Biotransformation and Clearance of 3-(Phenylamino)propane-1,2-diol, a Compound Present in Samples Related to Toxic Oil Syndrome, in C57BL/6 and A/J Mice

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In May 1981, a massive food-borne intoxication occurred in Spain. The so-called toxic oil syndrome (TOS) was associated with the consumption of aniline-denatured and refined rapeseed oil that was illegally sold as edible olive oil. Fatty acid anilides and fatty acid derivatives of 3-(phenylamino)propane-1,2-diol were detected in oils and implicated as potential toxic agents and markers of toxic oil batches. Epidemiological evidence points to 3-(phenylamino)propane-1,2-diol derivatives as the putative toxic agents, which were generated during the refining process at the ITH refinery. Here we present the biotransformation and clearance of 3-(phenylamino)propane-1,2-diol (PAP) administered intraperitoneally to A/J and C57BL/6 mice that have been proposed as a murine model for the immunological features of TOS. Mice eliminated 6 μCi of [U-^{14}C]PAP during a 24 h period, mostly in urine. Animals exhibited urine elimination rates of 70 and 36% in A/J and C57BL/6 strains, respectively. A/J mice exhibited no increase in the elimination rate when induced with β-naphthoflavone, whereas C57BL/6 did increase the rate of elimination to 57%. Feces contributed to a lesser extent to the elimination rate (0.6 and 3.3% in A/J and C57BL/6 mice, respectively). Radioactivity remaining in organ tissues was lower than 1% (liver, lung, kidney, spleen, heart, and muscle). Metabolic species in urine were identified by HPLC coupled to UV and radioisotope detectors and further GC/MS analyses. 2-Hydroxy-3-(phenylamino)propanoic acid metabolite was the major chemical species excreted in urine in both strains, in both control and induced animal groups. This compound was the main urinary metabolite of PAP, and unmetabolized PAP excreted in urine constituted less than 1% of the total administered dose. Two additional highly polar metabolites also detected in urine were identified as 3-[(4′-hydroxyphenyl)amino]propane-1,2-diol and 2-hydroxy-3-[(4′-hydroxyphenyl)amino]propanoic acid. These findings are the first reported on PAP metabolism and clearance in mice strains and suggest that PAP can be extensively metabolized in vivo and potential reactive species can be generated.

Introduction

The so-called toxic oil syndrome (TOS)[1] was widespread in 1981 in central and northern areas of Spain and represented a huge food-borne chemical intoxication affecting more than 20 000 people and causing more than 400 deaths (*1*). Epidemiological studies provided conclusive evidence that the epidemic resulted from the consumption of adulterated rapeseed oil purchased from street vendors (*2*−*6*). Most of the patients recovered after a long period of time; however, many continue to suffer severe sequelae or mild symptoms (*7*, *8*). International investigation efforts have been coordinated through an International Scientific Commission sponsored by the World Health Organization and the Spanish government aimed at elucidating the pathogenesis of the disease, identifying the causal toxic agent, and clarifying the chemical processes that generated the toxic species in the oil batches (*1*, *9*). Despite these efforts, the full pathogenic aspects and the exact causal toxic agent of the syndrome remain unknown.

Rapeseed oil, denatured with 2% aniline, was imported for industrial use and fraudulently refined and derived

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[1] Abbreviations: TOS, toxic oil syndrome; EMS, Eosinophilia-Myalgia syndrome; OLA, oleanilide; PAP, 3-(phenylamino)propane-1,2-diol; MPAP, DPAP, and TPAP, mono-, di-, and triacyl derivatives of PAP, respectively; PAA, 3-(phenylamino)-L-alanine; PhG, *N*-phenylglycine; TEAA, triethylammonium acetate; PBS, phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.4)]; BSTFA, *O*,*N*-bis(trimethylsilyl)trifluoroacetamide; ca, complex absorption; EI-MS, electron impact mass spectrometry; Ah, aryl hydrocarbon.

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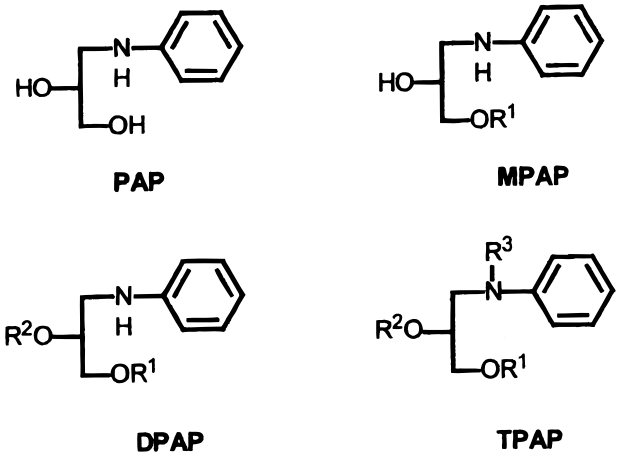
Chart 1. PAP and Its Mono-, Di-, and Triacyl Derivatives



as pure olive oil for human consumption (*1*, *2*, *10*). Investigations of oil batches detected several chemical species derived from aniline (*1*). Fatty acid anilides were first identified as the causal agent (*2*) and were clearly identified and characterized in the suspected toxic oils (*11*, *12*). The concentration of these anilides ranged from 500 to 2000 mg/L in oil batches (*1*, *2*, *11*, *12*). However, their association with TOS was not clear (*1*, *2*), and in vitro and in vivo experiments were not conclusive with respect to their toxicity in several animal species (*1*, *13*). In fact, many toxicological research studies with these chemicals and case-associated oil samples have failed to reproduce the full spectrum of symptoms observed in humans. Further epidemiological investigations of well-characterized oil batches which caused several cases established fatty acid anilides as good indicators of the adulterated toxic oil (*14*, *15*); however, they could not have been the toxic agent itself since they were present in oils distributed in other areas (Catalonia) with no reported toxicity in consumers (*14*, *15*). Nevertheless, aniline derivatives other than fatty acid anilides were found in toxic oils (*16*) and identified as 3-(phenylamino)-propane-1,2-diol (PAP) and its mono-, di-, and triacyl derivatives (MPAP, DPAP, and TPAP, respectively) (Chart 1). Recently, further epidemiological investigations established a good correlation between DPAP concentrations in oils and associated cases (*17*−*19*). Furthermore, these compounds were absent in oils distributed in Catalonia, which had fatty acid anilides at concentrations lower than those of the toxic oils. These studies point to these compounds as the likely potential toxic aniline derivatives implicated in TOS. Furthermore, they suggest that these compounds were generated by the chemical process involved in oil refining at the ITH company in Seville, since the epidemic is believed to have emerged from a single source in that refinery (*10*). To date, it is assumed that TOS patients were exposed to both substances, fatty acid anilides and PAP esters, present in their edible toxic oils; these chemical species are the aniline derivatives identified so far in oil batches. The aniline moiety of these compounds shares common features that make them susceptible to chemical biotransformation (*20*, *21*). In this respect, it was reported that oleanilide exhibited a strong and selective inhibition of benzo[*a*]pyrene hydroxylation in human liver microsomes (*22*).

PAP derivatives were toxic in Swiss mice, particularly when administered intraperitoneally. PAP and its monoester at C1 exhibited the highest toxicity; however, they lacked toxicity when administered orally or in chow (*23*). Extended experiments with such mice strains established that PAP given intraperitoneally was highly toxic (*24*), and accumulation in tissues was reported by means of TLC analyses in organs; however, metabolites were not identified. In more recent studies, DPAP administered orally to Wistar rats underwent intestinal hydrolysis to MPAP and PAP, and these compounds were absorbed into the general circulation (*25*). Conversely, a lack of toxicity of these compounds in other mouse and rat strains has been reported (*26*). The rate of DPAP conversion to PAP in vivo remains to be determined, as do the potential toxicity targets of both substances in an appropriate animal model.

These controversial data on animal toxicity might suggest a species-specific toxicity of these compounds for humans and/or a metabolic or clearance factor underlying its biological actions. Therefore, to obtain an animal model for in-depth analysis of TOS pathogenic mechanisms, full comprehension of the metabolism and clearance of the putative substances is required.

On the other hand, major efforts to clarify the immunological aspects of TOS have been fruitful in determining a specific TH2 profile in TOS patients (*27*, *28*). Moreover, experiments in mice have promisingly reproduced such a lymphocyte activation pattern under oleanilide administration; A/J and C57BL/6 mice, slow and fast acetylator strains, have been used as an autoimmune murine model (*29*). These animals exhibited a toxic response to oleanilide administered intraperitoneally, and this toxicity resembled the wasting syndrome reported in TOS; however, the interesting finding was that the T-lymphocyte activation pattern occurring in mice was similar to that reported in TOS patients.

To date, no studies have been conducted on PAP clearance and/or identification of metabolites in A/J and C57BL/6 mice. In the study presented here, we sought PAP metabolic species and clearance when administered to control and β-naphthoflavone-induced animals.

Materials and Methods

Chemicals and Reagents. Aniline (Aldrich, 99%) and glycidol (Merck, 98%) were previously distilled and stored at −20 °C. *O*,*N*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Aldrich. Olive oil, *N*-phenylglycine (PhG), β-glucuronidase from *Helix pomatia* or from *Escherichia coli*, and β-naphthoflavone were obtained from Sigma (St. Louis, MO). [U-^{14}C]Aniline hydrochloride (129 mCi/mmol) was from Amersham. Methanol, acetonitrile, dichloromethane, and other common solvents were HPLC quality and were purchased from Merck (Darmstadt, Germany). *N*,*N*-Dicyclohexylcarbodiimide, 4-(dimethylamino)-pyridine, and triethylammonium acetate (TEAA) buffer (1 M, pH 7.0) were obtained from Fluka. General laboratory reagents were obtained from local sources and were of high analytical grade. PAA was a kind gift from R. H. Hill (Centers for Disease Control and Prevention, Atlanta, GA). OptiPhase "HiSafe" cocktail was purchased from Fisons Chemicals.

Animals. A/J and C57BL/6 mice (9−10 weeks old) that were used in the experiments were obtained from Charles River and Criffa, respectively. Animals were housed in standard box cages on a 12 h light−dark schedule with food and water ad libitum for 3−4 days. The experiment began with three mice of each strain being placed in metabolic cages (Techniplast) for 3 days of acclimatization. On day 4, they were intraperitoneally given

a single total dose of PAP (250 mg/kg, average animal weight in each group of 22−24 g). Specifically, each animal received a total of 6 μCi of [U-^{14}C]PAP, and the dose was completed with the unlabeled PAP. Urine and feces were collected over a 24 h period, and the animals were euthanized. Organs (lung, liver, kidney, heart, muscle, and spleen) were collected in cold PBS and stored at −80 °C until the radioactivity was measured. In a second set of experiments, animals were induced with β-naphthoflavone during the acclimatization period before administration of PAP (total dose of 80 mg/kg in olive oil distributed in three intraperitoneal injections).

Synthesis of Substrates and Potential Metabolites. PAP was synthesized as described previously (*30*). Unless stated otherwise, organic solutions obtained from the treatment of crude reaction mixtures were dried over $MgSO_4$. Purification of metabolites by flash chromatography was performed using 35−70 μm silica gel (SDS). Reactions were monitored by either GC, HPLC, or TLC. GC analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph, provided with a FID detector and a 15 m HP-5 capillary column. HPLC analyses were performed on a Hewlett-Packard 1100 system provided with a DAD detector and an HP ODS Hypersil column (5 μm, 125 mm × 4 mm), using mixtures of HPLC grade acetonitrile and 10 mM TEAA (pH 6.8) buffer as eluents. Thin-layer chromatography (TLC) analyses and purifications were performed on Merck Kielsegel 60 F_{254} plates (aluminum sheets, 0.2 mm thick, and glass plates, 0.5 mm thick) using mixtures of hexanes and EtOAc as eluents, unless otherwise stated, and were developed by UV irradiation at 254 nm. Radioactivity was determined with an LKB 1217 Rackbeta scintillation counter following the addition of 10 mL of OptiPhase "HiSafe" cocktail. The IR spectra were recorded with a MB model 120 Bomen apparatus, and absorptions are given in cm^{-1}. The ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded with a Varian Unity 300 spectrometer. Spectra were recorded in neutralized $CDCl_3$ unless stated otherwise. Chemical shifts are given in parts per million relative to tetramethylsilane for ^{1}H and deuteriochloroform for ^{13}C as internal standards. The EI-MS spectra (70 eV) were obtained using a Fisons model MD 800 mass spectrometer coupled to a Fisons GC 8000 apparatus equipped with a 25 m HP-5 capillary column. Elemental microanalyses were carried out at the Servei de Microanàlisi of the IIQAB using a 1108 Carlo Erba analyzer.

(1) Synthesis of [U-^{14}C]PAP. This compound was prepared following the general procedure described previously (*31*). All evaporations of solvents and excess volatile reagents were performed under a nitrogen stream in a well-ventilated hood provided with the adequate filter systems. Briefly, a solution of [U-^{14}C]aniline hydrochloride (160 μCi in methanol) was evaporated to dryness, and the residue was redissolved in *tert*-butyl methyl ether (1 mL). This solution was washed with 0.1 N NaOH (200 μL). The organic phase was separated and evaporated, yielding free [U-^{14}C]aniline. Aniline (10.5 mg, 0.11 mmol), glycidol (9.0 mg, 0.12 mmol), and methanol (200 μL) were added to this residue, and the mixture was stirred overnight at 50 °C. The temperature was then increased to 60 °C and the stirring prolonged for 4 h. The reaction course was followed by TLC. The residue obtained from the evaporation of solvents was redissolved in chloroform and purified by preparative TLC (6:1 chloroform/methanol with 0.5% triethylamine), yielding a fraction of pure [U-^{14}C]PAP (R_f = 0.35, 10.5 mg, 57% yield, specific activity of 1.5 mCi/mmol), which was stored at −20 °C until it was used.

(2) 2-Hydroxy-3-(phenylamino)propanoic Acid (1). This compound was prepared by epoxidation of methyl acrylate, followed by reaction with aniline and finally ester hydrolysis. Briefly, a solution of methyl 2,3-epoxypropionate, prepared by reaction of methyl acrylate with excess dimethyldioxirane (0.08 g, 0.8 mmol) in MeOH (2 mL), was treated with aniline (0.15 g, 1.6 mmol), and the mixture was heated under reflux for 2 h (TLC monitoring). The elimination of solvent under vacuum gave a residue which was purified by preparative TLC (3:1 hexanes/EtOAc), yielding 0.092 g of pure ester **1a** as a colorless oil (60% yield): IR ($CDCl_3$) 3275, 3056, 2925, 1681, 1645; ^{1}H NMR δ 7.18 (t, 2 H, J = 7.4 Hz, H-3′, H-5′), 6.74 (t, 1 H, J = 7.2 Hz, H-4′), 6.66 (d, 2 H, J = 7.8 Hz, H-2′, H-6′), 4.42 (m, 1 H, H-2), 3.77 (s, 3 H, CH_3), 3.54 (dd, 1 H, J_1 = 13.2 Hz, J_2 = 3.8 Hz, CHH-N), 3.41 (dd, 1 H, J_1 = 13.2 Hz, J_2 = 5.6 Hz, CHH-N); ^{13}C NMR δ 174.0 (CO), 147.5 (C-1′), 129.2 (C-3′, C-5′), 118.2 (C-4′), 113.5 (C-2′, C-6′), 69.5 (C-2), 52.7 (CH_3), 47.0 (C-3); EI-MS m/z 195 (M^+), 136, 118, 106 (base peak). Elemental analysis for $C_{10}H_{13}NO_3$: C, 61.53; H, 6.71; N, 7.17. Found: C, 61.43; H, 6.87; N, 7.16.

A solution of **1a** (0.055 g, 0.28 mmol) in 1:1 methanol/0.1 N NaOH was stirred for 30 min at 25 °C. When the reaction was completed (TLC monitoring), the crude reaction mixture was acidified to pH 6 and passed through a reverse-phase (C-18) cartridge. Elution with methanol afforded acid **1** as a solid in near quantitative yield (*32*): ^{1}H NMR δ 7.10 (t, 2 H, J = 7.8 Hz, H-3′, H-5′), 6.69 (d, 2 H, J = 8 Hz, H-2′, H-6′), 6.62 (t, 1 H, J = 7.4 Hz, H-4′), 4.14 (m, 1 H, H-2), 3.45 (dd, 1 H, J_1 = 12.6 Hz, J_2 = 3.4 Hz, CHH-N), 3.21 (dd, 1 H, J_1 = 12.6 Hz, J_2 = 7.2 Hz, CHH-N); ^{13}C NMR δ 164.4 (CO), 150.9 (C-1′), 130.8 (C-3′, C-5′), 119.2 (C-4′), 115.3 (C-2′, C-6′), 98.1 (C-2), 73.1 (C-3).

(3) 2-Hydroxy-3-(phenylamino)propyl Acetate (2). A mixture of PAP (0.101 g, 0.6 mmol), acetic acid (38 μL, 0.66 mmol), *N*,*N*-dicyclohexylcarbodiimide (0.15 g, 0.7 mmol), and 4-(dimethylamino)pyridine (0.008 g, 0.06 mmol) in dichloromethane (4 mL) was allowed to react for 3 h at 25 °C. Once the reaction was completed (TLC monitoring), the solvent was eliminated under vacuum and the residue resuspended in hexane to induce precipitation of the urea derivative. Final purification of the crude reaction mixture by preparative TLC (4:1 hexanes/EtOAc) afforded pure acetate **2** as an oil (0.09 g, 72% yield) IR ($CDCl_3$) 3402, 3053, 2927, 1737; ^{1}H NMR δ 7.19 (t, 2 H, J = 7.5 Hz, H-3′, H-5′), 6.74 (t, 1 H, J = 7.3 Hz, H-4′), 6.66 (d, 2 H, J = 7.5 Hz, H-2′, H-6′), 4.27−4.06 (ca, 3 H, H-1, H-2), 3.32 (dd, 1 H, J_1 = 13 Hz, J_2 = 4.2 Hz, CHH-N), 3.17 (dd, 1 H, J_1 = 13 Hz, J_2 = 7.2 Hz, CHH-N, 2.12 (s, 3 H, CH_3); ^{13}C NMR δ 172.0 (CO), 147.8 (C-1′), 129.3 (C-3′, C-5′), 118.2 (C-4′), 113.3 (C-2′, C-6′), 68.5 (C-2), 66.6 (C-1), 46.6 (C-3), 20.8 (CH_3); EI-MS m/z 209 (M^+), 106 (base peak), 43.

(4) (*N*-Acetyl-*N*-phenylamino)propane-1,2-diol (3). This compound was prepared by protection of PAP as dioxolane followed by acylation at the secondary amine and finally release of the protecting moiety. Briefly, a mixture of PAP (1.62 g, 9.7 mmol), 2,2-dimethoxypropane (31 mL), and anhydrous *p*-toluenesulfonic acid (0.20 g, 0.97 mmol) was stirred for 3 h at 25 °C (GC monitoring). The residue obtained after elimination of solvent was redissolved in water (pH 9−10), extracted with dichloromethane, and dried. Purification of the new residue by column chromatography on silica gel (4:1 hexanes/EtOAc) afforded a pale yellow oil identified as the corresponding dioxolane (1.02 g, 50% yield): ^{1}H NMR δ 7.17 (t, 2 H, J = 7.9 Hz, H-3′, H-5′), 6.71 (t, 1 H, J = 7.3 Hz, H-4′), 6.61 (d, 2 H, J = 7.8 Hz, H-2′, H-6′), 4.32 (m, 1 H, H-2), 4.05 (dd, 1 H, J_1 = 8.2 Hz, J_2 = 6.4 Hz, CHH-O), 3.96 (s, 1 H, NH), 3.72 (dd, 1 H, J_1 = 8.2 Hz, J_2 = 6.2 Hz, CHH-O), 3.26 (dd, 1 H, J_1 = 12.6 Hz, J_2 = 4.4 Hz, CHH-N), 3.15 (dd, 1 H, J_1 = 12.6 Hz, J_2 = 6.4 Hz, CHH-N), 1.44 (s, 3 H, CH_3), 1.36 (s, 3 H, CH_3); ^{13}C NMR δ 147.9 (C-1′), 129.1 (C-3′, C-5′), 117.6 (C-4′), 112.8 (C-2′, C-6′), 109.3 (H_3CCCH_3), 74.4 (C-2), 67.1 (C-1), 46.5 (C-3), 26.8 (CH_3), 25.2 (CH_3); EI-MS m/z 207 (M^+), 192, 106 (base peak).

A solution of this dioxolane (0.21 g, 1.0 mmol) in 1,1,2-trichloroethylene (2 mL) was mixed with acetic anhydride (92 μL, 0.97 mmol) and pyridine (80 μL, 0.99 mmol), and the mixture was stirred for 30 min at 45 °C (GC monitoring). The elimination of solvent and excess reagents under vacuum led to a residue which was identified as the expected *N*-acetyl derivative as a solid (0.20 g, 83% yield): mp 62−64 °C; IR (KBr) 3022, 2987, 2879, 1666; ^{1}H NMR δ 7.4 (ca, 3 H, H-3′, H-4′, H-5′), 7.26 (d, 2 H, J = 8.2 Hz, H-2′, H-6′), 4.37 (m, 1 H, H-2), 4.00 (ca, 2 H, CHH-O, CHH-N), 3.71 (ca, 2 H, CHH-O, CHH-N), 1.86 (s, 3 H, CH_3), 1.31 (s, 3 H, CH_3); ^{13}C NMR δ 170.6 (CO), 143.4 (C-1′),

129.4 (C-3′, C-5′), 127.9 (C-4′), 127.7 (C-2′, C-6′), 108.9 ($H_3C\underline{C}CH_3$), 73.6 (C-2), 67.5 (C-1), 52.1 (C-3), 26.6 (CH_3), 25.3 (CH_3), 22.5 ($CO\underline{C}H_3$); EI-MS *m*/*z* 249 (M^+), 234, 191, 106 (base peak).

Finally, a solution of the *N*-acetyl derivative described above (0.15 g, 0.6 mmol) in a 1:1 1 N HCl/aqueous THF mixture was stirred for 2 h at 25 °C. The crude reaction mixture was concentrated under vacuum, neutralized, extracted with EtOAc, and dried. The residue obtained after solvent elimination was purified by preparative thin-layer chromatography (2:1 hexanes/EtOAc) to give the *N*-acetyl PAP derivative **3** as an oil (0.025 g, 20% yield): IR (KBr) 3383, 3055, 2923, 1645; ^{1}H NMR δ 7.41 (ca, 3 H, H-3′, H-4′, H-5′), 7.20 (d, 2 H, J = 7.2 Hz, H-2′, H-6′), 3.83 (ca, 3 H, H-2, H-1), 3.62 (m, 2 H, H-3), 3.44 (s, 1 H, OH), 3.21 (s, 1 H, OH), 1.89 (s, 3 H, CH_3); ^{13}C NMR δ 173.1 (CO), 143.3 (C-1′), 130.0 (C-3′, C-5′), 128.4 (C-4′), 127.6 (C-2′, C-6′), 70.3 (C-2), 63.7 (C-1), 52.6 (C-3), 22.6 ($CO\underline{C}H_3$); EI-MS *m*/*z* 209 (M^+), 178, 135, 106 (base peak), 43.

(5) 3-[(4′-Hydroxyphenyl)amino]propane-1,2-diol (4). Glycidol (0.07 g, 0.72 mmol) was added dropwise to a solution of 4-benzyloxyaniline (0.21 g, 1.07 mmol) in MeOH (2 mL), maintained at 50 °C. The mixture was heated under reflux until the reaction was completed (4 h, HPLC monitoring). The residue obtained after elimination of solvent was purified by crystallization from EtOAc, yielding the expected diol (0.070 g, 36% yield). When the reaction was performed by addition of 10% LiOTf as catalyst and toluene as a cosolvent (*33*), a 45% yield of the diol was isolated: mp 80−82 °C; ^{1}H NMR δ 7.4 (m, 5 H″), 6.77 (d, 2 H, J = 8.8 Hz, H-3′, H-5′), 6.60 (d, 2 H, J = 8.8 Hz, H-2′, H-6′), 5.01 (s, 1 H, CH_2Ph), 3.90 (m, 1 H, H-2), 3.67 (dd, 1 H, J_1 = 11.7 Hz, J_2 = 3.3 Hz, C$\underline{H}$H-O), 3.55 (dd, 1 H, J_1 = 11.4 Hz, J_2 = 6.6 Hz, CH$\underline{H}$-O), 3.16 (dd, 1 H, J_1 = 13 Hz, J_2 = 4.2 Hz, C$\underline{H}$H-N), 3.05 (dd, 1 H, J_1 = 13 Hz, J_2 = 7.4 Hz, CH$\underline{H}$-N); ^{13}C N$\underline{M}$R δ 152.8 (C-4′), 144.8 (C-1′), 138.9 (C-1″), 129.3 (C-3″, C-5″), 128.6 (C-4″), 128.4 (C-2″, C-6″), 117.1 (C-3′, C-5′), 115.9 (C-2′, C-6′), 71.8 (CH_2Ph), 71.0 (C-2), 65.2 (C-1), 57.4 (C-3); EI-MS *m*/*z* 273 (M^+), 238, 182 (base peak), 120, 91.

A mixture of the benzyloxy derivative described above (0.094 g, 0.35 mmol) and 10% Pd/C (0.019 g) in 95% EtOH (5 mL) was stirred under a hydrogen atmosphere at 20 °C. When the reaction was completed (1.5 h, HPLC monitoring), the crude reaction mixture was filtered. The elimination of solvent afforded a dark semisolid (0.063 g), which was identified as the expected phenol **4**. Final purification by preparative HPLC afforded the pure compound as a pale yellow solid **4** (*34*): IR (KBr) 3386, 3100, 3022, 2923, 1616, 1515, 1435, 815; ^{1}H NMR (CD_3OD) δ 6.62 (s, 4 H, H-2′, H-3′, H-5′, H-6′), 4.91 (s, 4 H, OH, NH), 3.79 (m, 1 H, H-2), 3.55 (m, 2 H, H-1), 3.20 (dd, 1 H, J_1 = 12.6 Hz, J_2 = 4.4 Hz, C$\underline{H}$H-N), 2.95 (dd, 1 H, J_1 = 12.6 Hz, J_2 = 7.5 Hz, CH$\underline{H}$-N); ^{13}C N$\underline{M}$R (CD_3OD) δ 150.6 (C-1′), 142.9 (C-4′), 116.8 (C-3′, C-5′), 116.5 (C-2′, C-6′), 71.5 (C-2), 65.6 (C-1), 47.9 (C-3); EI-MS (trisTMS derivative) *m*/*z* 399 (M^+), 195, 194 (base peak), 193, 73.

(6) Methyl 2-Hydroxy-3-[(4′-hydroxyphenyl)amino]propanoate (5a). Methyl 2,3-epoxypropanoate (0.10 g, 0.98 mmol) was added dropwise to a solution of 4-benzyloxyaniline (0.30 g, 1.51 mmol) in MeOH (5 mL) and maintained at 50 °C. The mixture was heated under reflux until the reaction was completed (6 h, GC monitoring). The residue obtained after solvent elimination was purified by preparative TLC (3:1 hexanes/EtOAc) to give 0.21 g of the expected benzyloxy intermediate as a solid (71% yield): mp 74−76 °C; ^{1}H NMR δ 7.4 (m, 5 H″), 6.86 (d, 2 H, J = 9 Hz, H-3′, H-5′), 6.64 (d, 2 H, J = 9 Hz, H-2′, H-6′), 5.00 (s, 1 H, CH_2Ph), 4.41 (m, 1 H, H-2), 3.79 (s, 3 H, CH_3), 3.51 (dd, 1 H, J_1 = 13 Hz, J_2 = 3.8 Hz, C$\underline{H}$H-N), 3.37 (dd, 1 H, J_1 = 13 Hz, J_2 = 5.6 Hz, CH$\underline{H}$-N); ^{13}C N$\underline{M}$R δ 174.1 (CO), 151.8 (C-4′), 141.8 (C-1′), 137.4 (C-1″), 128.4 (C-3″, C-5″), 127.7 (C-4″), 127.4 (C-2″, C-6″), 116.0 (C-3′, C-5′), 115.1 (C-2′, C-6′), 70.6 (CH_2Ph), 69.5 (C-2), 52.8 (CH_3), 48.0 (C-3); EI-MS *m*/*z* 301 (M^+), 210 (base peak), 150, 91. Elemental analysis for $C_{17}H_{19}NO_4$: C, 67.76; H, 6.35; N, 4.65. Found: C, 67.51; H, 6.40; N, 4.64.

A mixture of the benzyloxy derivative described above (0.033 g, 0.11 mmol) and 10% Pd/C (0.011 g) in MeOH (3 mL) was stirred under a hydrogen atmosphere at 20 °C. When the reaction was completed (1 h, HPLC monitoring), the crude reaction mixture was filtered. The residue obtained after elimination of the solvent was purified by preparative TLC (6:1 $CHCl_3$/MeOH), yielding the expected debenzylated ester as a brown oil (0.020 g) in 87% yield (**5a**): IR (KBr) 3600−3100 (stretching N−H and O−H), 3033, 2956, 1731, 1515, 1440, 825; ^{1}H NMR δ 6.70 (d, 2 H, H-3′, H-5′), 6.59 (d, 2 H, H-2′, H-4′), 4.41 (m, 1 H, H-2), 3.79 (s, 3 H, CH_3), 3.49 (dd, 1 H, J_1 = 13 Hz, J_2 = 3.6 Hz, C$\underline{H}$H-N), 3.36 (dd, 1 H, J_1 = 13.1 Hz, J_2 = 5.7 Hz, CH$\underline{H}$-N); ^{13}C N$\underline{M}$R δ 174.1 (CO), 148.5 (C-1′), 141.5 (C-4′), 116.1 (C-3′, C-5′), 115.5 (C-2′, C-6′), 69.5 (CH_3), 52.8 (C-2), 48.3 (C-3); EI-MS *m*/*z* 211 (M^+), 194, 122 (base peak).

Caution: *As PAP derivatives have been implicated in TOS, special precautions should be taken when handling these substances to avoid potential risks (use gloves, masks, and ventilated hood cabinets when handling solutions and powder).*

All standards and their solutions were stored at −20 °C until they were used.

Analytical Determinations in Biological Fluids. (1) High-Performance Liquid Chromatography (HPLC). HPLC analyses were conducted on a Waters system equipped with a DAD detector coupled on-line to a Berthold LB 507 radioactivity monitor system. C-18 Kromasil 100 columns (5 μm particle size, 250 mm × 4.6 mm) were used with 10 mM TEAA (pH 6.8) (solvent A) and acetonitrile (solvent B) as mobile phases. A time gradient elution consisted of an initial isocratic step (from 0 to 5 min) at a rate of 0.9 mL/min with 95% A, followed by a linear ramp to reach a rate of 1.1 mL/min with 60% A (from 5 to 20 min), conditions which were maintained up to 35 min. The DAD detector was set at 235 nm. The eluted sample was pumped and mixed with scintillation counting solution at a rate of 3 mL/min by a Berthold LB 5035 HPLC pump prior to the inlet of the radioactivity monitor. A guard column cartridge packed with the identical material was replaced periodically.

Urine samples were used as follows for different analytical purposes. Animal urine was used directly for radioactivity count; methanol-diluted urine was used for HPLC and GC/MS for general metabolic profiles, and extraction of direct or hydrolyzed urine yielded aqueous and organic extracts for HPLC and GC/MS analyses of specific metabolites. Briefly, 50 μL of the urine samples was mixed with methanol (1:9 v/v) and the mixture centrifuged for 10 min at 13000*g* in an Eppendorf centrifuge. Supernatants were injected directly into the chromatographic system. For the separation of polar metabolites from PAP, urine samples were buffered with 100 mM sodium phosphate (pH 8.0) and extracted with a mixture of dichloromethane and isopropyl alcohol (9:1) containing 1% ammonia. The organic extracts were evaporated and redissolved in TEAA (10 mM, pH 6.8)/acetonitrile (80:20) prior to being analyzed, while the aqueous phases were analyzed directly.

Two types of procedures, chemical and enzymatic, were used for conjugate cleavage and metabolite derivatization. The chemical procedure consisted of treatment of the previously lyophilized urine or aqueous extract samples with 1 mL of 1 M HCl in methanol (HCl/MeOH) for 15 min at 90 °C followed by evaporation of the solvents with a stream of nitrogen. Samples treated in this manner were subjected either to HPLC or to GC/MS analysis. Enzymatic hydrolytic procedures consisted of sample treatment with 2000 enzyme units of the sample incubate of β-glucuronidase from *E. coli* or *H. pomatia* per milliliter (which also has sulfatase activity) in the appropriate buffers for 16 h at 37 °C. Hydrolysates were further extracted at pH 8.0 as described above.

(2) Gas Chromatography/Mass Spectrometry (GC/MS). Samples for GC/MS analysis were lyophilized, redissolved in acetonitrile, and treated with BSTFA, as described by Adachi et al. (*32*), prior to injection into the GC/MS system described above. Samples treated with HCl/MeOH as described above were also redissolved in acetonitrile after evaporation of the solvents

and either injected directly or further treated with BSTFA before injection into the GC/MS system.

^{14}C Radioactivity Measurements in Organic Tissue and Body Fluids. Urine samples (20 μL) were mixed with 10 mL of OptiPhase "HiSafe" scintillation cocktail and assessed. The radioactivity remaining in feces and tissues was counted in a similar manner after total organ homogenization in PBS buffer (pH 7.4). An aliquot of tissue homogenate (0.5−1 mL) was analyzed, and the total number of ^{14}C disintegrations per minute in organs was calculated.

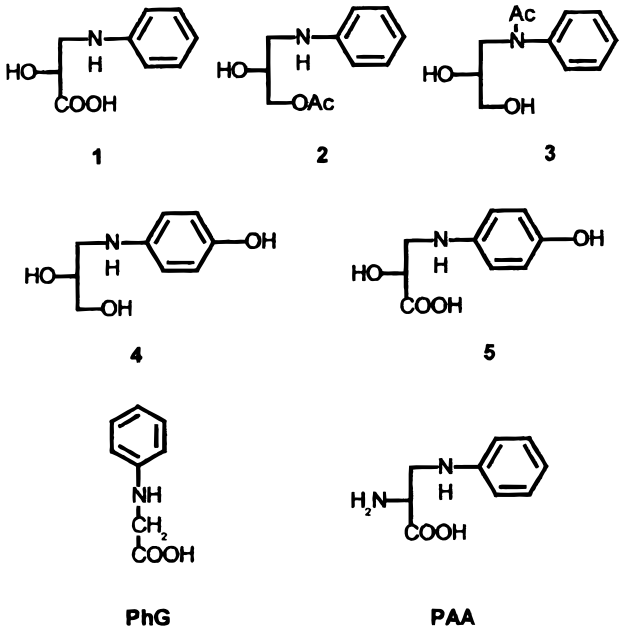
Results

Synthesis of Standards. Synthesis of the radioactive substrate [U-^{14}C]PAP was carried out by the regioselective opening of glycidol with [U-^{14}C]aniline in methanol, giving a 57% yield. Purification of the crude reaction mixture by preparative thin-layer chromatography was conducted with care to ensure separation of the unreacted aniline. On the other hand, the radiochemical purity of the injected PAP was established by HPLC under the elution conditions used for metabolism analysis. Furthermore, particular conditions for achieving a clean separation between aniline and PAP were developed (under the conditions described above, the separation was less than 1 min) to confirm that radioactive aniline was absent, within the detection limits, in the radioactive samples of PAP used for the experiments.

Synthesis of hydroxy acid **1** was accomplished via dimethyldioxirane epoxidation of methyl acrylate, followed by reaction with aniline in methanol and final saponification in basic medium. The epoxidation reaction was slow, but the mild conditions that were used and the easy workup of the dioxirane reactions made it possible to isolate this fairly reactive epoxy derivative in sufficiently pure form that it could be used in the subsequent step. On the other hand, it is worth noting that the methyl ester precursor of **1** exhibited higher lability due to hydrolysis compared with structurally related compounds lacking the free amino group (results not shown).

With respect to the acetyl derivatives **2** and **3**, conventional acetylation of PAP led only to the *O*-acetyl derivative **2**. Consequently, chemoselective acetylation of the secondary amino group required previous protection of the diol moiety as the dioxolane. As occurred with the methyl ester precursor of **1**, the *N*-acetyl derivative **3** also exhibited an enhanced tendency to undergo hydrolysis to yield PAP or acyl migration to give **2** under relatively mild acid conditions in comparison with other anilides. All these results suggest that the nitrogen atom at C-3 and the oxygen atom at C-1 of PAP derivatives could be promoting some kind of mutual interaction. Phenol **4** was synthesized by addition of 4-benzyloxyaniline over glycidol followed by hydrogenolysis of the benzyl ether protecting group. Under the standard conditions used for the HPLC analysis of the different extracts, compound **4** gave a unique peak at 3.5 min, but the profile became more complex (appearance of a peak at 7.9 min) when the sample was progressively diluted. In all cases, the GC/MS analysis of the respective trimethylsilyl derivative gave one peak consistent with the structure of **4**. Likewise, the synthesis of phenol acid **5** was attempted starting from methyl epoxypropionate by using a similar synthetic sequence, i.e., reaction with 4-benzyloxyaniline followed by hydrogenolysis and ester hydrolysis. However, this final step led to complex crude reaction mixtures due to the instability of compound **5**.

Chart 2. PAP Derivatives and Related Structures Investigated as Putative Metabolites of PAP in C57BL/6 and A/J Mice



The PAP derivatives and related structures investigated as putative metabolites in the study presented here are depicted in Chart 2.

Experimental Protocol. Throughout the experiments, the two groups of mice exhibited no obvious symptoms of toxicity after the single PAP dose or during β-naphthoflavone induction treatment. Metabolic cages served for collecting urine and feces, with no observable leaks. The two groups of animals (three A/J and three C57BL/6 mice in each cage) produced approximately 1.5−2 mL of urine and 3−5 g of feces in 24 h. Each animal group received a single PAP intraperitoneal dose in 0.2 mL of saline. Therefore, the urine and feces that were collected represented the production of each three-animal group. Similarly, organs were pooled separately by type for radioactivity measurement. When induction by β-naphthoflavone treatment was being performed, the chemical was administered during the 3 days of acclimatization to metabolic cages as described in Materials and Methods.

PAP Clearance in Urine of Controls and β-Naphthoflavone-Treated Animals. Radioactivity measured in urine and feces accounted for the total body clearance of PAP administered to each group, and the results are shown in Table 1. Percentages were established after calculation of the total radioactivity administered to each group. A/J mice had the highest urine clearance (68−70%) independent of induction treatment. Conversely, control C57BL/6 mice had lower urine clearance (36%) than the induced group (57%), which reached levels similar to those found in A/J mice. It is noteworthy that feces in neither of the groups exhibited significant clearance compared with urine; however, the radioactivity content in feces was higher than that observed in tissues (vide infra). Thus, significant clearance of PAP or metabolites was observed specifically in urine.

With respect to the radioactivity remaining in organs, only liver and kidney exhibited some significant remaining dose (Table 2), while lung, spleen, heart, and muscle contents were less than 0.01% of the total administered

Table 1. [^{14}C]PAP Clearance in Urine and Feces in a 24 h Period

	untreated mice[a]		β-NF-treated mice[a]	
	A/J	C57BL/6	A/J	C57BL/6
^{14}C-PAP dose[b]	3.9×10^{7} [b]	3.9×10^{7} [b]	4.7×10^{7} [b]	4.5×10^{7} [b]
urine (mL)	1.80	1.90	1.46	2.00
% of dose	70	36	68	57
feces (g)	3.95	4.46	3.36	5.19
% of dose	0.60	3.30	0.90	0.95

[a] Three mice in each group. [b] [^{14}C]PAP total dose (disintegrations per minute) administered to each animal group. Urine and feces samples were collected for a 24 h period in each metabolic cage housing three animals per group as described in detail in Materials and Methods.

Table 2. Percentages of the [^{14}C]PAP Dose Remaining in Tissues[a]

	untreated mice		β-NF treated mice	
tissue[b]	A/J	C57BL/6	A/J	C57BL/6
liver	0.08%	0.08%	0.05%	0.06%
kidney	0.03%	0.04%	0.02%	0.01%

[a] The [^{14}C]PAP total dose administered to the animals (Table 1) was considered to be 100%. Animals were euthanized after 24 h. [b] Organ types from each animal group were pooled together and homogeneized as described in detail in Materials and Methods.

dose. Accumulation of radioactivity in tissue reflected organ blood flow distribution, with liver and kidney exhibiting the highest remaining amount of radioactivity per gram of tissue. The radioactivity remaining in organs did not explain the differences observed in the urine clearance. PAP or its metabolites may accumulate in other organ tissues such as brain or skin that were not collected.

Analytical Determination of Metabolites in Urine Control Animals. Analytical conditions for determining very polar species derived from PAP were established with TEAA/acetonitrile as the mobile phase, as described in Materials and Methods. Under these conditions, PAP had a retention time of 19.8 min. HPLC profiles from methanol-diluted urine obtained from control animals are shown in Figure 1. Both strains exhibited similar chromatographic profiles, with a peak at 16.2 min as the major radioactive species, whose UV spectrum closely resembled that of PAP (Figure 1A, inset). Minor traces of untransformed PAP were in the detection limits as determined by the radiodetector (Figure 1B). To determine more accurately PAP traces, urine samples buffered at pH 8.0 were extracted using a mixture of dichloromethane and isopropyl alcohol (9:1) containing 1% ammonia. Previous experience with a PAP-spiked control urine showed 90% recovery under those conditions. Extracts exhibited a PAP peak at 19.8 min that accounted for 0.2% of the total radioactivity dose. The major metabolite remained in the aqueous phase. Its relative abundance, as derived from the integration of the radioactivity profile (Figure 1B), was estimated to be 57% in urine from both strain groups, and contributed to PAP clearance in 40 and 20% of the total administered dose in A/J and C57BL/6 mice, respectively (Table 3).

In addition, a second major radioactive peak was observed in the chromatograms in the 3−4 min region (Figure 1B). Peaks have a relative level of integration of 15−20% for both strain groups, which accounted for 10.5 and 7.2% of the total radioactivity administered to A/J and C57BL/6 mice, respectively (Table 3). This peak also remained in the aqueous phase on extraction of the urine

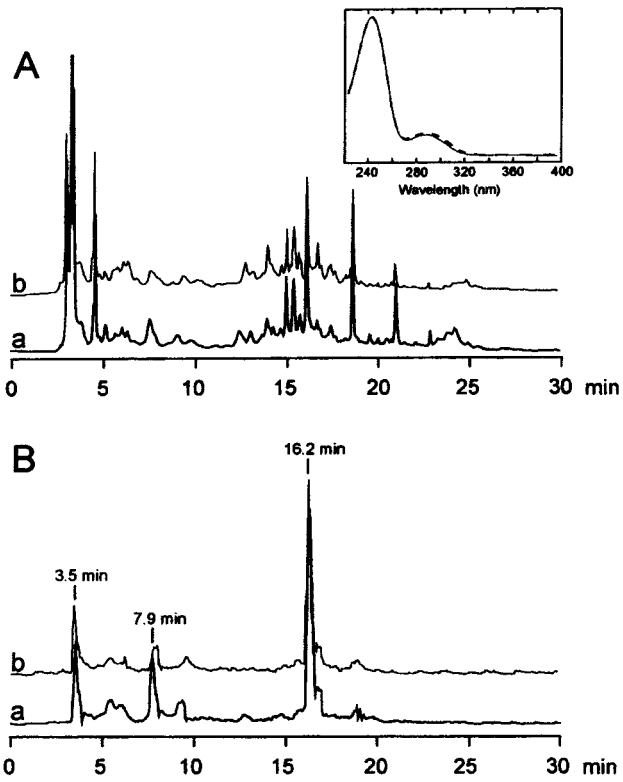


Figure 1. Typical HPLC chromatograms of methanol-diluted urine samples from [^{14}C]PAP-treated A/J (a) and C57BL/6 (b) mice. (A) HPLC tracings obtained by monitoring the UV absorbance at 235 nm. The inset shows the normalized spectra of the peak at 16.2 min (dashed line) and PAP (solid line). (B) HPLC tracings obtained by monitoring ^{14}C radioactivity. Both tracings are plotted 0.33 min to the left to correct for the dead volume between the on-line UV and radioactivity detectors. HPLC conditions are described in Materials and Methods.

Table 3. Estimation of the Metabolite Contribution to [^{14}C]PAP Elimination

		3−4 min peak[b]		16.2 min peak[b]	
	clearance factor[a]	% integration	% dose	% integration	% dose
untreated mice					
A/J	0.70	15	10.5	57	40
C57BL/6	0.36	20	7.2	57	20
β-NF-treated mice					
A/J	0.68	12	8.2	61	41
C57BL/6	0.57	15	8.6	65	37

[a] Urine clearance factor corresponds to the percentage of the radioactivity dose eliminated in urine, depicted in Table 1. [b] Integrated radioactive peaks are presented as a percentage of the total radioactivity detected in the urine sample as depicted in chromatograms (Figures 1 and 5). The peak contribution to elimination is estimated by correcting the integrated value with the clearance factor.

samples under the conditions mentioned above. This observation, together with the shape of the UV profile in the 0−5 min region (Figure 1A), suggested that the peak corresponds to one or more highly polar metabolites which proved to be difficult to separate chromatographically from other polar contaminants that eluted in the same region. Finally, aside from other minor radioactive peaks, a peak was observed at 7.9 min (Figure 1B) whose shape and integration appeared to be dependent on sample history.

Identification of Major Metabolites. Extractions at various pH values showed that the 16.2 min peak would start to partition to the organic phase at pH 5. Enzyme

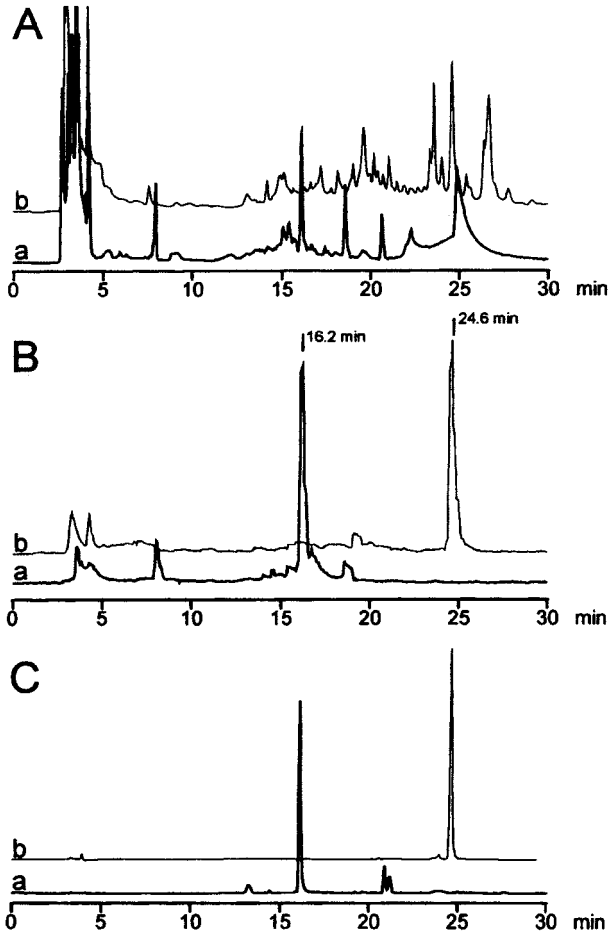


Figure 2. (A) HPLC chromatograms, obtained by monitoring the UV absorbance at 235 nm, of the aqueous phase of CH_2Cl_2-extracted urine samples from [^{14}C]PAP-treated A/J mice (a) and the same sample after chemical HCl/MeOH treatment (b). (B) HPLC chromatograms obtained by monitoring the ^{14}C radioactivity of samples described for panel A. (C) HPLC chromatograms of synthetic PAP acid **1** (a) and its corresponding methyl ester **1a** (b).

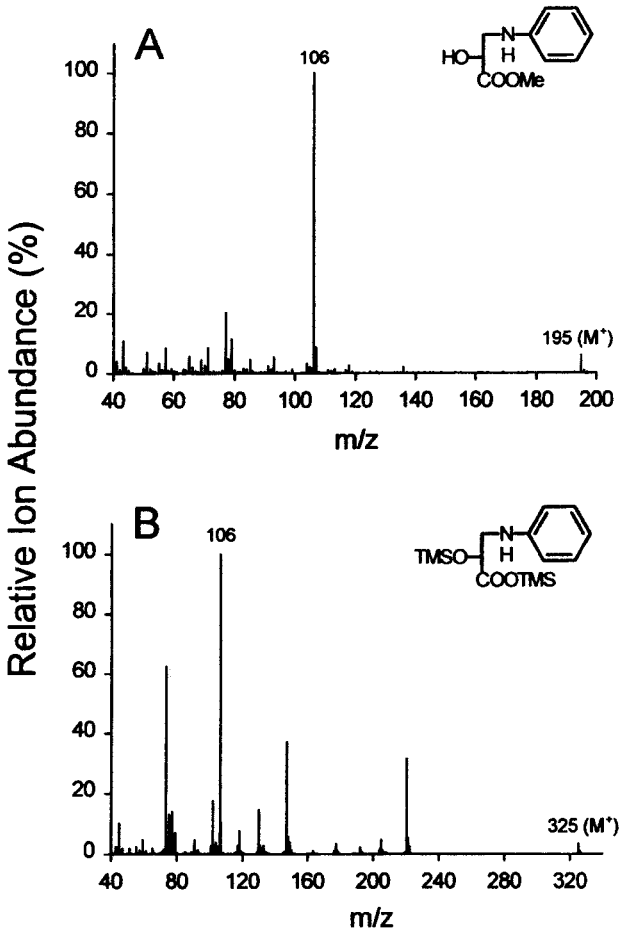


Figure 3. (A) EI-MS spectrum of the peak collected at 24.6 min. (B) EI-MS spectrum of the peak collected at 16.2 min after lyophilization and BSTFA treatment. The postulated structures of both products are shown.

hydrolysis attempts (glucuronidase and sulfatase) carried out on urine samples from both groups of mice failed to show any chromatographic change for that particular peak. These findings suggested an acid character for this compound which, if conjugated, would be resistant to enzyme hydrolysis. The fact that its UV spectrum was identical to that of PAP suggested that if any chemical modification had occurred, it should have taken place on the alkyl moiety without affecting the aromatic ring.

Tentative chemical conjugate cleavage and/or derivatization of metabolites was performed as follows. PAP was extracted from urine samples from both groups of mice as described above, and the corresponding aqueous fractions were lyophilized and further subjected to HCl/MeOH treatment, as described in Materials and Methods. After evaporation of the solvents, the residue was dissolved in acetonitrile and analyzed by HPLC. The chromatograms of the treated samples showed the disappearance of the 16.2 min peak in favor of a new peak with a retention time of 24.6 min (panels A and B of Figure 2) which did not show any changes in its UV spectrum. This peak was collected from semipreparative injections onto the HPLC system, and the fractions were lyophilized and redissolved in acetonitrile. Analysis of these solutions by GC/MS revealed a main product with an EI-MS spectrum (Figure 3A) that is compatible with the structure of methyl 2-hydroxy-3-(phenylamino)propanoate (**1a**). Furthermore, collection of the 16.2 min peak and derivatization of the lyophilized fractions with BSTFA, followed by GC/MS analysis, yielded the spectrum shown in Figure 3B which can be attributed to the corresponding bis(trimethylsilyl) derivative of acid **1**. Final confirmation of the identity of these two peaks was obtained from the comparison of the chromatographic mobilities in HPLC with those from synthetic standards (Figure 2C). Therefore, we conclude that the main 16.2 min peak present in urine samples from both mice strains corresponds to the PAP-derived acid **1**.

Similar attempts to characterize the most polar metabolites that appeared in the 3−4 min region of the HPLC chromatograms were carried out. Neither enzyme hydrolysis nor HCl/MeOH treatment apparently affected the chromatographic mobility of these compounds. The collected peak was subjected to derivatization with HCl/MeOH, followed by BSTFA treatment and analysis by GC/MS. This allowed the detection of two products which were identified as the trimethylsilyl derivatives from structures **4** and **5a**, on the basis of the identity of their EI-MS spectra (panels A and B of Figure 4) and the identity between HPLC and GC retention times and those from synthetic standards. We had expected the presence of phenolic species from the observation of pH-dependent color formation in urine samples. It is worth noting that when the peak appearing at 7.9 min (Figure 1B) was collected, lyophilized, and reinjected into the

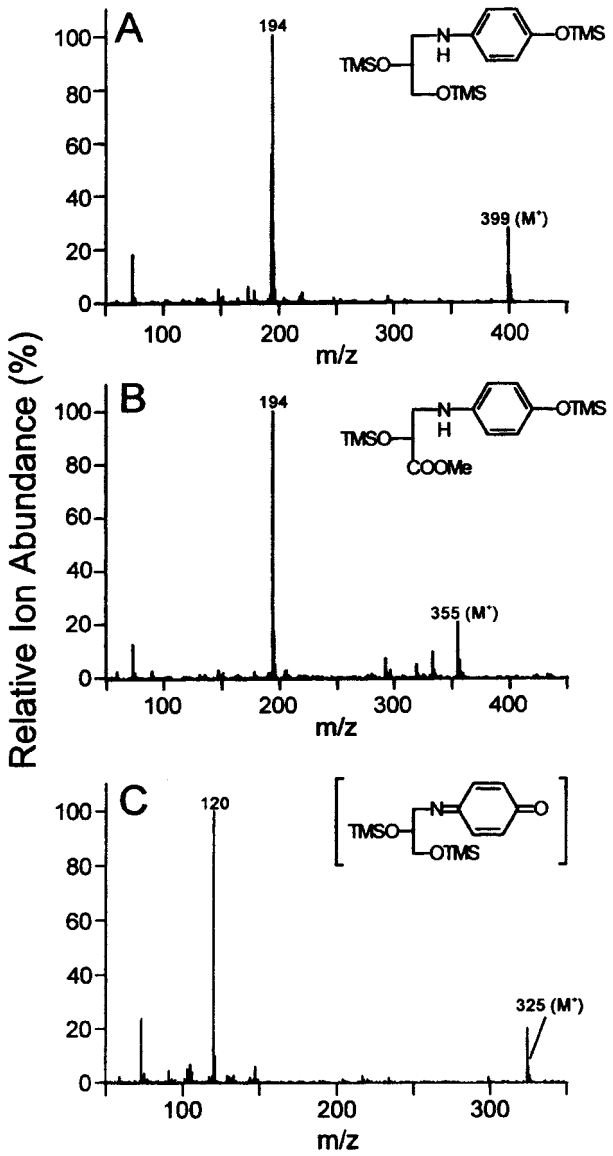


Figure 4. (A and B) EI-MS spectra of products identified in the 3−4 min region of the HPLC chromatograms, after HCl/MeOH and BSTFA treatment. (C) EI-MS spectrum of a minor component detected in direct urine samples which had been subjected to HCl/MeOH and BSTFA treatment. This component was tentatively assigned as the iminoquinone shown in brackets.

HPLC system, surprisingly only a peak at 3.5 min was observed. Furthermore, on derivatization of this fraction with BSTFA and analysis by GC/MS, only the trimethylsilyl derivative of phenol **4** could be identified. These observations seem to agree with our previous experience with synthetic standard **4**, suggesting that under the HPLC conditions used this product exhibits a complex chromatographic profile.

In addition, urine samples from both mouse groups were subjected to similar HCl/MeOH and BSTFA treatments so that analysis by GC/MS could be performed. Aside from the corresponding peaks of PAP, and metabolites **1**, **4**, and **5**, a peak with an EI-MS spectrum (Figure 4C) compatible with the structure of the iminoquinone derived from phenol **4** was observed at very low abundance.

Other potential PAP metabolites that we considered were the *O*- and *N*-acetylated derivatives **2** and **3** (Chart 2), which under our chromatographic HPLC conditions

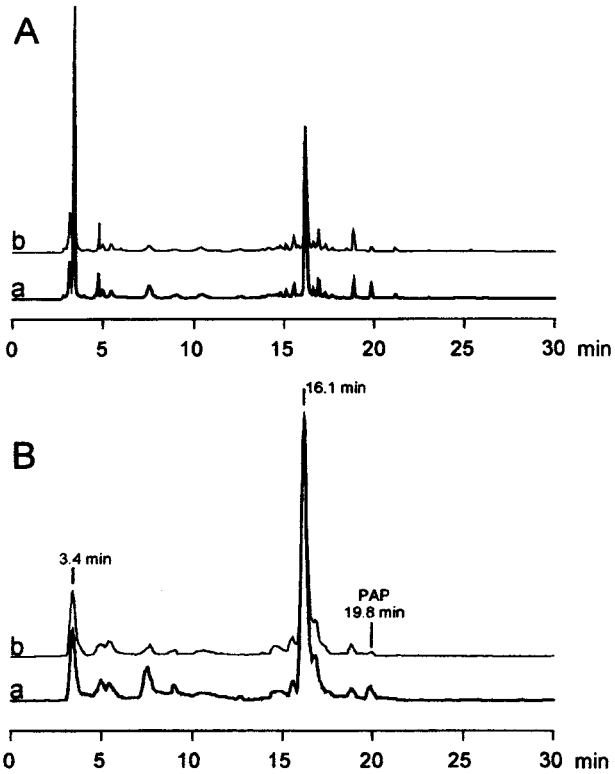


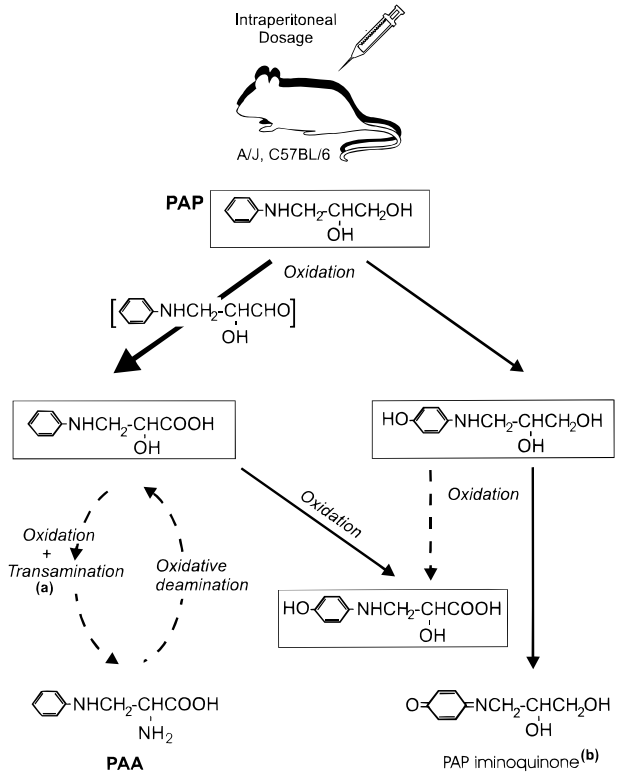
Figure 5. Typical HPLC chromatograms of methanol-diluted urine samples from [^{14}C]PAP-treated β-naphthoflavone-induced A/J (a) and C57BL/6 (b) mice. (A) HPLC tracing obtained by monitoring UV absorbance at 235 nm. (B) HPLC tracing obtained by monitoring the ^{14}C radioactivity.

eluted at 25.2 and 18.7 min, respectively. Neither of these compounds was identified in urine, nor was PAA or its potential decarboxylated metabolite PhG (Chart 2), whose retention times under our analytical HPLC conditions were 13.5 and 16.1 min, respectively. Despite the difficulty in separating the peak from PhG from that of acid metabolite **1** by HPLC, the results from the GC/MS analyses confirmed the absence of the PhG metabolite.

Urine Metabolites in the β-Naphthoflavone-Induced Animals. After induction treatment, both strain groups still exhibited a higher clearance in the form of the PAP acid metabolite **1**, constituting approximately 40% of the total dose administered in both animal groups (Figure 5 and Table 3). The highly polar chemical species in the 3−4 min zone were also observed in both strains and accounted for 8.2 and 8.6% of the total dose in A/J and C57BL/6 mice, respectively (Table 3).

Tissue and Feces Metabolites. As depicted in Table 2, the amount of radioactivity remaining in tissues was small, rendering analysis of compounds difficult. Concentrating chemical species in tissue samples would require the future development of solid-phase extraction procedures since the metabolites are very polar substances and remain in the aqueous phase. On the other hand, the low level of radioactivity limited the analyses. However, we were able to observe polar 3−4 min peak species in liver supernatants, and no PAP or other compounds were present. With regard to feces, traces of PAP were found, and practically the total amount of radioactivity present was in the form of polar 3−4 min peaks. These polar peaks observed in feces and liver were not further identified.

Scheme 1. PAP Elimination Pathways in Mice



[a] Pathways proposed by Mayeno et al. (*37*). [b] Molecular structure postulated from EI-MS spectral data.

Discussion

Major PAP metabolites excreted in urine in A/J and C57BL/6 mice were identified for the first time. These findings imply that PAP, a very polar substance, is highly metabolized in mice and excreted principally in urine in the form of 2-hydroxy-3-(phenylamino)propanoic acid (**1**); other species oxidized at the aromatic ring were also identified in urine. Thus, the major route of PAP elimination in these mice strains is alkyl chain oxidation (Scheme 1). PAP resynthesis to MPAP or DPAP derivatives was not detected either in urine or in the tissues that were examined. The urine profile was similar in both strains, and PAP-acetylated metabolites were not formed; thus, the strain acetylator status seems not to play a role in PAP metabolism. The C57BL/6 mouse strain exhibited a significant increase in the urine elimination rate after β-naphthoflavone treatment. An explanation of this phenomenon is that activation of the Ah receptor battery induced efficiently the expression of enzymes involved in PAP metabolism. Activation of the Ah receptor mediates the expression of several enzymes, including menadione oxidoreductase, glucuronyltransferase, glutathione *S*-transferase, CYP1A1, CYP1A2, CYP1B1, and aldehyde dehydrogenase (*35*, *36*). This latter enzyme could be involved in the formation of the PAP acid metabolite. The unresponsiveness of A/J mice to the inducing treatment may be related to Ah differential activation observed between mouse strains (*37*, *38*), and the fact that a constitutive aldehyde enzyme is present in microsomes (*39*).

The PAP dose administered in this study was chosen according to the work of Maestro Duran et al. (*24*), who reported PAP toxicity when it was administered intraperitoneally at higher doses (465 mg/kg) in Swiss mice. These authors observed a high mortality rate in male and female animals after a 3-fold dose regimen, whereas there were no fatalities among animals administered half-doses for 8 days; however, other types of motor and sensorial impairment and organ alterations were observed. Moreover, these authors detected PAP by TLC analysis in several organs. MPAP and DPAP resynthesis was observed only when PAP was administered orally. Our data are not in contradiction with their findings as they failed to identify polar metabolites since they used organic extracts analyzed by TLC. In a later work, they reported metabolite species formed in the rat intestinal tract which were not identified, and confirmed the existence of hydrolysis and resynthesis (*25*).

Much attention has been paid to mono- and diesters of PAP administered orally or intraperitoneally to animals (*23*, *24*); in any event, hydrolysis and conversion of these derivatives to PAP is to be expected. Therefore, elucidation of the PAP metabolism is considered to be an important step toward understanding PAP derivative disposition in animals. This knowledge would allow investigators to look for elimination and/or formation pathways of reactive metabolites with potential toxicity implications.

Eosinophilia-Myalgia syndrome (EMS), a physiopathological syndrome with clinical features similar to those of TOS (*40*), occurred in the United States as a result of contaminants present in tryptophan vitamin tablets. 3-(Phenylamino)-L-alanine (PAA) was a contaminant identified and associated with EMS (*41*). In particular, a metabolic conversion link was reported between these compounds (*42*) since metabolic conversion of PAP to PAA was observed in human liver slices and rat hepatocytes; however, the rate of formation seemed very low in comparison to those of other metabolic peaks that were not specified. Mayeno et al. (*42*) suggested the formation of a 2-hydroxy-3-(phenylamino)propanoic acid metabolite as the realistic pathway of conversion of PAP to PAA, but did not mention or identify such chemical species in their incubates. In a previous work, Adachi et al. (*32*) observed urine excretion of 2-hydroxy-3-(phenylamino)-propanoic acid and its *p*-hydroxyl metabolite after PAA oral administration in Wistar rats, suggesting that PAA exhibited phenylalanine-like behavior in its biotransformation, excretion, and accumulation in tissues.

We did not detect the PAA or PhG metabolite in our samples. If PAA had been formed from the PAP acid metabolite (as suggested by the work of Mayeno), the enzymatic deamination of PAA by monoamine oxidase enzyme would have recovered the PAP acid metabolite in urine, as suggested by Adachi et al. (*32*). Thus, our findings suggest that PAP has more chance of being eliminated through the acid metabolite **1** than being converted to PAA in vivo; however, the formation of PAA cannot be ruled out, and its possible accumulation in the nervous system has been reported (*43*). In this particular respect, we did not collect brain tissue and could not therefore exclude the possible formation of PAA, as it accumulates mainly in that tissue (*43*). The metabolites identified in the study presented here suggest that PAA and PAP share chemical features and undergo similar biotransformations, probably by different enzymes, and in fact have a differential clearance (refs *32*, *42*, and *43* and this study) (Scheme 1).

Our data indicate that oxidized reactive chemical species could be expected from PAP, either from aldehyde

intermediates in the formation of PAP acid **1** or from the iminoquinones derived from the hydroxylation at the aromatic ring. This point may be important in explaining the potential toxicity of these aniline derivatives when several doses have been given (*24*). Reactive metabolites derived from xenobiotic metabolism have long been claimed to underlie the pathologic mechanism of autoimmunity associated with xenobiotic exposure (*44*, *45*). In particular, aromatic oxidized species and iminoquinones have proved to induce some type of lymphocyte transformation (*44*−*46*). Oxidized species derived from oleanilides have also been proposed in the context of toxic oil syndrome because of their clinical features (*2*), and recently by the data of Berking et al. (*29*) obtained with the same animal strains that were used in this study. This last interesting study emphasized the possibility of reactive metabolites from oleanilides being involved in the TH2-lymphocyte pattern observed in those animals, which was similar to that reported in TOS patients (*28*).

When the latest considerations are taken into account, the study presented here would add a further step in the construction of an animal model for in-depth investigation of these autoimmunity features, since to date it has been assumed that TOS patients had ingested both oleanilides and PAP derivatives. The possibility of in vivo interaction or mutual synergism of these compounds in their biological actions is intriguing. The fact that PAP can be extensively metabolized and excreted in urine in the form of its acid metabolite points to a high detoxification pathway in mice. However, potential reactive species (aldehyde intermediate and iminoquinones) may be formed as well, and this affords another way of considering the potential toxicity of these compounds via their metabolites. Hypothetically, TOS patients could have had an enzyme activity impairment on pathways dealing with detoxification, resulting in accumulation of reactive species. Therefore, further studies on the disposition of PAP derivatives administered orally should clarify the potential contribution of intestinal lumen to DPAP hydrolysis and resynthesis, as well as the initial PAP metabolic species. In this respect, it is worth noting that a major intestinal PAP metabolite was identified when the compound was administered orally (*25*).

Efforts to identify the enzymes involved in the formation of the metabolites reported herein and clarify their occurrence in human tissues are in progress. These studies would add several clues to approximating the potential toxicity of these compounds in humans and help to build animal or in vitro models for ascertaining the mechanisms that are involved.

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References

(1) Gradjean, P., and Tarkowski, S., Eds. (1984) *Toxic Oil Syndrome: mass food poisoning in Spain. Report of a WHO Meeting: Madrid, 21*−*25 March, 1983*, World Health Organization Regional Office for Europe, Copenhagen, Denmark.

(2) Tabuenca, J. M. (1981) Toxic allergic syndrome caused by ingestion of rapeseed oil denatured with aniline. *Lancet* **2**, 567−568.

(3) Diaz de Rojas, F., Castro-García, M., Abaitua-Borda, I., Alonso-Gordo, J. M., Posada de la Paz, M., Kilbourne, E. M., and Tabuenca-Oliver, J. M. (1987) The association of oil ingestion with Toxic Oil Syndrome in two convents. *Am. J. Epidemiol.* **125**, 907−911.

(4) Cañas, R., and Kilbourne, E. M. (1987) Oil ingestion and the Toxic Oil Syndrome. Results of a survey of residents of the Orcasur neighbourhood in Madrid Spain. *Int. J. Epidemiol.* **16**, 3−6.

(5) Rigau-Perez, J. G. (1984) Summary of case-control studies and case or cluster investigations. In *Toxic Oil Syndrome: mass food poisoning in Spain. Report of a WHO Meeting: Madrid 21*−*25 March, 1983* (Gradjean, P., and Tarkowski, S., Eds.) pp 47−51, World Health Organization Regional Office for Europe, Copenhagen, Denmark.

(6) Rigau-Perez, J. G., Pérez-Alvarez, L., Dueñas-Castro, S., Choi, K., Thacker, S. B., Germain, J. L., González-de-Andrés, G., Canada-Royo, L., and Pérez-Gallardo, F. (1984) Epidemiologic investigation of an oil-associated pneumonic paralytic eosinophilic syndrome in Spain. *Am. J. Epidemiol.* **119**, 250−260.

(7) Abaitua-Borda, I., and Posada de la Paz, M. (1992) Clinical findings. In *Toxic Oil Syndrome: current knowledge and future perspectives* (Nadal, J., and Tarkowski, S., Eds.) pp 26−36, WHO Regional Publications, European Series 42, World Health Organization Regional Office for Europe, Copenhagen, Denmark.

(8) Gómez de la Cámara, A., Posada de la Paz, M., Abaitua-Borda, I., Barainca-Oyagüe, M. T., Abraira-Santos, V., Diez-Ruiz-Navarro, M. D., and Terracini, B. (1998) Health Status Measurements in Toxic Oil Syndrome. *J. Clin. Epidemiol.* **51** (10), 867−873.

(9) Nadal, J., and Tarkowski, S. (1992) *Toxic Oil Syndrome: current knowledge and future perspectives*, WHO Regional Publications, European Series 42, World Health Organization Regional Office for Europe, Copenhagen, Denmark.

(10) Posada de la Paz, M., Philen, R. M., Abaitua Borda, I., Bernert, J. T., Bada-Gancedo, J. C., DuClos, P. J., and Kilbourne, E. M. (1996) Toxic Oil Syndrome: traceback of the toxic oil and evidence for a point source epidemic. *Food Chem. Toxicol.* **34** (3), 251−257.

(11) Ventura Diaz, M. (1982) Conventional analysis of the available samples of purportedly toxic oils. *Grasas Aceites (Seville)* **33**, 73−77.

(12) Bernert, J. T., Kilbourne, E. M., Akins, J. R., Posada de la Paz, M., Meredith, N. K., Abaitua-Borda, I., and Wages, S. (1987) Compositional analysis of oil samples implicated in the Spanish toxic oil syndrome. *J. Food Sci.* **52**, 1562−1569.

(13) Aldridge, W. N. (1992) Experimental studies. In *Toxic Oil Syndrome: current knowledge and future perspectives* (Nadal, J., and Tarkowski, S., Eds.) pp 63−93, WHO Regional Publications, European Series 42, World Health Organization Regional Office for Europe, Copenhagen, Denmark.

(14) Kilbourne, E. M., Bernert, J. T., Posada de la Paz, M., Hill, R. H., Abaitua-Borda, I., Kilbourne, B. W., Zack, M. M., et al. (1988) Chemical correlates of pathogenicity of oils related to the toxic-oil syndrome epidemic in Spain. *Am. J. Epidemiol.* **127**, 1210−1227.

(15) Posada de la Paz, M., Philen, R. M., Abaitua-Borda, I., Diez-Ruiz Navarro, M., Abraira-Santos, V., Pozo, F., Pla-Mestre, R., Pollan-Santamaría, M., Sicilia-Socias, J. M., Azpeitia-Gamazo, P., Woodruff-Dyer, R., and Kilbourne, E. M. (1994) Factors associated with pathogenicity of oils related to the Toxic Oil Syndrome epidemic in Spain. *Epidemiology* **5**, 404−409.

(16) Vázquez-Roncero, A., Janer del Valle, C., Maestro-Duran, R., and Graciane-Constante, E. (1983) New aniline derivatives in cooking oils associated with the toxic oil syndrome. *Lancet* **2**, 1024−1025.

(17) Hill, R. H., Schurz, H. H., Posada de la Paz, M., Abaitua-Borda, I., Philen, R. M., Kilbourne, E. M., Head, S. L., Bailey, S. L., Driskell, W. J., Barr, J. R., and Needham, L. L. (1995) Possible etiologic agents for Toxic Oil Syndrome: fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol. *Arch. Environ. Contam. Toxicol.* **28**, 259−264.

(18) Schurz, H. H., Hill, R. H., Posada de la Paz, M., Philen, R. M., Abaitua-Borda, I., Bailey, S. L., and Needham, L. L. (1996) Products of aniline and triglycerides in oil samples associated with the Toxic Oil Syndrome. *Chem. Res. Toxicol.* **9**, 1001−1006.

(19) Posada de la Paz, M., Philen, R. M., Schurz, H. H., Hill, R. H., Gimenez-Ribota, O., Gómez de la Cámara, A., Kilbourne, E. M., and Abaitua-Borda, I. (1999) Epidemiologic evidence for a new class of compounds associated with Toxic Oil Syndrome. *Epidemiology* **10** (2), 130−134.

(20) Nelson, S. D. (1985) Arylamines and Arylamides: oxidation mechanisms. In *Bioactivation of Foreign Compounds* (Anders, M. W., Ed.) pp 349−374, Academic Press, New York.

(21) Hanna, P. E., and Banks, R. B. (1985) Arylhydroxylamines and arylhydroxamic acids: conjugation reactions. In *Bioactivation of Foreign Compounds* (Anders, M. W., Ed.) pp 375−402, Academic Press, New York.

(22) Roots, I., Hildebrandt, A. G., Hirner, A., and Heinemeyer, G. (1982) High sensitivity of cytochrome P-450 reactions in human liver microsomes towards oleylanilide, the presumptive toxin in the Spanish oil epidemic 1981. *Naunyn-Schmiedebergs Arch. Pharmacol.* (Suppl.), R-30.

(23) Vázquez-Roncero, A., Maestro-Duran, R., and Ruiz-Gutierrez, V. (1984) New aniline derivatives in oils related to the Toxic Syndrome. Toxicity in mice of 3-phenylamino-1,2-propanediol and its fatty acid mono- and diesters. *Grasas Aceites (Seville)* **35**, 330−331.

(24) Maestro-Duran, R., Ruiz-Gutierrez, V., and Vazquez-Roncero, V. (1985) Estudio toxicologico en ratones de nuevos derivados de la anilina presentes en aceites tóxicos. *Rev. Toxicol. (Spain)* **2** (3), 168−183.

(25) Ruiz-Gutierrez, V., and Maestro-Duran, R. (1992) Lymphatic absorption of 3-phenyalmino-1,2-propanediol and its esters. *Exp. Toxic. Pathol.* **44**, 29−33.

(26) Carthew, P., Edwards, R. E., Dorman, B. M., and Verchoyle, R. D. (1995) A comparison of the acute pathology induced by 3-phenyalmino-1,2-propanediol (PAP) and its mono-oleoyl ester in rodents with the toxic oil syndrome in man. *Hum. Exp. Toxicol.* **14**, 217−220.

(27) Gallardo, S., Del Pozo, V., Cardaba, B., de Andrés, B., Martín-Orozco, E., Fernández, J. C., Tramon, P., Posada, M., Abaitua, I., Palomino, P., and Lahoz, C. (1994) Immunological basis of toxic oil syndrome (TOS). *Toxicology* **93**, 289−299.

(28) del Pozo, V., de Andrés, B., Gallardo, S., Cardaba, B., de Arruda-Chaves, E., Cortegano, I., Jurado, A., Palomino, P., Oliva, H., Aguilera, B., Posada, M., and Lahoz, C. (1997) Cytokine mRNA expression in lung tissue from Toxic Oil Syndrome patients: a Th2 immunological mechanism. *Toxicology* **118**, 61−70.

(29) Berking, C., Hobbs, M. V., Chatelain, R., Meurer, M., and Bell, S. A. (1998) Strain-dependent cytokine profile and susceptibility to oleic acid anilide in a murine model of the Toxic Oil Syndrome. *Toxicol. Appl. Pharmacol.* **148**, 222−228.

(30) Ferrer, M., Galceran, M., Sanchez-Baeza, F., Casas, J., and Messeguer, A. (1993) Synthesis of aniline derivatives with potential toxicological implications to the Spanish Toxic Oil Syndrome. *Liebigs Ann. Chem.*, 507−511.

(31) Guardiola, M., and Messeguer, A. (1998) Synthesis of tritium and carbon-14 labelled linoleic acid esters of 3-(phenylamino)-1,2-propanediol, compounds potentially involved in the etiology of the Toxic oil Syndrome. *J. Labelled Compd. Radiopharm.* **41**, 75−80.

(32) Adachi, J., Mio, T., Ueno, Y., Naito, T., Nishimura, A., Fujiwara, S., Sumino, K., and Tatsuno, Y. (1994) Identification of four metabolites of 3-(phenylamino)alanine, a constituent in L-tryptophan products implicated in eosinophilia-myalgia syndrome, in rats. *Arch. Toxicol.* **68**, 500−505.

(33) Chini, M., Crotti, P., Favero, L., Macchia, F., and Pineschi, M. (1994) Lanthanide(III) trifluoromethanesulfonates as extraordinarily effective new catalysts for the aminolysis of 1,2-epoxides. *Tetrahedron Lett.* **35**, 433−436.

(34) Benson, L. M., Tomlinson, A. J., Mayeno, A. N., Gleich, G. J., Wells, D., and Naylor, S. (1996) Membrane preconcentration-capillary electrophoresis-mass spectrometry (mPC-CE-MS) analysis of 3-phenylamino-1,2-propanediol (PAP) metabolites. *J. High Resol. Chromatogr.* **19**, 291−294.

(35) Okey, A. B., Riddick, D. S., and Harper, P. A. (1994) Molecular biology of the aromatic hydrocarbon (dioxin) receptor. *Trends Pharmacol. Sci.* **15**, 226−232.

(36) Vasiliou, V., Puga, A., and Nebert, D. W. *(*1993) Mouse class 3 aldehyde dehydrogenases: positive and negative regulation of gene expression. *Adv. Exp. Med. Biol.* **328**, 131−139.

(37) Tukey, R. H., Hannah, R. R., Negishi, M., and Nebert, D. W. (1982) Quantitation of hepatic cytochrome P_1-450 mRNA with the use of cloned DNA probe. Effects of various P-450 inducers in C57BL/6N and DBA/2N mice. *Mol. Pharmcol.* **22**, 779−786.

(38) Landers, J. P., and Bunce, N. J. (1991) The Ah receptor and the mechanism of dioxin toxicity. *Biochem. J.* **276**, 273−287.

(39) Vasiliou, V., Kozak, C. A., Lindahl, R., and Nebert, D. W. (1996) Mouse microsomal class 3 aldehyde dehydrogenase: AHD3 cDNA sequence, inducibility by dioxin and clofibrate, and genetic mapping. *DNA Cell Biol.* **15**, 235−245.

(40) Philen, R., and Posada de la Paz, M. (1993) Toxic Oil Syndrome and Eosinophilia-Mialgia Syndrome: May 8−10, 1991 World Health Organization Meeting Report. *Semin. Arthritis Rheum.* **23** (2), 104−124.

(41) Mayeno, A. N., Belongia, E. A., Lin, F., Lundy, S. K., and Gleich, G. J. (1992) 3-(Phenylamino)alanine, a novel aniline-derived amino acid associated with the eosinophilia-myalgia syndrome: a link to the toxic oil syndrome? *Mayo Clin. Proc.* **67**, 1134−1139.

(42) Mayeno, A. N., Benson, L. M., Naylor, S., Colberg-Beers, M., Puchalski, J. T., and Gleich, G. J. (1995) Biotransformation of 3-(phenylamino)-1,2-propanediol to 3-(phenylamino)alanine: a chemical link between Toxic Oil Syndrome and Eosinophilia-Myalgia Syndrome. *Chem. Res. Toxicol.* **8**, 911−916.

(43) Adachi, J., Gómez, M., Smith, C. C., and Sternberg, E. M. (1995) Accumulation of 3-(phenylamino)alanine, a constituent in L-tryptophan products implicated in eosinophilia-myalgia-syndrome, in blood and organs of the Lewis rats. *Arch. Toxicol.* **69**, 266−270.

(44) Griem, P., Wulferink, M., Sachs, B., Gónzalez, J. B., and Gleichmann, E. (1998) Alergic and autoimmune reactions due to xenobiotics: how do they arise? *Immunol. Today* **19** (3), 133−141.

(45) Hess, D. A., and Rieder, M. J. (1997) The role of reactive drug metabolites in immune-mediated adverse drug reactions. *Ann. Pharmacother.* **31** (11), 1378−1387.

(46) Kubicka-Muranyi, M., Goebels, R., Goebel, C., Uetrecht, J., and Gleichmann, E. (1993) T Lymphocytes ignore procainamide, but respond to its reactive metabolites in peritoneal cells: demonstration by the adoptive transfer popliteal lymph node assay. *Toxicol. Appl. Pharmacol.* **122**, 88−94.

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