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Peak AAA fatty acid homolog contaminants present in the dietary supplement L-Tryptophan associated with the onset of eosinophilia-myalgia syndrome

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ABSTRACT

The eosinophilia-myalgia syndrome (EMS) outbreak that occurred in the USA and elsewhere in 1989 was caused by the ingestion of Showa Denko K.K. (SD) *L*-tryptophan (L-Trp). "Six compounds" detected in the L-Trp were reported as case-associated contaminants. Recently the final and most statistically significant contaminant, "Peak AAA" was structurally characterized. The "compound" was actually shown to be two structural isomers resulting from condensation reactions of L-Trp with fatty acids derived from the bacterial cell membrane. They were identified as the indole C-2 *anteiso* (AAA_1-343) and *linear* (AAA_2-343) aliphatic chain isomers. Based on those findings, we utilized a combination of on-line HPLC-electrospray ionization mass spectrometry (LC–MS), as well as both precursor and product ion tandem mass spectrometry (MS/MS) to facilitate identification of a homologous family of condensation products related to AAA_1-343 and AAA_2-343. We structurally characterized eight new AAA_1-XXX/AAA_2-XXX contaminants, where XXX represents the integer molecular ions of all the related homologs, differing by aliphatic chain length and isomer configuration. The contaminants were derived from the following fatty acids of the bacterial cell membrane, 5-methylheptanoic acid (*anteiso*-C8:0) for AAA_1-315; *n*-octanoic acid (*n*-C8:0) for AAA_2-315; 6-methyloctanoic acid (*anteiso*-C9:0) for AAA_1-329; *n*-nonanoic acid (n-C9:0) for AAA_2-329; 10-methyldodecanoic acid (*anteiso*-C13:0) for AAA_1-385; *n*-tridecanoic acid (*n*-C13:0) for AAA_2-385; 11-methyltridecanoic acid (*anteiso*-C14:0) for AAA_1-399; and *n*-tetradecanoic acid (*n*-C14:0) for AAA_2-399. The concentration levels for these contaminants were estimated to be 0.1–7.9 µg / 500 mg of an individual SD L-Trp tablet or capsule The structural similarity of these homologs to case-related contaminants of Spanish Toxic Oil Syndrome (TOS) is discussed.

1. Introduction

In late 1989 the USA Food and Drug Administration (FDA) issued a nationwide alert that advised consumers to stop consumption of manufactured *L*-Tryptophan (L-Trp) food products. The FDA also requested a recall of all L-Trp dietary supplements sold over-the counter. The resultant cause of such precipitous action was an outbreak of what became known as eosinophilia-myalgia syndrome (EMS) (Belongia et al., 1990; Belongia, 2004; Eidson et al. 1990; Kilbourne, 1992; Swygert et al., 1990;). EMS is a chronic, multisystemic disorder characterized by peripheral eosinophilia and sub-acute onset myalgia (Hertzman et al., 2001; Martin et al., 1990). In the aftermath over 1500

Abbreviations: AAA_1-XXX, all fatty acid homologs of AAA_1 family; AAA_2-XXX, all fatty acid homologs of AAA_2 family; CDC, USA centers for disease control and prevention; CE, collision energy; CES, collision energy spread; CID, collision induced dissociation; DoU, degree(s) of unsaturation; ESI, electrospray ionization; EMS, eosinophilia-myalgia syndrome; FDA, USA food and drug administration; HPLC, high performance liquid chromatography; IDA, information-dependent acquisition; LC–MS, microcapillary HPLC-mass spectrometry; LC-UV, HPLC with UV detection; L-Trp, *L*-tryptophan; MS/MS, tandem mass spectrometry; MS^n, multi-stage mass spectrometry; NMR, nuclear magnetic resonance; O-PAP, 1-Oleyl ester of PAP; OO-PAP, 1,2-Di-Oleyl ester of PAP; PAA, 3-(phenylamino)alanine; PAP, 3-(phenylamino)-1,2-propanediol; SD, showa denko K.K; TOF, time-of-fligh,; TOS, toxic oil syndrome

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patients were afflicted with EMS, and 38 deaths were directly attributed to the consumption of L-Trp in the USA alone (Swygert et al., 1993). Numerous other EMS cases were reported in Canada, UK, Germany, Belgium, France, Israel and Japan (Hertzman et al., 1991; COT-UK, 2004). In subsequent analyses by individual USA State health departments, and the US Centers for Disease Control and Prevention (CDC), it appeared that EMS was triggered by the consumption of the dietary supplement, L-Trp. Further investigation indicated that L-Trp produced by a single company, Showa Denko K.K. (SD) of Japan was primarily responsible for the EMS outbreak (Belongia et al., 1990; Slutsker et al., 1990).

The SD L-Trp was manufactured by a fermentation process that used a genetically engineered strain of *Bacillus amyloliquefaciens* (Belongia et al., 1992; Mayeno and Gleich, 1994). The epidemic was essentially curtailed when the FDA removed the suspect L-Trp from the retail market. Analyses of the SD L-Trp by high performance liquid chromatography (HPLC) and HPLC coupled on-line with mass spectrometry (LC–MS) revealed the presence of over sixty contaminants (Toyo'oka et al., 1991; Trucksess, 1993; Williamson et al., 1997, 1998a). Careful and exhaustive epidemiological studies as well as sample lot analyses of contaminated SD L-Trp determined that six contaminants were case-associated with the onset of EMS. These case-associated contaminants were identified as Peaks UV-5, E, 200, C, FF and AAA and named/labeled as a function of their unique HPLC retention times (Hill et al., 1993; Philen et al., 1993).

Structural characterization of all "six" case associated contaminants in SD L-Trp has now been completed. Peak UV-5 was identified as 3-(phenylamino)alanine (PAA) (Goda et al.,1992; Mayeno et al.,1992). Peak E was determined to be an acetaldehyde-tryptophan condensation reaction product, namely 1, 1' ethylidenebis(tryptophan) using a combination of MS, tandem mass spectrometry (MS/MS), nuclear magnetic resonance (NMR), and synthetic organic chemistry (Mayeno et al., 1990; Smith et al., 1991). Peak 200 was identified as 2-(3-indolylmethyl)-tryptophan using both NMR (Muller et al., 1991), and a combination of LC–MS and LC–MS/MS (Williamson et al., 1997). Peak C was determined by accurate mass LC–MS, LC–MS/MS and multistage mass spectrometry (MS^n) to be 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-[2,3-b]-indole-2-carboxylic acid (Williamson et al., 1998b). Peak FF was also subjected to the same analytical protocols as Peak C and identified as 2-(2-hydroxy indoline)-tryptophan (Williamson et al., 1998b). Recently we reported the structure determination of "Peak AAA", which Hill and coworkers had described as the most statistically significant contaminant in terms of association with EMS cases (Hill et al., 1993; Philen et al., 1993). This contaminant was determined to be actually two different fatty acid derived structural isomers. The structural isomers were identified as AAA_1-343 (*S*)-2-amino-3-(2-((*S,E*)-7-methylnon-1-en-1-yl)-1*H*-indol-3-yl)propanoic acid, and AAA_2-343 (*S*)-2-amino-3-(2-((*E*)-dec-1-en-1-yl)-1*H*-indol-3-yl)propanoic acid (Klarskov et al., 2018).

The efforts to determine causal onset of EMS have focused primarily on the structure determination of SD L-Tryptophan case-associated contaminants. However, there have been alternative suggestions as to the cause of EMS. Noakes and colleagues have argued that Quinolinic Acid may play a role in "cutaneous eosinophilic disorders" and hence by association EMS (Noakes et al., 2006). Others have argued that high doses of L-Trp alone were potentially responsible for EMS onset (Gross et al., 1999; Smith and Garrett, 2005). However, it is difficult to reconcile these findings with the original epidemiological work (Belongia et al., 1990; Slutsker et al., 1990; Swygert et al., 1993) and the subsequent analytical work and conclusions of Hill and Philen on SD L-Trp (Hill et al., 1993; Philen et al., 1993). We have argued that all the current EMS data in the literature supports the original consensus that the contaminants of SD L-Trp were responsible for the EMS outbreak (Naylor, 2017). This hypothesis is further reinforced by consideration of an earlier, disease related outbreak of Spanish Toxic Oil Syndrome (TOS).

The TOS outbreak was a related manifestation of elevated eosinophils associated with food and dietary supplement consumption that occurred in 1981 (Gelpi et al. 2002). TOS was caused by the ingestion of aniline-adulterated cooking oil, fraudulently sold by Spanish street-vendors as olive oil. The clinical symptoms manifested by TOS patients closely resembled those of EMS patients and were characterized by incapacitating myalgias and elevated peripheral eosinophils. The health impact was dramatic since in excess of 20,000 individuals were affected and over 300 deaths occurred in the first twenty months of the TOS epidemic. It was further estimated that an additional ~1690 premature deaths due to the use of the tainted oil occurred during the time period 1983–1997 (Gelpi et al., 2002).

In the present work we report the structure identification of a series of fatty acid condensation product homologs produced during the fermentation process. They are derived from the reaction of SD L-Trp with the *Bacillus amyloliquefaciens* lipid membrane fatty acids. We quantify all ten AAA_1-XXX/AAA_2-XXX contaminants present in the SD L-Trp ingested by patients at the time of the epidemic. Note that "XXX" represents the integer molecular ions of all the related homologs, differing by aliphatic chain length and isomer configuration. Finally, we discuss the structural similarities of these new contaminants with those of case-related contaminants from toxic oil shown to cause TOS.

2. Materials and methods

2.1. Chemicals and reagents

LC–MS grade water, methanol and acetonitrile were purchased from Millipore-Canada Ltd (Etobicoke, ON, Canada). Formic acid and L-Trp were obtained from either Sigma (Markham, ON, Canada) or Millipore-Canada. The synthesis and structural characterization of the standard *anteiso* AAA_1-343 is described in detail elsewhere (Klarskov et al., 2018). Solid phase Sep-Pak™ C-18 cartridges were obtained from Waters Corporation (Mississauga, ON, Canada).

2.2. Showa Denko L-Trp

Dr. Rossanne Philen (CDC) provided SD case-implicated L-Trp. This sample lot was manufactured between January-June 1989, and had previously been demonstrated as case-implicated in EMS onset (Hill et al., 1993; Mayeno and Gleich, 1994; Philen et al., 1993). Sample storage and handling at the CDC has been described elsewhere (Hill et al., 1993; Philen et al., 1993). We received these samples on September 10th, 1996. All samples were kept in Fisher Scientific polypropylene centrifuge tubes with screw caps, under Nitrogen and further sealed with parafilm. These sample tubes were kept either at room temperature or at −20 °C in assorted commercial freezers and out of contact with direct light except in brief instances of sample handling and preparation for analyses. In the case of sample analyses, all samples were prepared fresh on each occasion as described in section 2.3 below.

2.2.1. Composition and stability of SD L-Trp

The L-Trp sample lots originally analyzed by Hill and Philen were provided by SD and were manufactured between January 1987, and November 1989 (Hill et al., 1993; Philen et al., 1993). Dr. Philen (CDC) provided one such sample lot to us. Our sample is now almost thirty years old. However, we have regularly evaluated the sample integrity and stability. The CDC performed the initial HPLC analysis of the sample in 1993 and they published a HPLC UV chromatogram complete with an internal standard (Hill et al., 1993). In the intervening years we have evaluated the same sample using similar HPLC conditions with either simple UV detection (Williamson et al., 1998a) or MS (Mayeno et al., 1995) and MS/MS detection (Klarskov et al., 2000, 2018; Williamson et al., 1997; Williamson et al., 1998b). In all cases the relative composition of the chromatogram has not discernibly changed based on the number of peaks detected or the relative peak intensities