevetoxin B (**2**) derived from a C_{42} fatty acid, which forms a terminal δ-lactone fused to ten oxacycles ranging in size from 6 to 8-membered.



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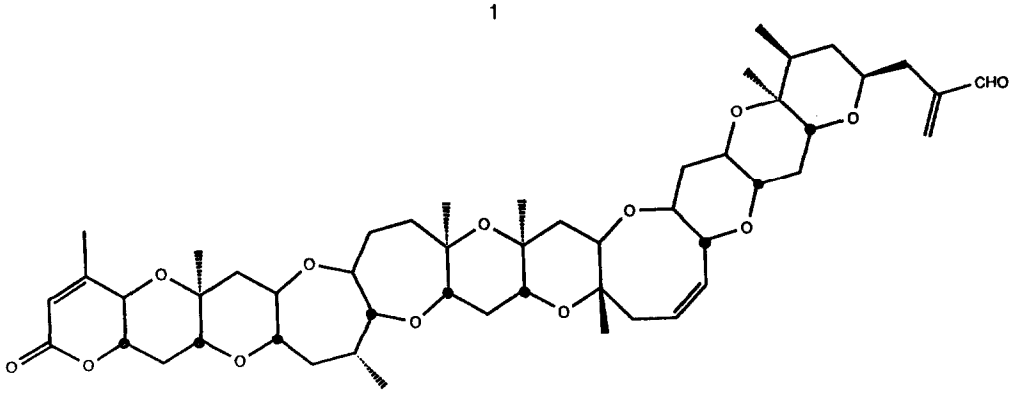

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Despite these structural similarities the biological activities of these two toxins vary widely. Massive fish kills are the most characteristic phenomenon associated with a *G. breve* bloom. The most common human symptoms are respiratory, believed to be caused by brevetoxins that are carried into the air by salt spray. Because of the tell-tale red tide caused by a *G. breve* bloom it is rare that filter-feeding shellfish which may accumulate the toxins will be eaten. Both toxins, ciguatoxin (**1**)

and brevetoxins (e.g. **2**) appear to affect sodium channels of nerve and muscle membranes, [26,27] no doubt a reflection on their structural similarities.

Origin of the toxin

Beginning with the earliest written accounts of ciguatera intoxications the random aspects of ciguatera incidents with regard to time, place, and species of fish carried the implication that fishes acquire the toxin through their diet (Fig. 3). This hypothesis was first put forth in coherent fashion by Randall.[28] It was based on field observations in the Caribbean and in French Polynesia and was buttressed by Randall's extensive knowledge of the feeding habits of herbivorous reef fishes.[29] These fishes had to be the focal point of any research since carnivorous fishes are at the next higher trophic level and acquire toxicity from feeding on herbivores. In addition to herbivores, coral detritus feeders, e.g. parrotfishes, constituted a significant source of intoxications in French Polynesia, which had to be accommodated by the hypothesis. Randall postulated that "the toxic organism would most likely be an alga, a fungus, a protozoan, or a bacterium. A herbivorous fish might ingest any of the latter three categories with its algal food, and the detritus-feeder would obtain any of those incidentally in its feeding."[28] Based on his knowledge of surgeonfishes Randall further reasoned that the alga had to be fine, most likely a blue-green (cyanophyte), since certain species of ciguatoxic surgeonfishes are unable to feed on coarse algae. For nearly twenty years, Randall's hypothesis had a profound effect not only on the direction of ciguatera research, but it also spawned inter alia the discovery of palytoxin[30] and the aplysiatoxins.[31]

Although Randall stated unequivocally, "The basic organism is benthic,"[28] the search for the toxin source concentrated for many years on sublittoral biota. To some extent this bias may have been influenced by the fact that two well-understood marine intoxications, paralytic shellfish poisoning and the Gulf of Mexico red tides, were known to be caused by dinoflagellates and were characterized by a highly visible surface phenomenon—a red tide. No such indicator had ever been associated with a ciguatera outbreak. It is indeed this lack of a warning signal that has contributed so markedly to the dread of ciguatera poisoning, particularly in the islands of Oceania. The breakthrough came in 1977;[32,33] it had been adumbrated by a suggestion, albeit one based on primitive (and subsequently shown to be misleading) analytical data, that the causative organism might be a dinoflagellate.[34] Yasumoto *et al.* in their investigation of stomach and gut contents of toxic *maito*[12] (the Tahitian name of a surgeonfish, *Ctenochaetus striatus*) encountered a water-soluble toxin, which they named maitotoxin. It was clearly distinct from the lipid-soluble ciguatoxin which had been extracted from the flesh and viscera of toxic carnivores.[11] Preliminary analytical data for maitotoxin were reminiscent of those attributed to a hemolytic ichthyotoxin isolated from a dinoflagellate *Prymnesium parvum* and known to be responsible for fishkills in brackish water and estuaries.[35] While Yasumoto and coworkers were able to show much later[36] that no structural relationship existed between maitotoxin and *Prymnesium* toxin, the apparent similarity prompted a search for dinoflagellates as the originating organism of ciguatera toxins.

Another fruitful observation that led to the discovery of a new toxic dinoflagellate emerged from Yasumoto's study of the gut contents of a parrotfish, *Scarus gibbus*.[37] Since parrotfishes feed on coral, chlorophyll in the gut would be a good indicator of an algal precursor. Hence finding only a low concentration of chlorophyll in the gut of the parrotfish was compelling evidence that the toxin originator could not be a fine benthic alga such as a blue-green, which had been the foremost suspect for nearly twenty years.

The final and crucial piece of evidence was provided by a microscopic examination of algae and coral detritus from the Gambier islands in French Polynesia: the most toxic samples contained much larger quantities of dinoflagellates than did mildly toxic or non-toxic specimens. The dinoflagellate was tentatively identified as *Diplopsalis* sp. *nov.*[32] Yasumoto *et al.*[33] demonstrated a quantitative relationship between the number of dinoflagellate cells and toxicity. Moreover, they separated the

crude toxin into an ether-soluble fraction with properties resembling ciguatoxin and an acetone-soluble one with properties reminiscent of maitotoxin. The organism, which had been assigned to the dinoflagellate genus *Diplopsalis,* was later found to represent a new genus and was named *Gambierdiscus toxicus.*[38] During a careful ecological survey of two reefs in Tahiti, Yasumoto *et al.*[39] made two significant observations. One, the level of toxicity was much lower there than it was in the Gambier islands. Secondly, *G. toxicus* settles preferentially on macroalgae. *Turbinaria ornata,* a brown alga, was the preferred substrate by far, while a calcareous red alga, *Jania* sp., was most heavily settled by the microalgae at another site, where no *Turbinaria* sp. was growing. The basis of this apparent chemotaxis is still unknown.

A diagnostic test

How can a fisherman or a consumer distinguish a ciguatoxic fish from one that is safe to eat? Appearance, or species of fish offer no clue, nor does the reef where it was caught. Hiyama used cats or kittens in his historical survey of toxic fishes of the South Pacific;[8] these animals found limited use in Japan and in French Polynesia during the 50's and 60's. We employed the mongoose to screen fish[14] and the mouse to monitor toxin purification.[15] None of these assays could be developed into a vehicle for rapid screening of large numbers of fishes by technically untrained individuals. Our goal was an assay comparable to litmus paper—an instant color change to distinguish toxic from non-toxic fishes. During the mid-1970's, when limited amounts of partially pure toxin became available from extraction of eel viscera, immunological techniques became feasible for the development of a reliable and rapid assay. Hokama *et al.*[40] conjugated semipure ciguatoxin to human serum albumin and injected the conjugate into rabbits and sheep to raise antiserum. The resulting antiserum was radio-labelled with ^{125}I and its reliability assessed by comparison with the mongoose feeding assay. Large (≥ 9 kg) amberjacks (*Seriola dumerilii, kahala*) were used to demonstrate the feasibility of the immunological approach, while at the same time providing evidence that this radio-immunoassay (RIA) would not be an assay that could be used routinely for large numbers of fishes of all sizes outside a laboratory.

The next stage in the development of a bioassay replaced radio-iodine with horseradish peroxidase as the indicator,[41] thus substituting a UV monitor for a radioactivity counter. The new enzyme-linked immunosorbent assay (ELISA) clearly distinguished between clinically documented (i.e. having caused human illness) and non-toxic fishes, including amberjacks *(Seriola dumerilii),* jacks *(Caranx* sp., *ulua),* and parrotfishes *(Scarus* sp., *uhu).*

The final goal, a rapid visual color test, was achieved by coating a bamboo stick that had been inserted into fish flesh with sheep anti-ciguatoxin coupled to horseradish peroxidase. After a ten minute incubation the color of the stick is evaluated visually, ranging from colorless (non-toxic) to intense bluish purple (highly toxic).[42] By rejecting fishes with borderline (slightly bluish-purple) test scores, it has been possible to avoid ciguatera poisoning from any of the fishes that were tested in preliminary trials in Hawaii.

Therapy

A victim of ciguatera poisoning usually experiences the onset of the illness some four or five hours after the meal. Gastrointestinal symptoms, abdominal pain, vomiting, and diarrhea, common to other forms of food poisoning, are followed by neurological symptoms that include general weakness, numbness and tingling of the lips, and a bizarre sensation of temperature reversal. The condition can last for weeks and often is reinforced even when eating fish that has no toxic effect on previously unaffected individuals. A visit to a clinic would offer no relief as none of the various "treatments" would help.[43] The first effective therapy, massive infusion of mannitol, was reported by physicians on Majuro, Marshall Islands, who suspected cerebral edema in two patients who were comatose after ciguatera poisoning.[44] The treatment, intravenous infusion of 1.0 g/kg of mannitol

over 45 minutes, was subsequently successfully administered to twelve ciguatera patients in Australia.[45] To conjecture what the mechanism of action of the mannitol treatment might be is premature. Because of the scarcity of pure ciguatoxin its pharmacology is still largely unexplored. One of its properties is the opening of voltage-dependent sodium channels in cell membranes.[46] The wide distribution of sodium channels in nerve and muscle tissues helps to explain the broad spectrum of symptoms in intoxicated patients, but the equation also includes mannitol and the stereostructure of the toxin.

WHAT OF THE FUTURE?

Despite intensive research spanning more than thirty years and despite impressive progress on many fronts, a number of fundamental questions remain.

Origin of the toxin

Randall's 1958 hypothesis,[28] Yasumoto's discovery of *Gambierdiscus toxicus* and its link to ciguatera in 1977,[33] and the ciguatoxin structure in 1989 represent major benchmarks in the history of ciguatera. But what are the parameters, ecological and/or genetic that trigger a population explosion of the dinoflagellates to render algae- and detritus-feeding fish toxic to humans? Answers to this vital question have yet to be found. It appears that the make-up of the total benthic community from corals to diatoms influences population density of *G. toxicus*.[47]

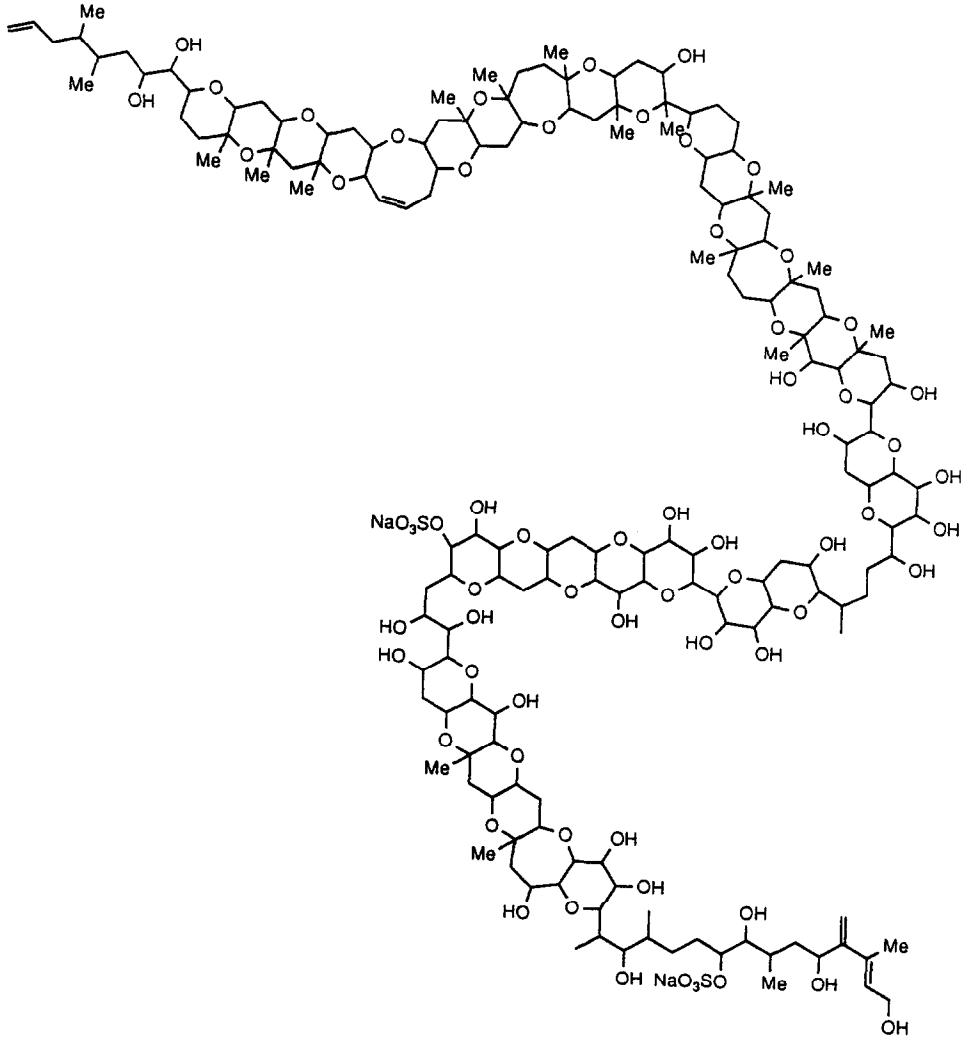
Evidence that genetic factors play an important role in ciguatera outbreaks comes from Australia. Holmes *et al.*[48] examined *G. toxicus* specimens from various sites in Queensland, Australia, and found that most of them did not produce detectable amounts of ciguatoxin. The phenomenon of dinoflagellate blooms alternating with a dormant encysted stage is well known for *Gonyaulax* spp, which are responsible for the red tides in temperate waters,[49] but has not been documented for ciguatera-producing dinoflagellates. An even stranger life cycle of a new species of marine dinoflagellate has recently been reported from the coast of North Carolina: an organism which requires live finfish or their fresh excreta to metamorphose from a dormant cyst to a toxin-producing stage.[50]

Even without having to deal with a "phantom" dinoflagellate[50] a considerable research effort is required before we will know what combination of genetic and environmental factors will culminate in a ciguatera outbreak.

Molecular structure

As has been pointed out earlier, the unavailability of pure ciguatoxin for chemical and biological research has been a limiting factor throughout the 35 year history of this effort. Hopes were high in 1977 that the toxin famine had ended and that ciguatoxin could be produced from cultured *G. toxicus*.[33] These hopes faded rapidly. The know-how of culturing the saxitoxin-producing *Gonyaulax* spp, which are surface organisms, could not be translated to the culture of *G. toxicus*, a benthic dinoflagellate. Once the parameters for *G. toxicus* culture were established, an even more discouraging facet of the problem became apparent: cultured *G. toxicus* cells would elaborate only the water-soluble maitotoxin (previously isolated from the guts of herbivorous fishes, *uide supra*),[12] but would produce no or only traces of ciguatoxin, while both toxins could be extracted from wild organisms. Despite efforts in many laboratories, successful culture of a *G. toxicus* clone from Rangiroa atoll, Tuamotu archipelago, French Polynesia, producing a ciguatoxin congener has only now been achieved.[51] From 1,100 L of culture, 0.7 mg of a close relative of ciguatoxin was isolated and its complete two-dimensional structure determined by NMR techniques.

Still unknown is the full stereochemistry of the moray eel-derived ciguatoxin or any of its congeners. In an ingenious attempt to short-circuit eventual x-ray diffraction studies or a total synthesis, Tohoku University scientists[52] have enantioselectively constructed a 14-carbon fragment corresponding to the glycol terminus of ciguatoxin (1) and including three chiral centers of rings A and B. And, finally, we know the complete structure of maitotoxin (3), which fortunately has been available from *G. toxicus* cultures for a number of years. It is a much larger molecule (3,424 da) and even more lethal than ciguatoxin. It is a bis-sulfate ester and hence water-soluble. Its C_{142} chain is dominated by 32 ether rings which are arranged in contiguous banks of two, two, five, six, seven and ten rings, mostly six-membered. Structural elucidation of this formidable molecule was greatly facilitated by the fortunate circumstance that twelve of its 28 hydroxy groups occur as vicinal diols,



which could be cleaved by periodate oxidation. Twenty-one methyl groups, two terminal methylenes, and two additional double bonds are the remaining structural features of maitotoxin (**3**).[53] Once stereostructures of the ciguatoxins and of maitotoxin are fully established, a number of important questions can be examined. Foremost perhaps is the relationship of ciguatoxin and maitotoxin in herbivorous fish. It had been shown sometime ago[12,54] that maitotoxin is the predominant toxin in the stomach and gut contents of *Ctenochaetus striatus* (maito), that maitotoxin and ciguatoxin are present in the liver, while the flesh of the fish contains only ciguatoxin. Availability of both toxins from laboratory cultures will enable production of isotope-labelled compounds for *in vivo* experimentation.

Exciting developments in Yasumoto's laboratory have high-lighted the crucial role of genetic factors in dinoflagellate chemistry and bioactivity. Additionally, this recent work has demonstrated how preoccupation with toxic properties affecting fish or mammals can preclude discovery of other biological activities. An observation of antifungal activity in marine phytoplankton led to the isolation of the gambieric acids from a Gambier Islands strain of *G. toxicus*.[55,56] Contrary to conventional wisdom that extraction of cultured cells yields all secondary metabolites, careful bioassay showed that the antifungal activity is released to the culture medium. In this fashion Yasumoto and coworkers isolated four polyether compounds which are devoid of mammalian toxicity, but exceed the activity of amphotericin B against *Aspergillus niger* by three orders of magnitude.

From a different *G. toxicus* strain, collected at Rangiroa atoll in the Tuamotus, Yasumoto and coworkers[57] isolated in the conventional way gambierol, a highly toxic polyether compound resembling ciguatoxin, thereby reinforcing the long-held supposition that *G. toxicus* is indeed the "culprit" in ciguatera as first suggested in 1977.[33]

Because of the co-occurrence of ciguatoxin and maitotoxin in herbivorous fish it is difficult to pinpoint the human symptoms which are evoked by the ingestion of pure cigua- or pure maitotoxin. In laboratory experiments it was shown that maitotoxin stimulates the movement of Ca^{2+} across biomembranes[58] rather than Na^{+}, which is the mechanism that characterizes ciguatoxin.[26,27]

Diagnostic test

Superficially, it would seem that this problem has been solved by the development of a simple and rapid stick test based on an enzyme immunoassay.[40] However, a number of problems remain to be examined. A more thorough world-wide evaluation is necessary to ascertain which of the toxins can be detected by the test and which cannot. If, as one might suspect, not all of the toxins respond to the specific antibody in the present test kit, antibodies of the individual toxins will have to be produced by conjugating a derivative based on a unique functional group in the molecule. Despite these caveats, distinction of ciguatoxic and untainted fishes on the boat or in the market is a nearly achieved reality.

Therapy

The recent serendipitous breakthrough with the discovery of the mannitol infusion treatment of ciguatera[44,45] is a long-awaited "miracle drug" for ciguatera-endemic areas. It needs to be followed up with scientific studies into its mechanism of action, which may have the additional benefit of increasing our fragmentary knowledge of the modes of action and binding sites of the toxins involved in ciguatera.

RESEARCH BEGETS RESEARCH

A truism in scientific research is the unfolding of new questions to be studied once an important question has been answered. The foregoing account includes a number of pertinent examples. An intriguing aspect of ciguatera research is its role as the initiator of significant research in marine natural products that bear no formal relationship to ciguatera. The following examples are largely drawn from research at the University of Hawaii, but others could no doubt be found elsewhere.

Palytoxin

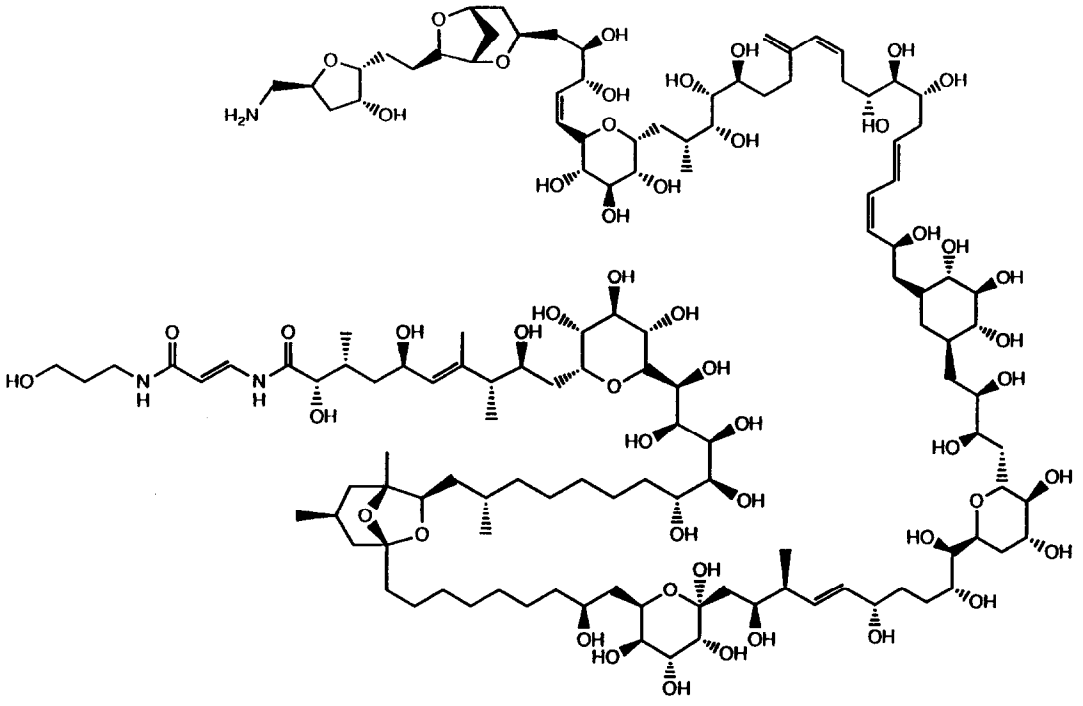
The first edition of a modern Hawaiian-English dictionary was published in 1957.[59] My late colleague and coworker in ciguatera research, Hank Banner, was an avid reader, even of dictionaries. Being aware of Randall's theory of the origin of ciguatera[28] (although it was not yet in print) he looked up *limu,* the Hawaiian word for seaweed, and came across an entry, *limu-make-o-Hana,* a seaweed reported to be deadly poisonous, from Hana, Maui. Additional information on the "reddish moss" could be found in a translation of David Malo's "Hawaiian Antiquities",[60] but securing a guide who knew the location of the tidepool (and was willing to divulge it) proved to be difficult since a strict *kapu* (taboo) associated the revealing of the location with impending disaster. Finally, biologists from the Hawaii Institute of Marine Biology succeeded in making a collection on December 31, 1961, a memorable day, indeed, since on that same afternoon a fire of undetermined origin destroyed the main laboratory building at the marine laboratory on Coconut Island, O'ahu. The collectors realized already in the field that the "*limu*" was not a plant, but an invertebrate animal, a zoantharian hexacoral, subsequently named *Palythoa toxica.* Injection into mice of the ethanol solution in which the specimen had been preserved proved dramatically that the animal contained a powerful toxin, but that it was a fast-acting toxin, which could have no bearing on ciguatera research as ciguatoxin is a notoriously slow-acting toxin.

As a result of this presumed dead-end lead, the jar containing the *P. toxica* sample and its ethanolic extract sat on the laboratory shelf for several years until R. E. (Dick) Moore's interest was aroused when he moved to Hawaii in 1963. Our initial publication[30] barely hinted of the formidable structural problem that lay ahead. When it was solved,[61] palytoxin (**4**), a molecule of composition $C_{129}H_{223}N_3O_{54}$ without repeating units and containing 64 chiral carbons, represented a monumental achievement in structure elucidation.[62] This was to be followed by Kishi's total synthesis, an equally challenging undertaking.[63] Total syntheses of significant natural products, have always been more than a convergence of creativity, organized knowledge, and discipline. They bring forth new insights, new reagents, and reveal new facets of carbon chemistry. Kishi's palytoxin synthesis has continued in this noble tradition. In addition to the unprecedented task of creating a chain of 115 carbon atoms, much of the stereochemistry had to be determined by synthesis. Among major achievements are an understanding of the conformation of C-oligosaccharides; the discovery of the coupling of an aldehyde with an iodoolefin by a $Cr^{II}Cl_2$–$Ni^{II}Cl_2$ catalyst; and a thallium hydroxide-mediated stereospecific formation of a *cis-trans*-diene from an iodoolefin and a vinylboronic acid.

The common structural feature among these powerful marine toxins is the long-chain aliphatic backbone, a C_{115} chain in the case of palytoxin. Yet palytoxin is not a polyether; none of its eight oxygen cycles are contiguous; it possesses three nitrogen atoms, a primary amine and two amides; and there are two unfunctionalized C_7 segments. The lethality of palytoxin (LD_{50}, i.p. mice) of 0.45 μg/Kg is of the same order of magnitude as those of cigua- (0.35 μg/Kg) and maitotoxin (0.13 μg/Kg), while brevetoxin B is less toxic in mammalian systems by three orders of magnitude.

Ciguatera research in Japan also spawned the discovery of toxic *Palythoa* spp. In the course of field work in the Ryukyus, Hashimoto learned that a filefish, *Alutera scripta,* contained toxic viscera and was reputed to cause ciguatera when its flesh was eaten.[64] Following this lead Hashimoto *et al.*[65] isolated from the viscera of the filefish a potent water-soluble toxin, aluterin. The gut content

consisted of a crushed zoantharian identified as *Palythoa tuberculosa.* Extraction of *P. tuberculosa* proved that the ingested zoantharian was indeed the source of aluterin, which was subsequently shown to be identical with palytoxin.[66,67] These early reports sparked Hirata's interest in palytoxin research,[68] culminating in full structural elucidation[69–71] parallel with Moore's.[30,72] Indeed, Kishi's early years in Hirata's Nagoya Laboratory anticipated his subsequent involvement in palytoxin research.



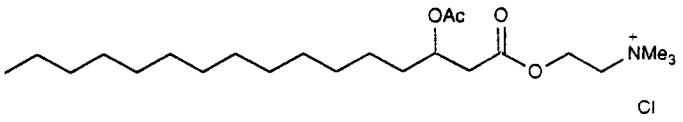
4

Pahutoxin

Halstead and Bunker[73] had reported that a boxfish (or trunkfish), *Ostracion cubicus,* was moderately ciguatoxic. Two reasons prompted us to investigate this purported boxfish toxicity. First, Halstead's experimental protocol[74]—blending of fish tissue in water—would be unlikely to extract a toxin which we knew to be lipid-soluble. Secondly, Brock[75] had shown earlier that a boxfish, *Ostracion lentiginosus,* when stressed, will release an ichthyotoxin that will kill other aquarium fishes without the usual gasping associated with fish deaths. There was a hint that the toxin causes foaming of the water. This description clearly differed from fish behavior associated with ciguatera, which had never been associated with fish distress, let alone mortality.

Brock's observations were substantiated and led to the isolation and characterization of the first marine allomone, pahutoxin.[76,77] It was indeed a saponin, but not a conventional steroidal glycoside. It proved to be a choline ester of β-acetoxypalmitic acid (5). In a later comprehensive study of eight species of trunkfishes in Australia it was shown that choline esters of β-acyloxypalmitic acid were the predominant constituents of their skin secretions.[78] These compounds

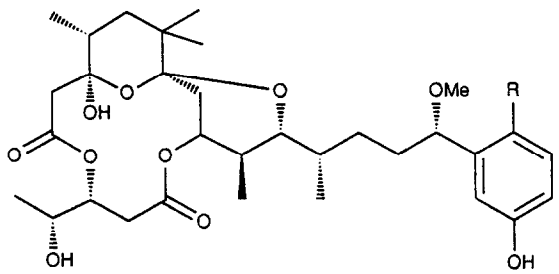
are hemolysins, but their ichthyotoxicity presumably is the result of the "soap" which blocks the gills.



5

Swimmers' itch and the aplysiatoxins

During the summer of 1958 swimmers at a few O'ahu beaches encountered a strain of a filamentous blue-green alga, *Lyngbya majuscula,* which caused contact dermatitis, particularly after the seaweed was lodged inside swimsuits.[79,80] A suggestion that there might be a connection between ciguatera and *Lyngbya* could readily be disproven by bioassay and limited experimental work. The molecular structure of the *Lyngbya* toxin, debromoaplysiatoxin (**6**) became known much later as a result of a general investigation of the chemistry of blue-green algae by Moore and coworkers.[81] The same compound, together with the related aplysiatoxin (**7**) had been isolated earlier from the sea hare *Stylocheilus longicauda* and their structures had been determined.[82,83] The dermatitis-causing property of aplysiatoxins became evident during isolation, but no connection to swimmers' itch was suspected at the time. The origin of the toxins from an algal diet had been assumed,[84] but was never proven or even seriously investigated. The finding by Fujiki *et al.*[85] that the aplysiatoxins are tumor promoters is perhaps not surprising for compounds that are known to be skin irritants.



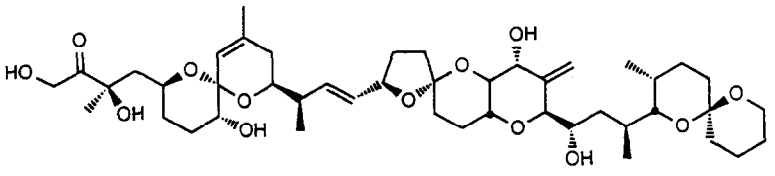
6 R = H

7 R = Br

Okadaic acid

The foregoing examples have revealed the fertile role of ciguatera research in having stimulated other studies in marine natural products. Investigation of the final topic, okadaic acid, began without the slightest inkling that a vital link with ciguatera research might develop.

Okadaic acid had been isolated from a sponge, *Halichondria okadai,* by scientists of the Fujisawa Pharmaceutical Company in Japan. Seemingly unexciting biological properties made structural elucidation commercially unattractive. Hence the pure compound (initially named halichondrin) was sent to Hawaii and studied by Tachibana, who determined its structure (**8**) as a polyether derived from a C_{38} fatty acid.[16,86] Simultaneously, the compound had also been isolated from a Caribbean *Halichondria.* [86]



8

Coincidentally, Yasumoto and coworkers were conducting surveys of benthic dinoflagellates in French Polynesia and in Okinawa.[87,88] Their aim was to determine whether any of the organisms were toxic and, if so, whether any toxins might contribute to the multi-faceted ciguatera syndrome. *Prorocentrum lima,* when grown in unicellular culture, produced three toxins, two of them soluble in ether. The major compound could be isolated as a colorless crystalline solid, which proved to be identical with the recently described okadaic acid (**8**).[89,90]

Remarkable as this turn of events was, its impact and ramifications were even more significant. Parallel chromatographic behavior which Murakami *et al.*[89] observed for okadaic acid and ciguatoxin provided the first tangible evidence that ciguatoxin also belonged to the polyether class of compounds. Secondly, the Tohoku workers also noted a striking resemblance between the chromatographic behavior of okadaic acid and toxic constituents of another dinoflagellate, *Dinophysis fortii,* the causative agent of diarrhetic shellfish poisoning.[90] A number of dinophysistoxins have since been isolated; all of them are close structural relatives of okadaic acid and have been encountered in filter-feeding shellfish in Europe and in Japan.[91,92]

While this okadaic acid saga was unfolding, Fujiki and coworkers[93] discovered yet another non-traditional (i.e. non-phorbol ester) tumor promoter: okadaic acid (**8**). It also proved to be a powerful inhibitor of phosphatases-1 and -2A *in vitro.*[94] During the past few years okadaic acid (**8**) has become a widely used probe for the study of cellular regulation.[95] It is the Cinderella story of a molecule that as halichondrin had been thought to be without much merit after its initial isolation from the sponge *Halichondria okadai.*

Okadaic acid, a C_{44} compound fashioned from a C_{38} fatty acid was the first free carboxylic acid among the polyether toxins; the gambieric acids are very recent additions.[55,56] In solution okadaic acid is strongly hydrogen-bonded to a hydroxyl and behaves more like an ester than a free acid. It is difficult to esterify. Only two of its seven ether rings are fused; five participate in spiro ketal linkages. Its lethality (i.p. mice) is comparable to that of brevetoxin B (**2**). Remarkably, its chromatographic mobility mirrors that of ciguatoxin (**1**), which proved to be a fortunate coincidence.

CONCLUSION

Natural products, isolation, molecular structures, their physical and biological properties mark the historical development of organic chemistry.

Isolation engendered few problems since alkaloids with their basic properties, hence easily isolated,—morphine, quinine, strychnine and many more—were the principal targets, based on the

conventional wisdom that bioactivity in mammalian systems is linked to the presence of nitrogen. After World War II the focus shifted to steroids, nicely coinciding with the development of chromatographic techniques, which rapidly transformed and continue to widen the scope of natural product exploration.

The road from a complex pure compound to a verifiable structure used to span decades of degradation and eventually synthesis. During all these many years melting points and mixed melting points were the cornerstone of physical properties, joined at the turn of the century by primitive UV spectrometers, and shortly after WW II by single beam IR spectrometers. (The penicillin structure during WW II was advanced greatly by the use of home-built instruments constructed by physicists.)

Long and arduous as this road was, it was rich with the discovery of new reactions, new theoretical concepts (Woodward's Rules, the nature of alicyclic rings, chiroptical theories and methods, to name but a few), and new physical methods, thus enriching all of chemistry as well as other natural sciences.

All of this has changed! Clever separation techniques, fantastic instrumental methods, sophisticated bioassays, computerized search tools, allow us to decipher a three-dimensional structure in days or weeks rather than decades.

Or has it? The foregoing Perspective shows that elucidation of a structure can still be a major challenge. It still encourages new developments in separation and instrumental techniques and offers new synthetic targets. But it also shows that while a structure is being born, seemingly unrelated events will intrude, perhaps contribute, and in the end assume lives of their own.

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