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Case Report

Identification of the potent toxin bongkrekic acid in a traditional African beverage linked to a fatal outbreak

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ABSTRACT

In January 2015, 75 people died and 177 were hospitalized in the Mozambique village of Chitima after attending a funeral. The deaths were linked to the consumption of a traditional African beverage called pombe. Samples of the suspect pombe were subjected to myriad analyses and compared to a control sample. Ultimately, non-targeted liquid chromatography–mass spectrometry screening revealed the presence of the potent toxin bongkrekic acid, and its structural isomer, isobongkrekic acid. Quantitative analysis found potentially fatal levels of these toxins in the suspect pombe samples. Bongkrekic acid is known to be produced by the bacterium *Burkholderia gladioli* pv. *cocovenenans*. This bacterium could not be isolated from the suspect pombe, but bacteria identified as *B. gladioli* were isolated from corn flour, a starting ingredient in the production of pombe, obtained from the brewer's home. When the bacteria were co-plated with the fungus *Rhizopus oryzae*, which was also isolated from the corn flour, synergistic production of bongkrekic acid was observed. The results suggest a mechanism for bongkrekic acid intoxication, a phenomenon previously thought to be restricted to specific regions of Indonesia and China.

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1. Introduction

Bongkrekic acid, or 20-(carboxymethyl)-6-methoxy-2,5,17-trimethyl-2*E*,4*Z*,6*R*,8*Z*,10*E*,14*E*,17*S*,18*E*,20*Z*-docosaheptaenedioic acid (CAS Registry number 11076-19-0), is a colorless tricarboxylic fatty acid with molecular formula $C_{28}H_{38}O_7$ and molecular weight 486.61 g/mol. There also exists an isomer, isobongkrekic acid, which exhibits *E* stereochemistry at the position 20 double bond. The structures of bongkrekic and isobongkrekic acid are shown in Fig. 1. These molecules are extremely potent toxins produced under certain conditions by the bacterium *Burkholderia gladioli* pv. *cocovenenans* [1], with isobongkrekic acid being produced at roughly 5% abundance relative to bongkrekic acid [2]. Current literature indicates that poisoning events involving bongkrekic acid have been reported in two specific geographic regions: the Banjumas province of Central Java, Indonesia and the Heilongjiang, Jilin, and Liaoning provinces of northeastern China [1]. These poisonings were due to the consumption of two locally-produced fermented foods: tempe bongkrek and fermented corn flour, respectively. Tempe bongkrek refers to coconut presscake that is fermented with *Rhizopus microsporus* var. *oligosporus* or *Rhizopus oryzae* fungus [1,3,4]. It is from tempe bongkrek that *B. gladioli* pv. *cocovenans* was first isolated and its toxin discovered [1].

Consumption of sufficient quantities of bongkrekic acid initially results in hyperglycemia, followed quickly by hypoglycemia that depletes the glycogen reserves in various tissues, including the heart and liver [5]. A variety of symptoms are observed 4–6 h after consumption before lapsing into a coma, with death occurring 1–20 h after symptom onset in fatal cases [5]. In such cases, there are no known treatments capable of preventing death [1]. The oral LD_{50} of bongkrekic acid in mice has been reported as 3.16 mg/kg [6]. It has been observed that several milligrams of bongkrekic acid can be produced per gram of food in only 48 h [5]. It is important to note that cooking foods contaminated with bongkrekic acid does not render them safe for consumption: although the bacteria are destroyed, the toxin itself is heat-stable [7,8].

This case report describes the unexpected identification and quantitation of bongkrekic acid in samples originating from an entirely different continent than those where such intoxications have been previously reported. The isolation and identification of the bacterium *B. gladioli* is also described. The results suggest a natural means of contamination, as opposed to intentional

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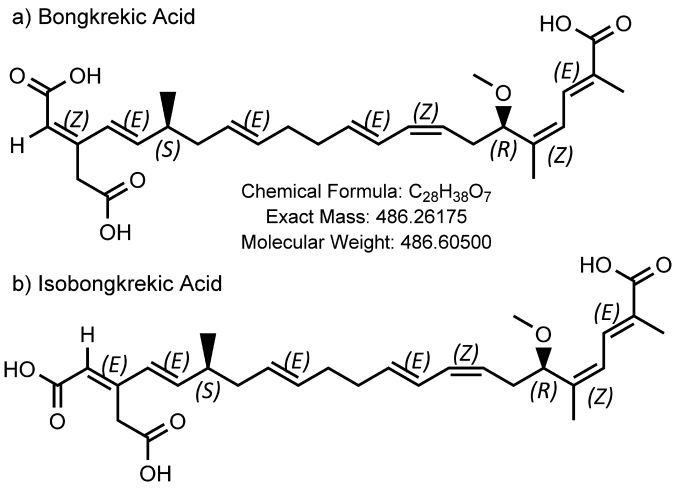

Fig. 1. Structures of (a) bongkrekic acid and (b) isobongkrekic acid.

contamination, as was originally hypothesized. The results also have far-reaching public health implications, given the potency of the toxin, the difficulty in its detection, the lack of an effective treatment, and the manner in which it is produced.

2. Case history

On January 9, 2015, over 200 people fell ill in the village of Chitima in the Tete province of western Mozambique. Symptoms included gastrointestinal distress, diarrhea, vomiting, muscle pain, and labored, rapid breathing [9–11]. Within a short time, 75 of these people had died. The illnesses and deaths were linked to the consumption of a traditional beer called pombe at a funeral [11]. The brewer, who was regarded to have made the best pombe in the area, was among the victims who died and initial suspicion was directed toward one of her competitors. This speculation was fueled by the discovery of an open, unlabeled, 200-mL bottle weighted to the bottom of the drum used to brew the pombe, and by the subsequent disappearance of the drum [10–12]. This suspicion was propagated by the suggestion that the poison in question was crocodile bile [12]. However, it has been established that crocodile bile is not toxic [13]; it was subsequently proposed that either organophosphate pesticides used in the region or toxic plant species could have caused the symptoms exhibited by the victims [10]. Despite the consistency of the symptoms exhibited, the cause of those symptoms and associated deaths could not be rapidly determined.

3. Materials and methods

3.1. Samples

Pombe brewed in Tete, Tete Province, Mozambique was collected in two 50-mL conical centrifuge tubes as a control sample. Five portions of suspect pombe were collected from the brewer's home and a vending stall in Chitima, Tete Province, Mozambique. The suspect pombe was collected in 15-mL conical centrifuge tubes and shipped frozen. Upon receipt, all samples were stored at approximately 0 °C. A sample of corn flour from the brewer's home was also collected and shipped to the laboratory for analysis.

3.2. Standards

Bongkrekic acid was purchased as a solution from Sigma–Aldrich (St. Louis, Missouri, USA); per the certificate of analysis, this solution had a concentration of 1.2000 mg/mL with 96.60% purity. A corrected concentration of 1.1592 mg/mL was used for all quantitative determinations. A 10.433 μg/mL stock standard solution was prepared by dilution with HPLC-grade acetonitrile (CH_3CN). Toxoflavin was purchased as a powder from Sigma–Aldrich; per the certificate of analysis, this powder was 98.3% pure and a correction factor was not applied. A 987.1 μg/mL stock standard solution was prepared by dissolution of 3.646 mg of toxoflavin in 4.0629 g of dimethylsulfoxide (density at 20 °C = 1.1 g/mL). Preparation of all working standard solutions was carried out using 50/50 HPLC-grade CH_3CN/18.2 MΩ cm H_2O as the solvent.

3.3. Reagents

HPLC grade CH_3CN, 1 mL ampules of "Optima" LC–MS grade formic acid, and 99% purity ammonium formate were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Dimethylsulfoxide was purchased from Sigma–Aldrich. 18.2 MΩ cm H_2O was generated using a Millipore (Darmstadt, Germany) Milli-Q Advantage A10 water purification system. Trypticase soy broth was purchased from Remel (Lenexa, Kansas, USA). All prepared media, including *Burkholderia cepacia* (BC), oxidation/fermentation-polymyxin-bacitracin-lactose (OFPBL), and potato dextrose (PDA) agars, were purchased from BD Diagnostics (Franklin Lakes, New Jersey, USA).

3.4. Preliminary screening

The samples were subjected to extensive examination: microscopic and spectroscopic analyses; metals analysis; cyanide, fluoride, and formaldehyde analyses; targeted gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) screens for fluoroacetate, glycols, mycotoxins, pesticides, and common poisons; enzyme-linked immunosorbent assays for ricin and botulinum neurotoxin; headspace GC–MS for solvents including methanol and methylene chloride; non-targeted GC–MS and LC–MS screens; and microbiological testing. The targeted analyses detected only trace levels of aflatoxin B1, aflatoxin B2, and beauvericin. However, comparison of the non-targeted LC–MS data to that obtained for a control sample of pombe produced in another location revealed significant differences.

3.5. Non-targeted LC–MS analysis

Duplicate 500 μL portions of each pombe sample were combined with 500 μL of HPLC-grade CH_3CN in 15 mL conical centrifuge tubes. These solutions were mixed briefly with a vortex mixer, shaken for 20 min, and centrifuged at 2000 × *g* for 10 min. These solutions were then filtered through 17 mm, 0.2 μm PTFE syringe filters.

The analysis was performed on a Thermo Scientific Dionex (Germering, Germany) UltiMate 3000 liquid chromatograph (LC) equipped with an Agilent Technologies (Santa Clara, California, USA) ZORBAX SB-C18, 1.8 μm, 2.1 × 150 mm column. The LC was coupled to a Thermo Scientific (Bremen, Germany) Q Exactive mass spectrometer (MS). Data were acquired and analyzed using Xcalibur 2.2 software from Thermo Scientific. Mobile phase flowed at a constant rate of 0.200 mL/min. Gradient elution was performed with initial conditions of 95% A (18.2 MΩ cm H_2O with 0.1% formic acid) and 5% B (HPLC-grade CH_3CN with 0.1% formic acid), ramped to 95% B in 20 min, and held for 15 min. Each injection was preceded by a 7 min equilibration at the initial conditions. The injection volume was 2.0 μL and the column was held at 40 °C.

The MS was equipped with a heated electrospray ionization (HESI) source operated with sheath gas flow rate of 40 units, aux