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Identification of Decomposition Products of 1,1'-Ethylidenebis [L-Tryptophan], a Compound Associated with Eosinophilia-Myalgia Syndrome

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At the end of 1989 a new disease called eosinophilia-myalgia syndrome (EMS) appeared in America and West Germany. Epidemiologic investigators traced the cause to an unknown contaminant in L-tryptophan (LT) dietary supplement tablets (Swygert et al. 1990; Slutsker et al. 1990; Belongia et al. 1990). Of the more than 50 contaminants found in the LT material by high-performance liquid chromatography (HPLC) analysis, a contaminant identified as 1,1'-ethylidenebis[L-tryptophan] (EBT), also known as "peak 97" and "peak E", was the only one that had a marked association with EMS cases (Centers for Disease Control 1990; Mayeno et al. 1990; Smith et al. 1991). This suggested that EBT might be the EMS causal agent or, possibly, a marker for a change in the manufacturing process that produced the causal agent.

Others (Sakimoto et al. 1990) *(J. Ito, Y. Hosaki, Y. Torigoe, K. Sakimoto, personal communication)* have observed that within hours in an acidic environment EBT breaks down into LT, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MCCA), and an unidentified product. In this report we describe studies in which we incubated EBT in simulated gastric fluid, followed the course of its breakdown into other products, and identified all of those products.

MATERIALS AND METHODS

We used the methods of *Ito et al., (personal communication)* to synthesize EBT and MCCA. The simulated gastric fluid (SGF) was made by diluting 8 mL of concentrated HCl and 2.2 g of NaCl with 1 L of water. EBT (20 mg) was added to 10 mL of SGF and sonicated for 2 min. The solution was incubated in a 37°C water bath, and samples of 50 ul were removed at intervals during the incubation and analyzed by ultraviolet liquid chromatography and liquid chromatography-mass spectrometry (LC-MS).

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For the HPLC analyses a Whatman[1] Partisil 5 ODS-3 column (4.6 mm x 25 cm) was used with the Waters 600 solvent delivery system and the Waters 490 detector. Elution was by the following linear gradient:

time	solution A	solution B
initial	100.0	0.0
20 min	0.0	100.0
25 min	0.0	100.0
28 min	100.0	0.0
36 min	100.0	0.0

Solution A was 10.0:89.9:0.1 and solution B was 75.0:24.9:0.1 acetonitrile:water:trifluoroacetic acid (TFA). The flow rate was 1 mL/min; the column temperature was 22° C; the detector was set at 280 nm.

Peak 1, a product of EBT incubated in SGF, was isolated as described below. We dissolved 100 mg of EBT in 10 mL of SGF by sonication for 2 min. After 30 min incubation at 37° C, the solution was divided into 2 mL aliquots and frozen in dry ice. Each 2-mL aliquot, thawed just before analysis, was injected on a Waters C18 microBondapak semipreparative column (1.1 cm x 30 cm). The concave elution gradient (curve 7 on the Waters 600 system) was from 100% solution C to 100% solution D over 38 min. Solution C was 10.0% acetonitrile, 89.9% water, and 0.1% TFA; solution D was 50.0% acetonitrile, 49.9 % water, and 0.1% TFA. The flow rate was 4 ml/min. Peak 1 eluted in about 15 min between LT and EBT with baseline separation. About 0.5 mg of peak 1 was collected in a beaker as it emerged from the column. A few drops of 4 M ammonium acetate was added to bring the pH to 5.5 before the fraction was placed on dry ice. The collected material was dried by lyophilization. The dried peak 1 was not stable unless it was transferred to a desiccator with a drying agent immediately after lyophilization.

The LC-MS and LC-MS/MS analyses were performed with the Finnigan TSQ-70 triple quadrupole mass spectrometer with the thermospray LC-MS interface and the Waters 600 liquid chromatography system. For the LC-MS analyses, we used discharge ionization (DI) at 0.5 kV under the analytic chromatography conditions described above. For the flow injection analyses, we used buffer ionization with a mobile phase of 50.0% acetonitrile, 49.8% water, and 0.2% ammonium acetate. When DI was

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1. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services

used, the thermospray vaporizer was set at 120°C and the block was set at 240°C. When buffer ionization was used the vaporizer was set at 70°C and the block at 150°C. For the MS/MS analyses, the collision gas was argon; the collision cell pressure was 1.3 millitorr; and the collision offset was -10 V. The scan rate was 1 scan/sec in all modes.

Nuclear magnetic resonance (NMR) spectroscopic analysis was performed using a Varian Associates XL-300 NMR spectrometer equipped with a 7.0 Tesla superconducting magnet. All spectra were measured at the ambient temperature (24° C) in deuterium oxide. Water suppression allowed receiver gain to be increased. Other parameters included between 256 and 1024 scans collected, 4000 Hz sweep width, 7.0-microsec pulse width, and 1.0-sec delay.

Fourier transform infrared (FTIR) analysis was performed with a Nicolet Model 170SX FTIR spectrometer equipped with an array processor and mercury-cadmium-telluride (MCT) detectors. Samples were introduced to the system in KBr pellets. Spectra were recorded at 4 cm-1 resolution.

RESULTS AND DISCUSSION

Figures 1 and 2 show chromatograms of the products of EBT 15 min and 2.2 hrs, respectively, after exposure to SGF. Peak 1 was at its highest concentration between 15 min and 30 min after exposure, reaching about 5% of the total 280 nm absorbing material. After about 30 min peaks 2 and 2′ start to appear. EBT is almost completely converted to LT and peak 1 and peaks 2 and 2′ after 2 hrs. In Figure 1, the small peak that elutes immediately after EBT is an impurity in the EBT starting material. In Figure 2, the peak in front of EBT that is not baseline resolved is also probably an impurity in the starting material that is seen only after most of the EBT is broken down.

We analyzed peak 1 with Thermospray LC-MS. The LC-MS peak 1 mass spectrum contained two ions, a very strong 205 amu ion and a weak 231 amu ion. This showed that peak 1 is labile in the mass spectrometer source, breaking down to a significant degree to LT (205 amu). We did not know if the 231 amu ion was the molecular ion. Analysis of the 231 amu ion of peak 1 by LC-MS/MS produced a mass spectrum that was the same as the MS/MS mass spectrum of MCCA, the other decomposition product of EBT; this showed that peak 1 is converted to MCCA and LT in the source and indicated that the molecular ion of peak 1 was not being seen. We modified thermospray conditions by decreasing temperatures and

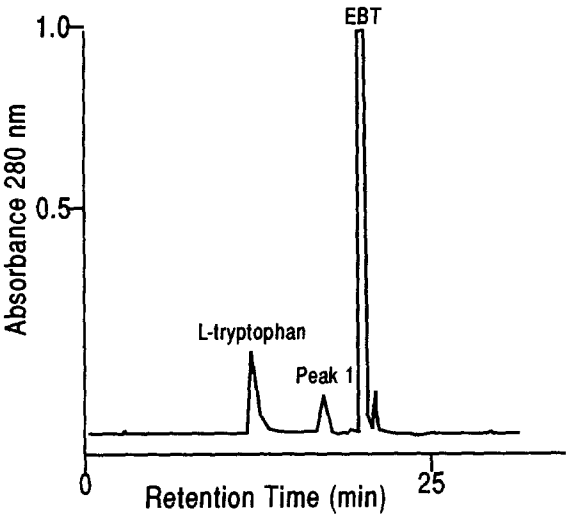

Figure 1. EBT 15 min after exposure to SGF, HPLC trace

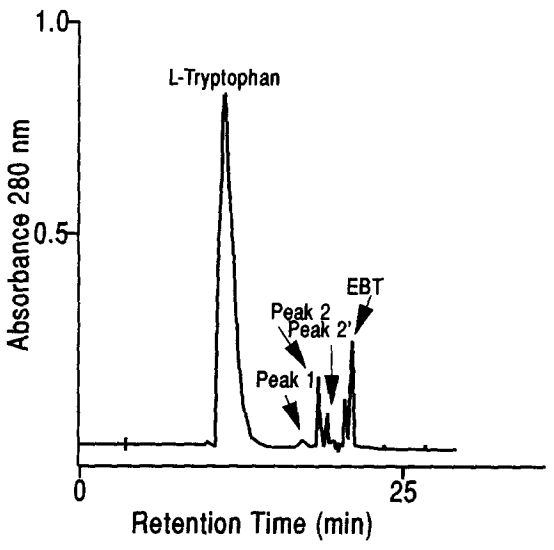

Figure 2. EBT 2.2 hr after exposure to SGF, HPLC trace

using buffer ionization instead of discharge ionization to try to avoid fragmentation of peak 1. Since sensitivity is very low under these conditions, a relatively large amount of sample was necessary. By using flow injection MS with a 0.02 mg sample of

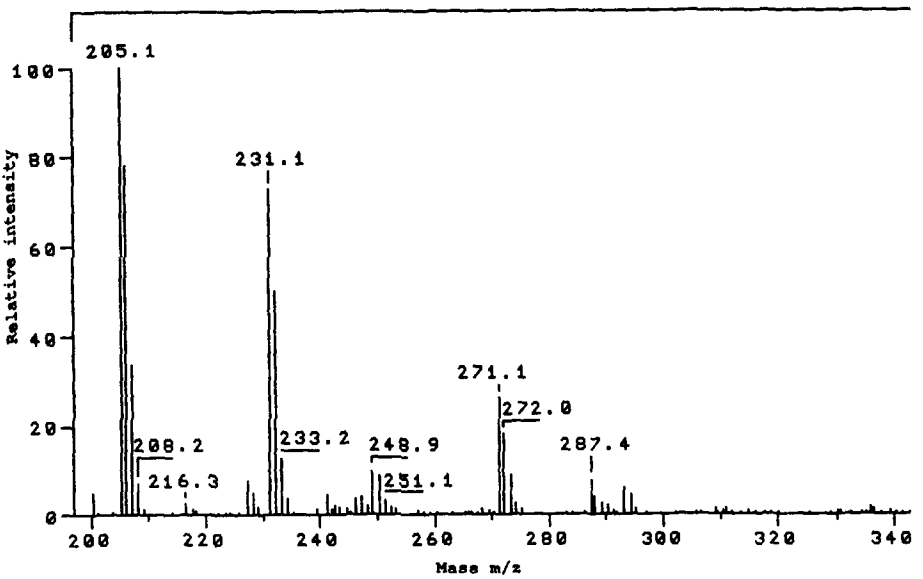

Figure 3. Mass spectrum of peak 1 analyzed at low thermospray temperatures by flow injection with buffer ionization.

purified peak 1, we obtained the mass spectrum, which is shown in Figure 3. We attributed the two highest masses (the 271 amu and 287 amu peaks) to sodium and potassium adducts of the molecular mass and identified the 249 amu peak as the MH+ ion. Our experience has been that sodium and potassium adduct ions are rarely seen with thermospray under normal conditions; evidently, the very low temperatures used were responsible for their preservation here. There are several reasons why the 249 amu peak is very likely to be the molecular ion: the mild thermospray conditions decrease fragmentation; sodium and potassium adducts are almost always an association of the the metal ion with an intact molecule; a 249 MH+ molecular ion implies a plausible reaction in acid solution, which is the hydrolysis of EBT to produce 1-(hydroxyethylidene)-L-tryptophan (HET) and LT as shown in Figure 4.

NMR measurement of pure peak 1 in D_2O gave the following results δ(ppm) [mult., nH, J(Hz)]: 1.8 [d, 3H (6.1)], 3.2 [m, 1H (8.2, 15.4)], 3.4 [m, 1H (4.8, 15.5)], 4.0 [dd, 1H (4.8, 8.0)], 6.2 [q, 1H (6.0)], 7.2 [dd, 1H (7.4, 8.5)], 7.3 [dd, 1H (7.2, 8.6)], 7.4 [s, 1H], 7.6 [d, 1H (8.4)], 7.7 [d, 1H (8.3)]. This spectrum was similar to both LT and EBT but differed in ways that suggested a possible structure. Resonances between 7.2 and 7.8 ppm for peak 1 show similar fine structure as that found for both LT and EBT in this

region, suggesting that the aromatic protons and the proton in the number 2 position are essentially the same for all three compounds. The same is true for the resonances found in the region between 3 and 4 ppm. This finding suggests that the aliphatic chain is also similar for all three compounds. The largest differences occurred in the doublet at 1.8 ppm and the quartet at 6.2 ppm. In LT these resonances did not appear. They were present in EBT but at different chemical shifts and a different relative intensity. These resonances were found at twice the relative intensity in peak 1 as compared to EBT, suggesting that peak 1 only contains one indole ring instead of two. Additional effects were seen for the chemical shifts of these protons. The doublet and quartet of peak 1 are both found at higher field than with EBT. Replacement of the indole moiety by a hydroxyl group can account for these differences.

Simulating gastric conditions by adding acid to EBT resulted in a transformation of EBT to LT through an intermediate step. At 35 min after the addition of acid, a strong doublet at 1.8 ppm and weak peaks at 6.1 ppm could be seen. These peaks grew in inverse proportion to the EBT peaks over the next 6 hours. During this time, the other resonances corresponding to peak 1 could be seen. After 24 hours, EBT and peak 1 were essentially gone and the solution chiefly contained LT. In addition to the resonances corresponding to LT, the final reaction mixture contained resonances that corresponded to resonances of acetaldehyde (1.2 and 9.6 ppm).

The infrared spectrum of peak 1 exhibits a medium intensity band at 3417 cm-1 (characteristic of O-H stretch) that was not observed in either LT or EBT. N-hydroxymethylcarbazole shows a similar band at 3425 cm-1. The strong absorption band at 3406 cm-1 associated with indole N-H stretch in LT was not observed in EBT or peak 1. Strong absorption bands characteristic of asymmetric NH_3^+ deformation (1675 cm-1) and asymmetric COO^- stretching (1636 cm-1) in amino acids (Colthup et al. 1964) were more differentiated than the coalesced bands (near 1615 cm-1) in EBT, but not as well differentiated as the corresponding bands (1667 cm-1 and 1591 cm-1) in LT.

The mass spectral, NMR, and FTIR results for peak 1 are consistent with each other and indicate that peak 1 is 1-(hydroxyethylidene)-L-tryptophan (HET) shown in Figure 4.

We analyzed peaks 2 and 2′at various incubation times by LC-MS. The spectra of peaks 2 and 2′ were identical

and had a strong peak at 231 amu and a weak peak at 158 amu. The retention time of peaks 2 and 2′, the constant peak-height ratio between them, and the strong signal at 231 amu are characteristic of the structural isomers of MCCA, which we have found to be an impurity in the tryptophan tablets. Synthetic MCCA had the same LC retention times and the same LC-MS spectrum as peaks 2 and 2′. The MS/MS spectra of peaks 2 and 2′ and synthetic MCCA were also the same. The principal peaks in the MS/MS spectra and the ions attributed to them are as follows with "M" standing for the molecular weight of peaks 2 and 2′: 231 amu, MH+; 214 amu, MH+ - NH3; 188 amu, MH+ - CO2; and 158 amu, MH+ - C(NH2)COOH. This evidence and the NMR results described below lead us to identify peaks 2 and 2′ as the MCCA structural isomers (Figure 4) -- as did *Ito et al., (personal communication)*.

While in SGF, Peak 1 rearranged to form a mixture of compounds with the following proton NMR spectra: δ(ppm) [mult., nH, J(Hz)]: 1.50 [d, 3H (6.8)], 1.52 [d, 3H (6.7)], 2.84 [ddd, 1H (2.5, 12.2, 16.5)], 2.95 [ddd, 1H (1.1, 9.1, 16.4)], 3.19 [ddd, 1H (1.4, 5.4, 16.5)], 3.21 [dd, 1H (5.7, 16.4)], 3.79 [dd, 1H (5.4, 12.2)], 4.16 [dd, 1H, (5.7, 9.1)], 4.43 [ddq, 1H (1.4, 2.5, 6.7)], 4.79 [dq, 1H, (1.0, 6.8)], 6.99 [dt, 2H (1.0, 7.8)], 7.08 [dt, 2H (1.0, 7.8)], 7.28 [dd, 2H, (1.0, 7.8)], 7.41 [d, 2H (7.8)]. These resonances showed excellent agreement with spectra taken individually of standards of the α-methyl and β-methyl isomers of MCCA. The spectrum of the α-methyl isomer of MCCA gave the following results: δ(ppm) [mult., nH, J(Hz)]: 1.50 [d, 3H (6.8)], 2.79 [ddd, 1H (1.2, 9.3, 15.3)], 3.07 [dd, 1H, (4.6, 15.3)], 3.69 [dd, 1H, (4.6, 9.3)], 4.79 [dq, 1H, (1.2, 6.8)], 6.91 [dt, 1H (1.1, 7.7)], 6.99 [dt, 1H (1.1, 7.7)], 7.26 [dd, 1H, (1.1, 7.7)], 7.35 [dd, 1H (1.1, 7.8)]. These peaks match very well with half of the resonances found in the peak 1 rearrangement mixture. The spectrum of the β-methyl isomer of MCCA gave these results: δ(ppm) [mult., nH, J(Hz)]: 1.50 [d, 3H (6.6)], 2.73 [ddd, 1H (2.5, 11.4, 15.2)], 3.10 [ddd, 1H (1.8, 4.4, 15.2)], 3.51 [dd, 1H (4.4, 11.4)], 4.17 [ddq, 1H (1.8, 2.5, 6.6)], 6.94 [dt, 1H (1.1, 7.5)], 7.02 [dt, 1H (1.1, 7.5)], 7.27 [dd, 1H, (1.1, 7.5)], 7.37 [dd, 1H (1.1, 7.5)].

These peaks correspond with the remaining resonances from the rearrangement mixture. Assigning resonances is straightforward, given the chemical shifts and coupling constants. The doublets at about 1.5 ppm correspond to the methyl group attached at the 1 position in MCCA. Methylene protons in the 4 position are found between 2.7 and 3.2 ppm. The protons attached to the 3 position are found between 3.5 and

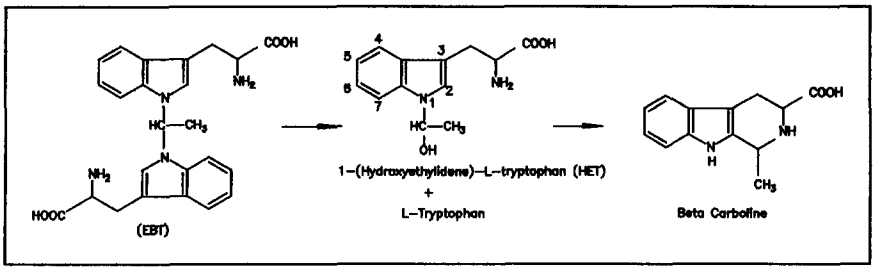

Figure 4. Decomposition Products of EBT

4.2 ppm. The protons attached to the 1 position correspond to resonances between 4.1 and 4.8 ppm. The protons in the 1 and 3 position are readily distinguished by their coupling to either the methyl or methylene groups. Finally, protons in the aromatic ring are found between 6.9 and 7.4 ppm.

The formation of MCCA from HET could occur via (1) an intramolecular rearrangement and cyclization reaction or (2) the elimination of acetaldehyde followed by a reaction with LT. Presumably, the protonation of the indole nitrogen of EBT followed by C-N bond scission results in an LT molecule and a resonance-stabilized carbenium-immonium ion, which yields the hemiaminal, HET, on hydrolysis. Formation of such hemiaminals during hydrolysis of aminals has been observed (Gross and Gloede 1966; Barrows et al. 1976). If the intramolecular reaction mechanism is correct, the hemiaminal would rearrange by migration of the hydroxyethyl group to indole carbon 2, and the ring would close to form MCCA. The identification of acetaldehyde in the reaction medium by NMR suggests that an intermolecular reaction is also possible. The formation of MCCA from a reaction of LT with acetaldehyde is well known (Brehm and Lindwall 1950). Further studies would be necessary to decide among the different reaction mechanism possibilities.

This study showed that under simulated stomach conditions, EBT is almost completely broken down into LT, HET, and MCCA within 2 hrs. *Ito et al., (personal communication)* also showed that EBT breaks down to tryptophan and two other substances in an acidic environment; apparently the substance we call peak 1 appears in their HPLC chromatograms, but they do not identify its structure. They do identify MCCA as the other product. If EBT is the causative agent for EMS, this study like Ito's study, suggests that EBT, HET, MCCA, or a combination of the three could be the primary circulating toxicant(s): EBT is not broken down so fast that it

could not be absorbed into the circulation; HET and MCCA are formed quickly enough that they also could be absorbed. As noted above we have found that MCCA appears in many of the L-tryptophan lots as an impurity. In a study of 18 case and control tryptophan lots, MCCA was present in all of the case lots and in 5 out of 9 control lots, suggesting that it may not be the toxic agent. If EBT is proven to be the causative agent for EMS, further studies will be necessary to find out whether EBT or one of the stomach products of EBT is the primary circulating toxicant.

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