Articles

Biotransformation of 3-(Phenylamino)-1,2-propanediol to 3-(Phenylamino)alanine: A Chemical Link between Toxic Oil Syndrome and Eosinophilia-Myalgia Syndrome

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During late 1989, the eosinophilia-myalgia syndrome (EMS) developed as an epidemic in the United States, with numerous additional cases reported in several other countries worldwide. Eight years earlier, a closely-related disease, the toxic oil syndrome (TOS), occurred in Spain as a massive food-borne epidemic. Although EMS was linked to the ingestion of tainted L-tryptophan, and TOS to aniline-denatured rapeseed oil, the etiologic agent(s) responsible for both diseases remains undetermined. Contaminants in these foodstuffs are believed to have triggered the diseases. Aniline contaminants, including 3-(phenylamino)-1,2-propanediol (PAP), have been reported in oil used by patients who developed TOS. A related aniline derivative, 3-(phenylamino)-L-alanine (PAA), was recently isolated from L-tryptophan associated with the onset of EMS. Here, we demonstrate the biotransformation of PAP into PAA by both rat hepatocytes and human liver tissue. The structural characterization of PAA was unequivocally determined using on-line HPLC coupled with atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS). This finding is the first reported chemical link between TOS and EMS and suggests that these two related diseases share a common etiology, namely, PAA.

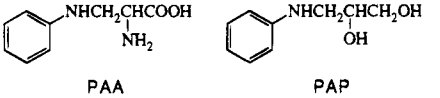
Introduction

The eosinophilia-myalgia syndrome (EMS)[1] is a multisystem inflammatory disease with characteristic features of myalgia and profound eosinophilia (*1–3*). The disease affected over 1500 persons in the United States during late 1989 and caused over 30 deaths (*4, 5*). Cases of EMS were also reported in Europe, Canada, and elsewhere (*6*). In Germany, over 100 persons became ill with EMS (*7*). Epidemiologic studies demonstrated an association between EMS and consumption of L-tryptophan (Trp) manufactured by one company (Showa Denko K.K.) (*8, 9*) and suggested that the illness was triggered by a contaminant and not Trp itself. Analysis of Trp by HPLC (*9–12*) revealed the presence of six contaminants associated with EMS. The structures of three are known: 1,1′-ethylidenebis[L-tryptophan] (EBT) (*13, 14*), 3-(phenylamino)-L-alanine (PAA) (*15, 16*), and 2-(3-indolylmethyl)tryptophan (*12*). The other three contaminants remain uncharacterized.

In many patients, the clinical manifestations of EMS evolved to a chronic phase resembling the Spanish toxic oil syndrome (TOS) (*17–21*). The TOS epidemic occurred in Spain during mid-1981 and was attributed to the ingestion of adulterated rapeseed oil (*22, 23*). The disease affected approximately 20 000 persons and caused several hundred deaths (*24*). The specific component of the toxic oil that triggered the illness has not been determined, although aniline and aniline derivatives were identified as markers of implicated cooking oil (*19, 22, 23, 25*). One aniline derivative, 3-(phenylamino)-1,2-propanediol (PAP), along with its fatty acid esters, has been isolated from implicated oil (*26*).

TOS and EMS share clinical features with eosinophilic fasciitis (*21, 27, 28*) and scleroderma-like syndromes (*29–31*). Although the causes of these diseases are unknown, scleroderma-like illnesses have been caused by exposure to various chemicals (*29–31*).

We have previously hypothesized that PAP and PAA, due to their structural similarity (see below for structures), may be metabolically interconvertible *in vivo*,



PAA PAP

thereby linking EMS and TOS to a common etiological agent (*16*). Here, we report the *in vitro* biotransformation

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[1] Abbreviations: APCI, atmospheric pressure chemical ionization; EBT, 1,1′-ethylidenebis[tryptophan]; EMS, eosinophilia-myalgia syndrome; HBSS, Hanks' balanced salt solution; MS/MS, tandem mass spectrometry; PAA, 3-(phenylamino)alanine; PAP, 3-(phenylamino)-1,2-propanediol; TOS, toxic oil syndrome.

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of PAP into PAA by rat hepatocytes and human liver tissue. The chemical structure of PAA was verified by utilization of the recently available technique of HPLC coupled to atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) (*32, 33*). This finding is the first reported chemical link between TOS and EMS and suggests that these two related diseases share a common etiology, namely, PAA.

Materials and Methods

Materials. (*R*)- and (*S*)-glycidol and racemic glycidol were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C(U)]-Aniline hydrochloride was obtained from NEN/DuPont (Boston, MA). Hanks' balanced salt solution (HBSS) (10×) was purchased from Gibco Laboratories (Grand Island, NY). (*R*)-, (*S*)-, and (*RS*)-PAP were synthesized from (*R*)-, (*S*)-, and racemic glycidol, respectively, as described previously (*34*). PAA was prepared as described previously (*16*).

Synthesis of ^{14}C-Labeled 3-(Phenylamino)-1,2-propanediol. [^{14}C]PAP was prepared from [^{14}C(U)]aniline hydrochloride (0.0379 mmol, 1 equiv; 0.25 mCi; in 0.5 mL of ethanol solution; specific activity, 6.6 mCi/mmol; 0.5 mCi/mL; 75.8 μmol/mL) and glycidol, based on a previously described method (*34*), and purified by HPLC. Briefly, the ethanolic aniline solution was transferred to a 1-mL reaction vial containing a stirring fin. With stirring, triethylamine (5.6 μL, 0.04 mmol, 1.1 equiv) was added, followed by glycidol (0.133 mmol, 3.5 equiv) which had been prediluted with methanol. Nitrogen gas was bubbled through the solution to prevent autoxidation. The reaction was allowed to proceed for 4 h, by which time the solvent had evaporated, leaving a brown residue. Deionized water (700 μL) was added, which led to the formation of a black precipitate. The solution was filtered (0.45 μm), giving a clear filtrate. [^{14}C]-PAP was purified by HPLC on a C-18 column with a H_2O/MeOH gradient. The yield was 1.24 mg (20% based on [^{14}C]aniline used).

Primary Hepatocytes and Human Liver Tissue. Isolated rat hepatocytes, generously provided by S. Bronk and G. J. Gores (Mayo Clinic), were obtained from Sprague-Dawley animals (Harlan Laboratories, Indianapolis, IN) by perfusion with collagenase as described previously (*35*). Viability, as determined by trypan blue exclusion, was ≥90%. Hepatocytes constituted >99% of the isolated cells. Normal human liver tissue was obtained within 30 min of liver resections, placed in saline, and kept on ice until use. Human liver was chopped into pieces of diameter ≤0.5 mm with a McIlwain Tissue Chopper (Brinkmann, Westbury, NY) and used immediately.

Incubations with Hepatocytes and Liver Tissue. For rat hepatocytes, unlabeled PAP or [^{14}C]-(*RS*)-PAP (4×10^{-4} M; specific activity, 6.6 mCi/mmol) was incubated with isolated hepatocytes (8×10^6/mL) in HBSS containing Mg^{2+} and Ca^{2+}, at 37 °C under an atmosphere of 95% O_2/5% CO_2 with gentle rocking to keep the cells well oxygenated. For human liver, PAP (4×10^{-4} M) was incubated with chopped liver tissue (~200 mg/mL; tissue size: 0.5 × 0.5 × 0.5 mm^3) in HBSS at 37 °C with 95% O_2/5% CO_2 bubbling through the suspension. After 6 h, the cells or tissue was pelleted by centrifugation. The supernatant was removed, syringe filtered (0.45 μm), and analyzed by HPLC. For analyses by LC-APCI-MS, the samples were desalted using a Sep-Pak C-18 cartridge (Millipore Corp., Milford, MA) before testing.

HPLC Analyses. HPLC was performed on a Beckman (Fullerton, CA) System Gold or Hewlett-Packard (Palo Alto, CA) Model 1090 liquid chromatograph using a C-18 column [Primesphere HC, 5 μm particle size, 110 Å pore size, 4.6 mm × 250 mm (Phenomenex, Inc., Torrance, CA)] at a flow rate of 1 mL/min with gradient elution (0–5 min, 5% B; 5–18.5 min, 5–22.5% B; 18.5–35 min, 22.5–100% B; 39–40 min, 100–5% B), and buffers (A = 5 mM triethylammonium acetate, pH 7; B = 90% methanol/10% water). The guard column, packed with material identical to the column, was replaced periodically. Radioisotope and UV detectors were connected in series after the column. On-line ^{14}C-radioactivity was monitored using a Beckman 171 radioisotope detector. UV spectral data were obtained from the HP 1090 chromatograph equipped with a diode-array detector (DAD).

LC-APCI-MS/MS Analyses. HPLC was performed on an Ultrafast Microprotein Analyzer System (Michrom BioResources, Inc., Auburn, CA). LC conditions were similar to those given above: column [Primesphere 5 μm C18 HC, 110 Å pore size, 3.2 mm × 250 mm (Phenomenex, Inc., Torrance, CA)], flow rate (0.4 mL/min), gradient (0–5 min, 5% B; 5–18 min, 5–22.5% B; 18.5–30 min, 22.5–95% B; 30–40 min, 95–95% B; 40–45 min, 95–5%B), and buffers (A = 5 mM ammonium acetate; B = 90% methanol/10% water).

MS and MS/MS analyses were performed on a Finnigan MAT 95Q (Bremen, Germany) mass spectrometer of BEQ_1Q_2 configuration (where B is the magnet sector, E is an electrostatic analyzer, Q_1 is a radiofrequency-only octapole collision cell, and Q_2 is a mass filter-only quadrapole). A Finnigan MAT APCI source was used in positive mode throughout with capillary and vaporizer temperatures of 200 and 475 °C, respectively, and the corona spray needle energy at ~2.5 kV. LC-MS experiments were performed while scanning from m/z 50 to 500 at a scan rate of 2 s/decade. For data presentation, partial ion current chromatograms, instead of total ion current chromatograms, are shown.

Protonated molecular weight ions (MH^+) were subjected to collision-induced dissociation in the octapole collision cell utilizing an argon gas pressure of ~6×10^{-5} mbar with collision energies of 20–30 eV. The product ions were analyzed in Q_2 with spectra collected over a mass range of 40–200 with 10–20 scans acquired and averaged to afford a composite product ion spectrum.

Results

In order to investigate an etiologic link between TOS and EMS, we studied the metabolic interconversion of PAP and PAA *in vitro*. [^{14}C]PAP (4×10^{-4} M) was incubated with isolated rat hepatocytes (8×10^6/mL) or chopped human liver tissue (~200 mg/mL) in HBSS for 6 h at 37 °C, and the supernatants were analyzed by HPLC using on-line UV and ^{14}C-radioisotope detectors. Panels A and B of Figure 1 show the HPLC chromatograms of the incubation mixture from rat hepatocytes, monitoring absorbance at 240 nm and ^{14}C-activity, respectively, whereas Figure 1C shows the ^{14}C-elution profile of the supernatant from human liver tissue. The chromatograms revealed that both rat hepatocytes and human liver tissue metabolize PAP into metabolites that were detectable in the supernatants. One of these metabolites eluted with the same retention time (t_R) as synthetic PAA (t_R = 18.4 min) and displayed an identical UV spectrum (λ_{max} 290 and 240 nm) (*16*), suggesting the conversion of PAP to PAA. PAP alone (t_R = 29.3 min) incubated in HBSS buffer under identical conditions did not afford any products in the absence of hepatocytes or liver tissue.

To unequivocally confirm the formation of PAA from PAP, LC-APCI-MS/MS analyses were performed on the incubation mixtures. LC-APCI-MS analyses of synthetic PAA and PAP were carried out first to determine their retention times and the optimal separation and detection conditions. LC conditions were similar to the HPLC conditions in Figure 1, with the following major modifications: (1) 5 mM ammonium acetate was used as the buffer instead of 5 mM triethylammonium acetate which interfered with the MS/MS analyses (*vide infra*), and (2) an HPLC column of a narrower inner diameter (3.2 mm *vs* 4.6 mm) was utilized to provide a reduced flow rate (0.4 mL/min *vs* 1 mL/min) for the APCI-MS interface.

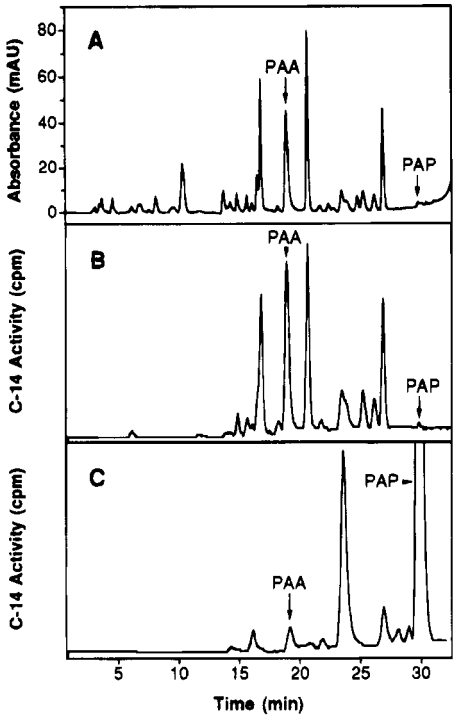


Figure 1. Typical HPLC chromatograms showing the biotransformation of PAP to PAA by (A) rat hepatocytes and monitoring UV absorbance at 240 nm, (B) rat hepatocytes and monitoring ^{14}C-radioactivity, and (C) human liver tissue and monitoring ^{14}C-radioactivity. The chromatogram in (A) is plotted 0.5 min to the left to correct for the dead volume between the on-line UV and radioisotope detectors. Synthetic PAA and PAP eluted with retention times of 18.4 and 29.3 min, respectively. [^{14}C]-PAP (4×10^{-4} M) was incubated with isolated rat hepatocytes (8×10^6/mL) or chopped human liver tissue (~200 mg/mL) in HBSS for 6 h at 37 °C, and the supernatants were analyzed. HPLC conditions are described in Materials and Methods.

These changes in the HPLC conditions resulted in shifts in the retention times of some metabolite peaks, as determined by the UV spectra of the peaks (using the on-line diode array detector). Several peaks eluted earlier with NH_4OAc as the buffer. Under these conditions, authentic PAA and PAP eluted with retention times of 18.1 and 27.4 min, respectively (data not shown).

After establishing LC-APCI-MS conditions, tandem mass spectrometry (MS/MS) analyses of synthetic standards PAA and PAP were carried out to provide product ion (or MS/MS) spectral data produced from each precursor ion. For product ion analysis, the precursor ions (m/z 181 and 168 for the protonated molecular ion [MH^+] of PAA and PAP, respectively) were selected in MS_1 (BE) and subjected to collision-induced dissociation in the octapole collision cell (Q_1), and the resulting product ions were mass analyzed and detected in MS_2 (Q_2). LC-APCI-MS/MS data for authentic PAP and PAA are listed in Table 1. The most intense product ion for both standards was at m/z 106, corresponding to the immonium ion, $[PhNH{=}CH_2]^+$, that forms after the characteristic cleavage of the C–C bond next to the nitrogen atom of alkylarylamines (*36*).

After the LC-APCI-MS/MS analysis of the standards was completed, supernatants from rat hepatocytes or human liver tissue incubated with PAP were analyzed for the presence of PAA. Panels A and C of Figure 2 show the full-scan partial ion current chromatograms (m/z 104–300) of PAP incubated with rat hepatocytes and human liver tissue, respectively. The range of m/z 104–300 was selected to focus on ions of potential interest.

Table 1. Product Ion MS/MS Spectral Data for Synthetic PAA and PAP Obtained by LC-APCI-MS/MS[a]

standard	MH^+	product ions, m/z (rel ion abundance, %) [likely loss]
PAA	181 (25)	164 (9) [$-NH_3$]; 146 (3); 136 (5) [$-COOH$]; 118 (27) [$-NH_3$, $-COOH$]; 106 (100) [$-CH(NH_2)COOH$]; 104 (38); 93(9); 88 (34) [$-PhNH_2$]; 76 (8); 70 (17); 41 (8)
PAP	168 (42)	150 (9) [$-H_2O$]; 132 (59) [$-2H_2O$]; 119 (18); 117 (12); 106 (100) [$-CH(OH)CH_2OH$]; 93(13); 77 (6); 57 (5); 44 (3)

[a] LC-APCI-MS/MS conditions are described in Materials and Methods.

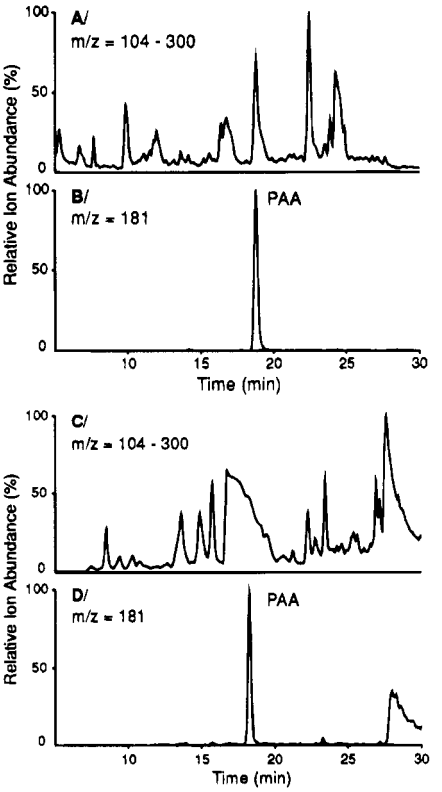


Figure 2. Typical LC-APCI-MS chromatograms of PAP incubated with rat hepatocytes or human liver tissue. (A) and (B) show the partial ion current (m/z range of 104–300) and the extracted ion current at m/z 181 (for PAA), respectively, of the supernatants from rat hepatocytes. (C) and (D) display the analogous data for human liver supernatants. Incubation conditions are identical to those described in Figure 1. LC-APCI-MS conditions are described in Materials and Methods.

Presumably, all positive ions within this range are represented in the partial ion current chromatogram. The presence of PAA was tentatively assessed by examining extracted ion current chromatograms at m/z 181, as shown in panels B and D of Figure 2 for rat and human supernatants, respectively. In contrast to total or partial ion current chromatograms that show the sum of all ions within a broad m/z range, extracted ion current chromatograms display only a selected mass range or even a single ion, e.g., m/z 181, enabling identification of an ion of a specific m/z. The presence of a single peak (t_R = 18.1) of m/z 181 in the extracted ion current chromatograms of both rat and human supernatants was highly suggestive of the formation of PAA.

LC-APCI-MS/MS analyses of the m/z 181 peak produced by rat hepatocytes and human liver tissue in the presence of PAP were subsequently carried out to determine its structure. Panels A and B of Figure 3 show the full-scan mass spectra obtained by MS/MS analysis of

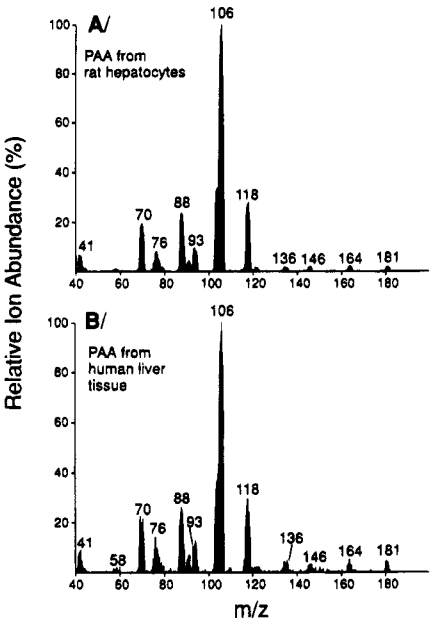


Figure 3. Product ion MS/MS spectra of the metabolite ion at m/z 181, formed after incubation of PAP with (A) rat hepatocytes and (B) human liver tissue. These spectra are essentially identical to that of synthetic PAA (see Table 1).

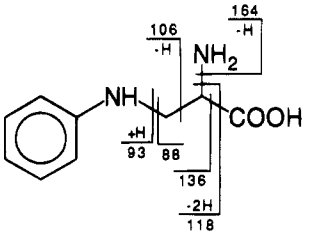


Figure 4. Structure of PAA (MH^+ = 181) showing fragment ions derived from collision-induced dissociation (MS/MS) analysis.

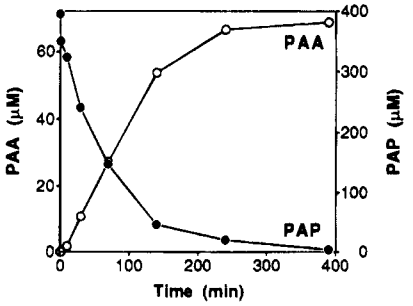


Figure 5. Time course for the formation of PAA and metabolism of PAP by rat hepatocytes. Incubation and analytical conditions were identical to those in Figure 1, except that samples were analyzed at the various times indicated.

this metabolite peak (precursor ion, m/z 181) produced by rat hepatocytes and human liver tissue, respectively. The spectra were essentially identical to each other and to that of synthetic PAA (Table 1). Figure 4 shows the proposed bond fragmentations of PAA that produce the major product ions. These LC-APCI-MS/MS analyses demonstrate definitively the biotransformation of PAP to PAA by both rat hepatocytes and human liver tissue.

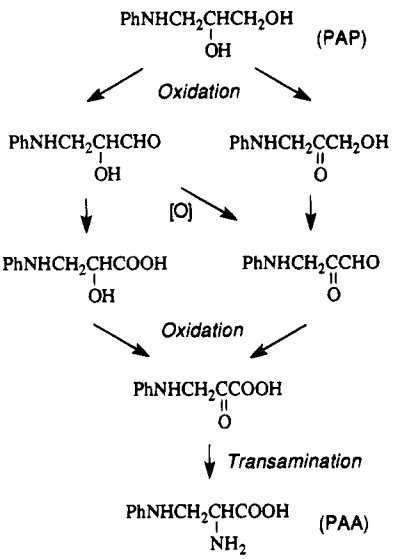
The time course for the formation of PAA and the metabolism of PAP by rat hepatocytes is shown in Figure 5. Essentially all of the PAP added was metabolized by 6.5 h. PAA formation began within 15 min. Integration of the peak areas of PAA and PAP indicated that more than 15% of the PAP was converted to PAA. Experiments using rat hepatocytes were repeated >15 times with similar results (data not shown). All data shown (Figures 1–3 and 5) represent the metabolism of racemic PAP. Racemic PAP is consumed entirely, indicating that both enantiomers are metabolized. However, incubation of the individual enantiomers, (*R*)-PAP or (*S*)-PAP, with rat hepatocytes revealed that the metabolism of PAP is stereospecific: PAA is formed from the *S*-enantiomer of PAP (data not shown). The reverse reaction, formation of PAP from PAA, did not occur: PAP was not observed in the supernatant when PAA alone was incubated with rat hepatocytes or human liver tissue for 6 h (data not shown). Thus, short term incubations with hepatocytes do not convert PAA to PAP. All freshly obtained human liver tissue tested (four individuals) were able to covert PAP to PAA. When a high concentration of PAP (10 mM) was incubated with isolated rat hepatocytes (8×10^6/mL), the metabolite profile more closely resembled that with human liver tissue, with a large peak at 22.9 min (data not shown).

Discussion

In this paper, we demonstrate the *in vitro* biotransformation of PAP to PAA, linking the related diseases EMS and TOS to a common chemical substance, PAA. The formation of PAA was confirmed using LC-APCI-MS/MS, a technique that has been employed to characterize a variety of small molecules (*37–39*). Both rat hepatocytes and human liver tissue metabolize PAP to PAA. PAA is metabolized from PAP and not from an endogenous substrate, as confirmed by using ^{14}C-labeled PAP, synthesized from [^{14}C(U)]aniline and glycidol (Figure 1 B,C). The metabolite profile using rat hepatocytes differed from that of human liver in the number and relative amounts of metabolites formed: PAA was formed by both human liver tissue and rat hepatocytes, but in a smaller proportion with human liver tissue relative to the other metabolites (Figure 1). This difference may reflect a difference between tissue slices and isolated cells. Metabolism by rat hepatocytes is rapid: PAP (4×10^{-4} M) was completely metabolized by rat hepatocytes in 6 h (Figure 5). Numerous other metabolites of PAP are formed, and structure elucidation of these compounds is currently in progress.

Little is known about the biological effects of either PAP or PAA. Intraperitoneal injection of PAP (465 mg/kg/day) has been reported to cause mortality in mice (*40*). The greatest pathologic changes occurred in the lungs, where vascular congestion and acute inflammatory infiltrates were observed. Oral doses of PAP given at the same rate caused no mortality. In another study, PAP (350 mg/kg) was toxic to rats when given intraperitoneally (*41*). Pulmonary thromboembolism was the major histopathologic finding; however, the pathology observed in rats was thought not to be representative of that observed in patients with TOS (*41*). Interestingly, respiratory symptoms were more prominent and severe during the early phase of TOS in comparison to EMS (*19*). Certain drugs, such a propranolol (which has some structural similarities to PAP), are known to accumulate in the lungs (*42*). Recent analyses of oil samples have determined that the 3-oleyl ester of PAP and the 1,2-dioleyl ester of PAP were found more frequently and at higher concentrations in TOS case-associated oils than in control oils (*43*). In addition, oral administration of PAP and its corresponding mono- and diesters with fatty

Scheme 1. Hypothetical Metabolic Pathway for the Biotransformation of PAP to PAA[a]



[a] [O] = oxidation.

acids to rats revealed that these compounds enter the blood by lymphatic absorption and that a metabolite is produced from them (*44*). The chemical nature of this metabolite was undetermined. Biological studies on PAA are currently limited. PAA metabolism in rats (*45*), as well as its tissue distribution and elimination (*46*), has been studied; however, its immunological and toxicological effects have not yet been reported.

The biotransformation of PAP to PAA must proceed through various intermediates (Scheme 1). The first steps most likely involve stepwise oxidation of the diol to an α-keto acid or some other keto intermediate, which then undergoes transamination to give PAA. It is likely that any one of these intermediates, in the presence of hepatocytes, can be metabolized to PAA, and thus, many molecules related to PAP may be channeled down this pathway to give PAA. This model suggests that numerous molecules with similar chemical structures to PAP will give rise to PAA, and if PAA is indeed responsible for EMS and TOS, the model predicts that an entire class of molecules, as shown in Scheme 1, can cause EMS/TOS-like diseases. Interestingly, cases of EMS not associated with Trp usage have been reported (*47, 48*), and an EMS-like syndrome has been associated with use of L-5-hydroxytryptophan (*49*).

Although the etiologies of the EMS and TOS epidemics have been under investigation for over half a decade and a decade, respectively, the agents responsible for the diseases remain undetermined. It was proposed very early that EMS and TOS share a related etiology and pathogenesis because of the many clinical and histopathological similarities (*20, 50*). PAA represents the first and only reported direct chemical link between TOS and EMS. Further studies of both PAA, PAP, and their metabolites are warranted to follow this provocative lead. Until the agent(s) responsible for EMS and TOS is identified, it is almost certain that another EMS/TOS-like epidemic will occur. Moreover, these or related toxins may be present as impurities in the diet and contribute to a low level occurrence of similar syndromes, such as eosinophilic fasciitis, scleroderma-like disorders, or EMS not associated with Trp usage.

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Note Added in Proof

Subsequent to submission of this paper, a toxicological study on PAA was reported [Sato, F., Hagiwara, Y., and Kawase, Y. (1995) Subchronic toxicity of 3-phenylamino alanine, an impurity in L-tryptophan reported to be associated with eosinophilia-myalgia syndrome. *Arch. Toxicol.* **69**, 444–449]. PAA (1, 10, and 100 mg/kg/day) was administered to Sprague-Dawley rats for up to 13 consecutive weeks; no EMS-like symptoms were observed, however.

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