Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam

Paul Alan Cox*[†], Sandra Anne Banack*[‡], and Susan J. Murch*

*Institute for Ethnobotany, National Tropical Botanical Garden, Kalaheo, HI 96741; and [‡]Department of Biological Science, California State University, Fullerton, CA 92834

We here report biomagnification (the increasing accumulation of bioactive, often deleterious molecules through higher trophic levels of a food chain) of the neurotoxic nonprotein amino acid β-methylamino-L-alanine (BMAA) in the Guam ecosystem. Free-living cyanobacteria produce 0.3 μg/g BMAA, but produce 2–37 μg/g as symbionts in the coralloid roots of cycad trees. BMAA is concentrated in the developing reproductive tissues of the cycad *Cycas micronesica*, averaging 9 μg/g in the fleshy seed sarcotesta and a mean of 1,161 μg/g BMAA in the outermost seed layer. Flying foxes (*Pteropus mariannus*), which forage on the seeds, accumulate a mean of 3,556 μg/g BMAA. Flying foxes are a prized food item of the indigenous Chamorro people who boil them in coconut cream and eat them whole. Chamorros who die of amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC), a neurodegenerative disease with symptoms similar to amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease, have an average of 6.6 μg/g BMAA in their brain tissues. The biomagnification of BMAA through the Guam ecosystem fits a classic triangle of increasing concentrations of toxic compounds up the food chain. This may explain why the incidence of ALS-PDC among the Chamorro was 50–100 times the incidence of amyotrophic lateral sclerosis elsewhere. Biomagnification of cyanobacterial BMAA may not be unique to Guam; our discovery of BMAA in the brain tissue from Alzheimer's patients from Canada suggests alternative ecological pathways for the bioaccumulation of BMAA in aquatic or terrestrial ecosystems.

The unique amino acid β-methylamino-L-alanine (BMAA) is previously known only to occur in cycads, nonflowering seed plants of ancient lineage. Discovered in the genus *Cycas* by Vega and Bell (1), BMAA is a nonprotein amino acid and structurally appears as a methylated alanine. The source of BMAA in cycads has been unknown. However, given its profound neurotoxic properties, BMAA may function in cycads as a chemical deterrent to herbivory. Because the indigenous Chamorro people consume tortillas made from cycad seed flour, Spencer and his coworkers (2) suggested that BMAA might be a cause of amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC), a progressive neurological disease of the Chamorro with aspects of amyotrophic lateral sclerosis, Alzheimer's disease, and parkinsonism dementia. Duncan *et al.* (3), however, argued that massive amounts of flour would have to be consumed to generate a progressive neurological disease among the Chamorro people.

Biomagnification (the increasing accumulation of bioactive, often deleterious molecules through successively higher trophic levels of a food chain) has recently been proposed by Cox and Sacks (4) as a mechanism that could generate sufficiently high doses of cycad neurotoxins to produce neurological disease among the Chamorro people. Flying foxes, large bats of the genus *Pteropus* that feed on cycad seeds (5), were hypothesized as a possible sources of biomagnified cycad neurotoxins in the Chamorro diet. This hypothesis has been partially corroborated by the recent discovery of high concentrations of BMAA in

museum specimens of the Guam flying fox *Pteropus mariannus mariannus* (6).

During an investigation of distribution patterns of BMAA in various tissues of the endemic Guam cycad *Cycas micronesica* Hill (7), we found BMAA in morphologically specialized, positively geotropic "coralloid" roots but not in unspecialized, negatively geotropic roots. Other trophic levels in the Guam food chain also show the presence of BMAA. We here sought to discover (*i*) the ultimate source of BMAA in cycads (*C. micronesica*), (*ii*) the mechanisms of ecological amplification of BMAA in the food chain, and (*iii*) whether BMAA present in the Chamorro diet can cross the human blood–brain barrier. We cultured the endosymbionts harbored in coralloid cycad roots (8) to see whether these nitrogen-fixing cyanobacteria produce BMAA. We also tested other cyanobacterially unrelated plant species with cyanobacterial endosymbionts to see whether they contained BMAA. Other nonprotein amino acids, but not BMAA, have been identified in cyanobacteria (9). We also tested the brain tissues of Chamorros who died from ALS-PDC for the presence of BMAA.

Materials and Methods

The cyanobacterial symbionts from the infected coralloid roots of *C. micronesica* Hill, harvested from vouchered specimens in the National Tropical Botanical Garden (Kalaheo, Kauai, HI), were grown as axenic cultures. Root tissues were surface sterilized by immersion in a solution of 70% ethanol for 3 min, followed by a 30-min immersion in 1.6% sodium hypochlorite with two drops of surfactant and three sequential washes with sterile deionized water. Root explants (1–2 cm long) were excised and cultured onto standard BG-11 medium, pH 7.1, solidified with gellam gum (Sigma). Cultures were incubated in a controlled environment room with a 16-h photoperiod at a light intensity of 35–45 μmol/m^2 per s and temperatures of 25–30°C. After 7–10 days of culture, the proliferation of cyanobacteria was clearly visible. The absence of residual BMAA from root tissue was ensured by the serial subculture of individual colonies. Histological verification of culture purity was conducted before chemical analyses. The cyanobacterial colonies seemed to be generally devoid of heterocysts, and prolific filamentous growth was observed. BMAA was quantified in 200-mg samples of actively growing cyanobacteria, cycad tissues, *Azolla* plants (collected near Hanapepe, Kauai, HI), *Gunnera* (collected from Mount Wailaleale, Kauai, HI), and samples of the superior frontal gyrus from the brains of six ALS-PDC patients from Guam, two Alzheimer's patients from Canada, and two asymptomatic Chamorros, as well as 13 individuals who died from causes unrelated to neurodegeneration. All samples were ho-
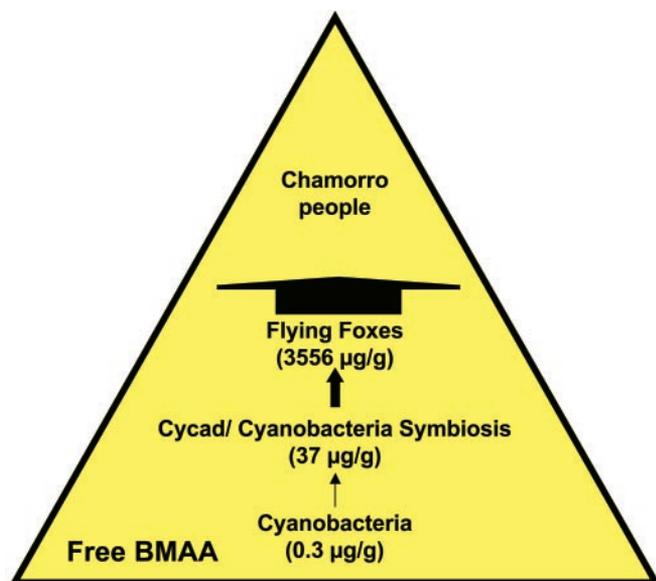

Fig. 1. Biomagnification of cyanobacterial BMAA in Guam. The widths of the arrows are proportional to the concentration of free BMAA delivered to the next higher trophic level.

mogenized twice in 0.1 N trichloroacetic acid and centrifuged at $15,800 \times g$ for 3 min to precipitate proteins and extract free amino acids. Sample extracts of free amino acids were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and amino acids quantified via HPLC separation as published (6). The presence of BMAA in the samples as well as the identity and purity of the BMAA peak in the HPLC separation was verified by liquid chromatography–mass spectroscopy by using an Agilent 1100 HPLC coupled with a variable wavelength diode-array detector and an SL single quadrapole mass spectrometer with an atmospheric pressure ionization source by using the electrospray ionization interface. Compounds were separated on a Waters SymetryShield RP-18 column heated at 30°C with a linear gradient elution of CH_3CN (10–40%) in water. The diode-array detector detected compounds at 254 nm with a full spectral scan from 190 to 450 nm and 0.5-nm resolution within a semimicro flow cell. Nitrogen gas was purified and supplied to the electrospray ionization interface with a nebulizing pressure of 35 psi, and two distinct modes were used for detection of compounds within the mass spectrometer. The initial signal was determined in positive scan mode with a 100- to 600-Da range at 50-V fragmentor voltage. BMAA was identified through selective ion monitoring in the positive ion mode with a dwell time of 45 ms and a 150-V fragmentor voltage. For both signals, the capillary voltage was 3 kV, and the electron multiplier voltage gain was 1 V. The cycle time was 0.82 s per cycle, split 50% for each of the two mass spectroscopy signals.

Results

The accumulation of BMAA in ascending trophic levels of the Guam ecosystem fits classic models of biomagnification (Fig. 1). We report the results by trophic level, beginning with the lowest.

Cyanobacteria. If nitrogen can be considered as a key ecological currency that flows through the ecosystem, then nitrogen-fixing cyanobacteria (10) are at the base of the nitrogen pyramid (Fig. 1). Axenic cultures of *Nostoc* isolated from coralloid roots of *C. micronesica* were found to produce 0.3 μg/g BMAA.

Root/Cyanobacterial Symbiosis. BMAA was not detected in roots with normal morphology, but is abundant in positively geotropic

coralloid roots, whose prolific branching causes them to resemble small clumps of coral near the soil surface (Fig. 2*b*). Cyanobacteria live as endosymbionts in the coralloid roots, invading a specialized region of the root (Fig. 2*c*). BMAA levels vary with root maturity; uninfected coralloid roots have no BMAA, coralloid roots with new flourishing infections have 37 μg/g, and coralloid roots with abundant but senescent infections have 2 μg/g (7). Before our work, BMAA had previously been found only in cycads, so to corroborate cyanobacteria as the source of BMAA in cycads, we examined two unrelated plant species with cyanobacterial symbionts (11). *Azolla filiculoides*, a floating fern with cyanobacteria symbionts in its leaves (12), has 2 μg/g BMAA, whereas *Gunnera kauaiensis*, a large-leafed angiosperm (13, 14) with cyanobacterial symbionts in the petiolar tissue, has 4 μg/g BMAA in the petioles. *Azolla* is commonly used in Southeast Asia as a green manure in rice paddies.

Cycads. Cycads have varying levels of BMAA in different tissues, with the highest concentrations in the developing reproductive organs (7). The fleshy sarcotesta of the seeds has a mean of 9 μg/g BMAA, and the outermost layer of the seed has a mean of 1,161 μg/g BMAA; both of these tissues are consumed by flying foxes. The gametophytes, or seed kernels, which are processed into flour by the Chamorro people (and are not typically eaten by flying foxes), have 240 μg/g BMAA before processing.

Flying Foxes. Cycad seeds are one of the top 10 food items of Guamanian flying foxes (5). Museum specimens of Guamanian flying foxes of the species *P. mariannus mariannus* have a mean of 3,556 μg/g BMAA (6). Flying foxes are served at traditional Chamorro feasts where they are prepared by boiling and then eaten in their entirety including brains, viscera, fur, and wing membranes (5). The immense appetite of the Chamorro people for flying foxes resulted in *P. mariannus mariannus* being placed on the endangered species list, after the other Guamanian flying fox species, *Pteropus tokudae*, went extinct (15).

Chamorro People. The Chamorro people celebrate the consumption of flying foxes as one of the key components of their culture. Consumption of flying foxes greatly accelerated when the Chamorros gained access to firearms and disposable incomes as Guam was developed as a military base (5). We found a mean of 6 μg/g free BMAA in brain tissues from the frontal cortex of six Chamorro patients who died from ALS-PDC. We also found a mean of 6.6 μg/g BMAA in brain tissues from the frontal cortex of two patients from Canada who died from Alzheimer's disease (Fig. 3). Our studies were based on a subset of tissues that have been extensively studied (16) and consisted of patients with long clinical histories and histochemical evidence of progressive neurodegeneration on autopsy. BMAA was not found in brain tissues of a group of 13 individuals who died of causes unrelated to neurodegeneration. Clinical details of these cases and data from controls will appear elsewhere.

Discussion

The biomagnification of BMAA through the Guamanian ecosystem, with two orders of magnitude increase of concentration at each organismic level (Fig. 1) from cyanobacteria (0.3 μg/g) to cycad (37 μg/g) to flying fox (3,556 μg/g), is similar to the biomagnification of DDT, PCBs, and other lipophilic pesticides or pollutants up the food chain. BMAA is not lipophilic, however, nor is it a consequence of pesticides or industrial pollutants. BMAA is a naturally occurring, nonprotein amino acid that is produced by cyanobacteria. Its possible ecological function in cyanobacteria is unclear. Although each molecule of BMAA has two nitrogen atoms, BMAA seems to play only a minor role as a mechanism of nitrogen transfer within the coralloid root/cyanobacterial symbiosis, as the ratio of the
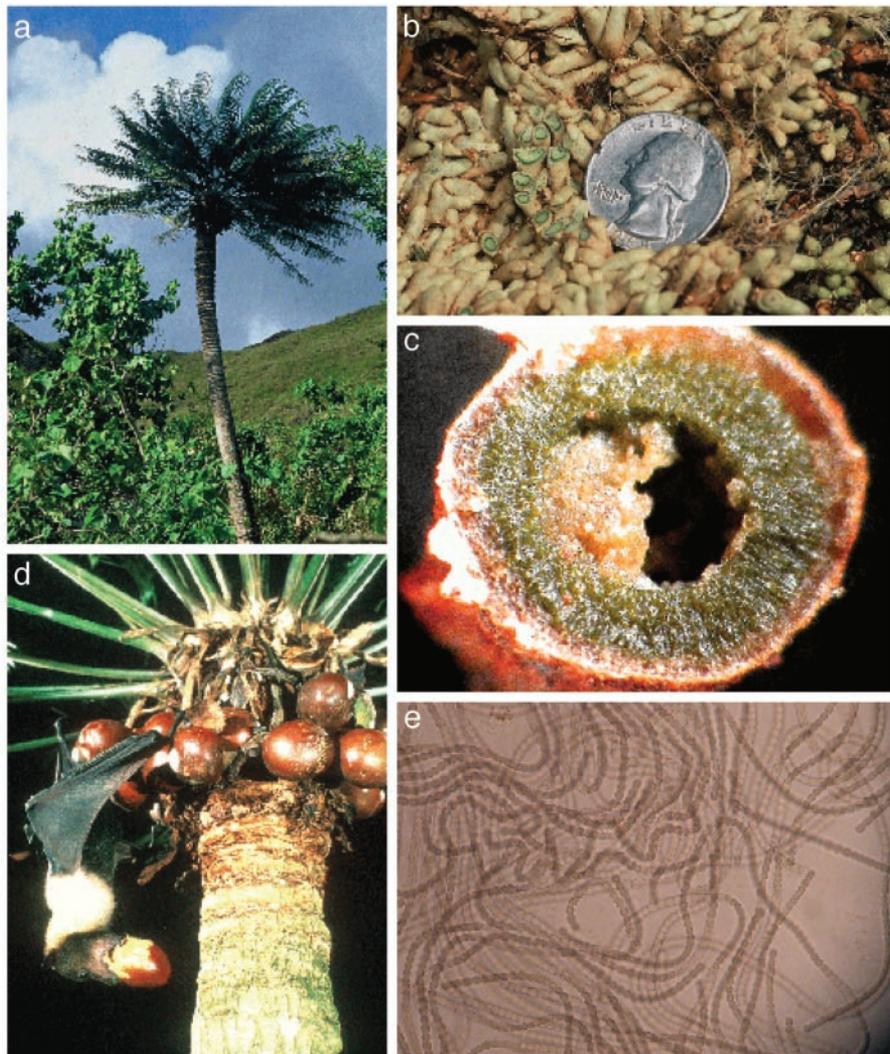
Fig. 2. *C. micronesica* Hill. (*a*) Habit in South Guam as a 4-m-tall unbranched tree. (*b*) Positively geotropic coralloid roots with tips cut to show zone of cyanobacterial invasion. (*c*) Cross section of coralloid root showing green ring of cyanobacterial growth. (*d*) *P. mariannus* feeding on fleshy sarcotesta of seed (photo courtesy of Merlin Tuttle, Bat Conservation International). (*e*) Cyanobacteria of the genus *Nostoc* cultured from the coralloid roots.

concentration of GLU to BMAA in coralloid roots is high, ranging from 255:1 to 11,494:1 (7). The high concentration of BMAA in the developing reproductive organs of *C. micronesica* (1,546 μg/g BMAA in the immature male sporangia and 1,161 μg/g BMAA in the outer integument layer of the seed sarcotesta) suggests that BMAA may serve as an antiherbivory compound in cycads (7). If true, this indicates that the few, but taxonomically diverse plant species that have established symbiotic relationships with cyanobacteria including primitive seed plants (cycads), aquatic ferns (*Azolla*), and flowering plants (*Gunnera*), may derive antiherbivory protection as well as nitrogen fixation as a benefit from the symbiosis.

Flying foxes, however, may suffer increased mortality or motor neuron impairment from the BMAA they accumulate. Because there are no native predators of flying foxes in Guam, there is little likelihood that BMAA functioned in them as an antipredator deterrent. However, if BMAA is indeed a "slow toxin" as suggested by Spencer and his coworkers (17), flying foxes in nature may not live long enough to succumb to progressive motor neuron disease from this potent excitotoxin. Controlled feeding experiments with living flying foxes, perhaps from a closely related species (as *P. mariannus* is highly endangered), may help to resolve these issues.

BMAA, which originates with cyanobacteria, accumulates through the Guam food chain (Fig. 1) and is then inadvertently ingested by the Chamorro people during their traditional feasting on flying foxes. We suggest that BMAA now be reinvestigated as a primary cause of progressive motor neuron disease among the Chamorro population. Given the high concentrations of BMAA in flying foxes (6), and given that the Chamorros are the only culture in the Mariannas islands to eat flying foxes, it is of interest that incidence rates of ALS-PDC among the Chamorros once peaked at 50–100 times the incidence of amyotrophic lateral sclerosis elsewhere (18). Interviews with Chamorros indicate that flying foxes are the most favored food item (5) with some informants reported eating multiple flying foxes per week when they were abundant. Consumption of *P. mariannus* waned, however, as the flying fox populations drastically declined because of overhunting.

The neurotoxin derived from eating a single flying fox, which could yield a BMAA dose equivalent to eating 1,014 kg of cycad flour, would eclipse any alternative source of BMAA in the Chamorro diet, particularly among those who ate flying foxes with some frequency. Comparing individual lifetime BMAA doses derived from flying foxes versus cycad flour is difficult, but consumption of flying foxes peaked during the mid-20th century
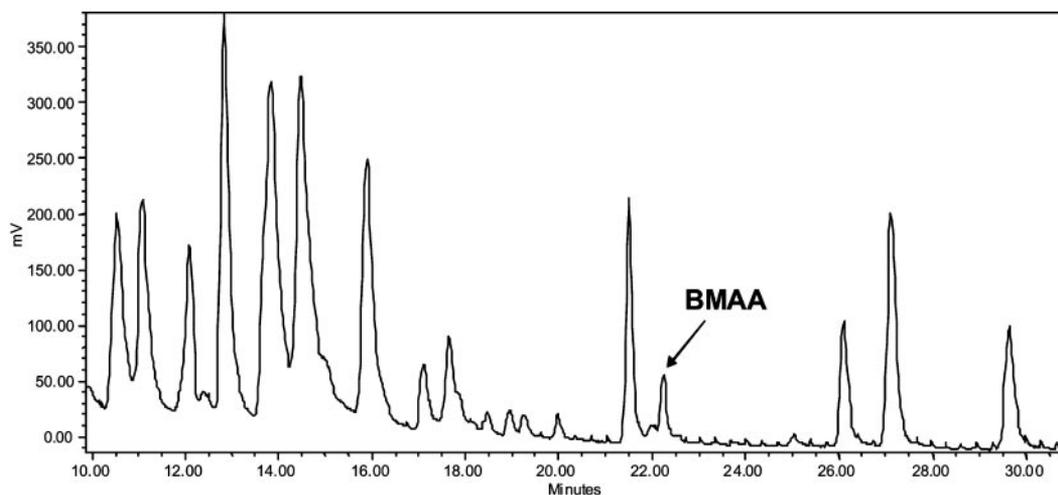
Fig. 3. Representative chromatogram depicting BMAA in the frontal superior gyrus tissue of a Canadian Alzheimer's patient; peak is consistent with BMAA peaks from cyanobacteria, cycads, flying foxes, and Chamorro ALS-PDC patients.

when both firearms and disposable cash were available to the Chamorros (5), whereas consumption of cycad flour likely remained constant from earliest European contact through the early 20th century. Hunter yields in pre-European contact times were likely low and success was infrequent. Netting animals at night time from trees or snagging sleeping animals during the day with thorns were probably highly uncertain enterprises (5), given that flying foxes have acute visual and auditory awareness. Our search of the records of early explorers and missionaries have failed to yield any account of a pandemic paralytic disease among the Chamorro before the 20th century, which suggests that the apparent pulse of ALS-PDC during the 20th century is likely due to an increase in the consumption of flying foxes in the aftermath of the military occupation of Guam. The demise of the flying fox population was mirrored after 10–20 years by the decline in incidence rate of ALS-PDC among the Chamorros.

Unlike mercury in Minamata Bay or PCBs in the Færoe islands (19, 20) BMAA in the Guam ecosystem is of natural origin, but unlike such lipophilic pollutants, BMAA must be biomagnified through a novel mechanism. Its increasing accumulation (Fig. 1) up the food chain suggests the existence of a novel endogenous reservoir of BMAA within plant and animal tissues. Earlier reports indicate that 90% of injected BMAA is

not eliminated from either urine or feces in rats (21). Epidemiologically, ALS-PDC is characterized by a pronounced latency; perhaps the BMAA reservoir functions as a ''slow toxin'' (16). A known GLU agonist at the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid site of motor neurons (22), BMAA also modifies N-methyl-D-aspartate receptors (23).

Our finding of BMAA in the frontal cortex of two Alzheimer's patients from Canada (7 μg/g) suggests that bioaccumulation of cyanobacterial BMAA may occur through different food chains in areas far from Guam. Because frequency of illness in a population exposed to neurotoxins is a function of dose (24), even low levels of progressive neurological illness might be related to low concentrations of BMAA in water supplies contaminated by cyanobacteria (25). As BMAA was known previously only from cycads, and cycads do not occur naturally in Canada, cyanobacteria should be investigated as a possible source of any BMAA found in Canadians suffering from progressive neurological disease.

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