Geranyl nitrile (3,7-dimethyl-2,6-octadienenitrile, GN) and citronellyl nitrile (3,7-dimethyl-6-octenenitrile, CN) are monoterpene nitriles used extensively as fragrance ingredients (Fig. 1). Commercial GN consists of an isomeric mixture of geranyl nitrile (E isomer) and neryl nitrile (Z isomer). These compounds are common components in a wide variety of consumer products, including soaps, shampoos, cosmetics, perfumes, detergents, and insect repellents. In 1995 and 1996, 238 metric tons of GN and 45 metric tons of CN were used worldwide (Potter et al., 2001). The primary expected route of human exposure for both compounds is via dermal contact. However, there is also potential for oral exposure from detergent residues on dinnerware and for inhalation exposure via volatilization or aerosol formation. The extent of systemic bioavailability of these compounds by various potential routes of human exposure has not been assessed.

The acute toxicity of both nitriles is low, with oral LD₅₀ values in rats of approximately 3 g/kg for both compounds and dermal LD₅₀ values in rabbits of approximately 4 g/kg (RIFM, 1974a,b). The acute toxicity of geranyl nitrile is also low by the inhalation route, with a 4-h LC₅₀ greater than 5.2 mg/l (BASF (1989) Acute toxicity studies on geranyl nitrile and citronellyl nitrile (unpublished report)). In a 28-day subchronic study, decreased body weight, minor changes in clinical chemistry and urinalysis parameters, and increased serum α-2 μglobulin were observed in rats given either 150 or 450 mg/kg/day for 28 days [BASF (2003) Subacute toxicity study with geranyl nitrile in Wistar rats; administration by gavage for 4 weeks and recovery period of 2 weeks (unpublished report)]. The no-observed-effect level for this study was 50 mg/kg/day. Long-term toxicity data are not currently available for either compound.

The toxicological endpoint of greatest concern for these compounds is genotoxicity. GN and CN were both negative in the Ames test, but GN produced chromosomal aberrations in V79 cells in the presence of metabolic activation in vitro and induced formation of micronuclei in mice in vivo [BASF (2002) In vitro chromosome aberration assay with geranyl nitrile in V79 cells (unpublished report); BASF (2003) Subacute toxicity study with geranyl nitrile in Wistar rats; administration by gavage for 4 weeks and recovery period of 2 weeks (unpublished report)]. The no-observed-effect level for this study was 50 mg/kg/day. Long-term toxicity data are not currently available for either compound.

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cyanoacrylate and other compounds, as indicated by excretion of urinary thiocyanate (Potter et al., 2001). However, whether this was due to low dermal penetration or lack of metabolism of the nitrile moiety to cyanide was not determined. Considering the potential for human exposure to GN and CN in cosmetics and consumer products and the apparent role of metabolic activation in the in vitro genotoxicity of GN, there is a need to understand the metabolism and disposition of these compounds in animals and humans.

As a preliminary step to whole animal studies, this report details our in vitro studies on the metabolism of GN and CN. The primary objectives of this study were to characterize the in vitro metabolic clearance of these compounds and to identify major metabolites of GN and CN in hepatocytes from mice, rats, and humans. Data from this study will be useful for designing definitive in vivo metabolism studies in animals and extrapolation of the results to humans exposed to GN and CN.

Materials and Methods

Chemicals and Biological Materials. GN and CN were provided by BASF AG, Ludwigshafen, Germany. All hepatocyte isolation reagents were obtained from Invitrogen (Carlsbad, CA). Percoll was obtained from Sigma-Aldrich (St. Louis, MO). All other reagents used were of HPLC grade or higher. Human hepatocytes were obtained from Cambrex Bioscience (Walkersville, MD) and from Tissue Transformation Technologies (Edison, NJ). Donor H1 was a 42-year-old Caucasian male with a history of smoking and heavy alcohol use. Donor H2 was a 28-year-old Caucasian female with a history of smoking and moderate alcohol use. Donor H3 was a 46-year-old Caucasian female with a history of smoking and moderate alcohol use. Human hepatocytes were purified by centrifugation through Percoll by the vendors before shipment.

Hepatocyte Isolation. Male Sprague-Dawley rats and CD mice (6–8 weeks old) were obtained from Charles River Laboratories (Raleigh, NC). Rat and mouse hepatocytes were prepared by two-stage collagenase perfusion by a modification of the method of Seglen (1976) using commercially available reagents. Isolated rat and mouse hepatocytes were purified by centrifugation through 40% Percoll at 4°C. Upon receipt, human hepatocytes were washed twice with L-15 medium, diluted to the desired concentration, and maintained on ice until used. Cell viability and yield in all species were determined by trypan blue exclusion. In all cases, hepatocyte viability was >85%.

Clearance Kinetics. Hepatocytes were suspended in L-15 medium at a concentration of 2.5 × 10⁶ cells/ml. Reactions were carried out in 10-ml headspace vials fitted with Teflon septa in a final volume of 1 ml/vial. Kinetic experiments were conducted using an automated assay system composed of a Gerstel MPS-2 autosampler (Gerstel Inc., Baltimore, MD) with a temperature-controlled shaking incubator coupled to a GC/MS system as described below. Hepatocyte suspensions were preincubated at 37°C for 5 min, after which the test compound was introduced into the liquid phase of the reaction mixture through the septum, resulting in an initial concentration of 25 μM. The concentration of acetonitrile in the incubation mixtures was 0.5% (v/v). At selected time points, the headspace above the reaction mixtures was sampled for 10 s by solid-phase microextraction (SPME) using a 1 cm × 100-μm nonbonded polydimethylsiloxane SPME fiber (Sigma-Aldrich, St. Louis, MO). Headspace samples were analyzed by GC/MS using selected ion monitoring as described below. Calibration standards were prepared by incubating varying concentrations of test compounds with heat-inactivated hepatocytes at 37°C in septum-cap vials.

Quantitative analysis of GN and CN was accomplished by GC/MS with electron impact (EI) ionization using selected ion monitoring. For both compounds, m/z 69 was used as the target ion for quantification. Ions m/z 134 and m/z 136 were used as qualifier ions for GN and CN, respectively (Fig. 1). Analyses were conducted using an HP6890 GC coupled to 5970 GC/MSD (Agilent Technologies, Wilmington, DE) equipped with a 30 m × 0.25 mm × 1-μm DB-5MS column (J&W; Agilent Technologies). The following chromatographic parameters were used for quantitative analysis of GN and CN: injection port temperature, 280°C; desorption time, 30 s, split ratio, 10:1; carrier gas, helium; flow rate, 0.4 ml/min; oven temperature, 280°C; MS transfer line temperature, 280°C. Under these conditions, GN (combined isomers) and CN eluted at 1.35 and 1.28 min, respectively.

Biotransformation Experiments. For biotransformation experiments, hepatocytes were suspended at a concentration of 5 × 10⁶ cells/ml in L-15 medium. Incubations were carried out in 24-ml reaction vials fitted with Teflon-lined septa in a final volume of 5 ml. Cell suspensions were preincubated for 5 min at 37°C before the introduction of the test compound to provide an initial concentration of 250 μM. Cell suspensions were incubated with shaking for 60 min, after which reactions were terminated by rapid cooling in an ice bath. Hepatocytes were lysed using an ultrasonic probe, saturated with NaCl (0.5g/ml), and extracted twice with 10 ml of ethyl acetate. The organic fractions were combined and concentrated to a final volume of approximately 0.25 ml under a gentle stream of nitrogen at room temperature. Organic extracts were stored at approximately –20°C for up to 2 days until analyzed by GC/MS. An aliquot of the aqueous phase was removed and stored at approximately 5°C until analyzed by LC/MS. Repeated analysis of stored samples confirmed sufficient stability of major metabolites for the duration of the experiments under these conditions. Although both GN and CN are sufficiently volatile to allow quantitative analysis in the headspace vapor, they are approximately 2000 times less volatile than the extraction solvent ethyl acetate (vapor pressure, 0.04 mg Hg at 25°C for GN versus 76 mg Hg for ethyl acetate). Moreover, all of the synthetic oxidative metabolites of GN and CN were substantially less volatile than the parent compounds (vide infra).

Thus, although recovery of test compounds and metabolites was not determined, it is unlikely that substantial loss of primary metabolites during concentration of extracts occurred.

Metabolite Identification. Phase I metabolites of GN and CN were identified by GC/MS using a scan range of 29 to 300 amu. The GC oven was
programmed as follows: initial oven temperature, 100°C; initial time, 1 min; temperature ramp, 20°C/min; final oven temperature, 280°C; final time, 5 min. In some cases, the molecular weights of metabolites were confirmed by GC analysis with methane chemical ionization time of flight MS detection. Analyses were carried out using a Micromass GC-time of flight (Micromass Ltd., Manchester, UK). The column and GC conditions used were the same as those described above for EI-GC/MS.

Phase II metabolites were identified by time of flight LC/MS using negative electrospray ionization. Analyses were carried out using a Waters Alliance 2790 HPLC system (Waters, Milford, MA) coupled to a Micromass Q-ToF-II hybrid quadrupole time-of-flight mass spectrometer (Micromass Ltd.). The purity of metabolites was accomplished using a Zorbax SB-C18 RP, 2.1 × 150-mm, 5-µm particle size column (Agilent Technologies). The mobile phase was 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B with a flow rate of 0.3 ml/min. The HPLC was programmed with a linear gradient starting at 3% B increasing to 100% B over 30 min, holding 5 min, returning to 3% B at 35.1 min, and holding for 5 min.

NMR Analysis. 1H NMR was performed on a 360-MHz Bruker AMX spectrometer (Bruker BioSpin, Bremen, Germany) at 23°C with a dual 1H/19F probe. Samples were measured in CDC13. One-dimensional 90°-pulse spectra were collected with 5-KHz sweep width, 16 K data points, and four scans. Two-dimensional correlation spectroscopy spectra were recorded with 512 complex t1 increments, 8192 t2 points, and 16 scans for each free induction decay. Chemical shifts were referenced to tetramethylsilane.

Synthesis of Selected Metabolites of GN and CN. Several proposed metabolites of GN and CN were synthesized via ozonolysis of the parent compounds with m-chloroperbenzoic acid (m-CPBA). In brief, 1 mmol of m-CPBA was dissolved in 10 ml of methylene chloride at room temperature. E-GN, Z-GN, or CN (1 mmol in 1 ml of methylene chloride) was added to the m-CPBA solution by drops, and the reaction vessel was sealed. The reaction mixture was stirred for approximately 2 h at room temperature, by which time all of the parent nitrile had been consumed as determined by GC/MS. The mixture was washed three times with 10 ml of iced-cold 0.1 M NaOH. Concentration of the organic phase to a constant weight by vacuum centrifugation yielded approximately 100 mg of clear oil for all three epoxides. The products were analyzed by GC/MS and 1H NMR. Z-5-(3,3-Dimethyl-oxiranyl)-3-methyl-pentanenitrile: 5.197 (s, 1 H, CH), 1.687 (s, 3 H, CH3), 1.077 (m, 3 H, CH3), 1.250 (s, 3 H, CH3), 1.280 (s, 3 H, CH3), 1.320 (s, 3 H, CH3), 1.530 (m, 4 H, 2-CH2), 1.920 (m, 1 H, CH), 2.310 (m, 2 H, CH2), 2.618 (m, 2 H, 2-CH2), 5.202 (s, 1 H, CH), 6.462 (t, J = 7.5 Hz, 1 H, CH), and 9.432 (s, 1 H, CHO).

In some cases, the molecular weights of metabolites were confirmed by GC/MS. For synthesis of 7-cyano-2,6-dimethylhepta-2,6-dienoic acid, 35 mg of 8-oxogonarnitriyl was dissolved in 1 ml of 50% aqueous dimethyl sulfoxide containing 16 mg of K2PO4, NaClO (50 mg/ml, 1.5 ml) was added dropwise, and the mixture was stirred overnight at room temperature (Diliberto et al., 1990). The mixture was made alkaline by the addition of 3 ml of 5% sodium bicarbonate and extracted three times with dichloromethane (10 ml). The aqeous phase was acidified with 10 N HCl, and the product acids were extracted into diethyl ether. The ethereal extract was washed three times with HPLC grade water, dried over sodium sulfate, and concentrated under vacuum to yield approximately 10 mg of white solid. Z-7-Cyano-2,6-dimethylhepta-2,6-dienoic acid: 81.866 (s, 3 H, CH3), 1.954 (d, J = 1.5 Hz, 3 H, CH3), 2.457 (q, J = 7.3 Hz, 2 H, 2-CH2), 2.573 (t, J = 7 Hz, 2 H, CH2), 5.187 (s, 1 H, CH), and 6.873 (t, J = 1.3 Hz, 1 H, CH).

Data Analysis. Noncompartmental kinetic analysis was carried out using WinNonlin version 4.0 (Pharsight, Mountain View, CA). Hepatocyte intrinsic clearance values were calculated by dividing the model-independent clearance (CLint) by the number of cells in the reaction mixture.

\[ CL_{int} = \frac{CL_{L}}{ \text{number of cells} } \]

(1)
Values were then scaled to estimate whole animal intrinsic clearance (CL\text{int}) assuming a liver weight equivalent to 5% of body weight (50 kg) and a hepatocellularity of 1.28 \times 10^6 cells/g liver for rodents (Seglen, 1976). The corresponding values for humans were a liver weight equivalent to 2.5% of body weight (25 kg) and a hepatocellularity of 1.37 \times 10^6 cells/g liver (Arias et al., 1982). Equation 2 illustrates the calculation for rodents.

\[ CL_{int} = \frac{CL_{L}}{ \text{number of cells/g liver} } \]

(2)
All data are presented as means ± S.D. unless otherwise indicated.

Results

Metabolic Clearance of GN and CN. Metabolic clearance rates for GN and CN in hepatocytes were estimated from the rates of disappearance of the parent compounds as described by Houston (1994). The volatility of the parent compounds allowed the use of automated headspace SPME, which provided a simple and efficient method for repeated sampling and analysis of serial headspace samples. Preliminary experiments in rat hepatocytes suggested that the rates of metabolic clearance of E and Z isomers of GN were similar (data not shown). For this reason, the GC oven was programmed such that the two isomers coeluted and clearance of total GN could be estimated.

Kinetic parameters for in vitro clearance of GN and CN are presented in Table 1. Within each species, metabolism of GN and CN occurred at similar rates. However, significant species differences in the clearance kinetics of both terpenes were evident. Clearance of GN in mouse hepatocytes was approximately 5 times more rapid than in rat hepatocytes. A similar species difference was also observed for hepatocyte clearance of CN but was less pronounced than with GN (approximately 2-fold faster in mouse compared with rat hepatocytes). Two of the three donors (HL1 and HL3) metabolized GN and CN much more slowly than rodents, whereas the third donor (HL2) metabolized the compounds at rates comparable with rat. Hepatocyte intrinsic clearance determined in vitro was used to estimate whole body intrinsic clearance by scaling the in vitro values up to the organ.
level using species-specific relative liver weight and hepatocellularity (Houston, 1994). For the species used in these experiments, the predicted in vivo intrinsic clearance values (Table 1) were well in excess of normal hepatic blood flows (approximately 90, 80, and 17 ml/min/kg body weight for mice, rats, and humans, respectively; Brown et al., 1997). For this reason, in vivo metabolic clearance of both compounds is expected to be blood flow-limited in all three species.

**Biotransformation of GN in Rat, Mouse, and Human Hepatocytes.** Phase I metabolites of GN and CN were extracted into ethyl acetate and analyzed by GC/MS, whereas phase II metabolites were identified in the aqueous phase by LC/MS. The EI mass spectrum of GN (Fig. 1) contains a small molecular ion at \( m/z \) 149, and a larger fragment at M-1, a pattern that is typical of monoterpenes (Budzikiewicz et al., 1967). The peak at \( m/z \) 134 (M-15) corresponds to loss of a methyl group. The base peak at \( m/z \) 69 results from cleavage at the doubly allylic C4–C5 bond, and the peak at \( m/z \) 41 represents \( C_6H_{14}^+ \), the formation of which must involve rearrangement. The latter two peaks are characteristic of allylic acyclic terpenes (Budzikiewicz et al., 1967), and their modification is diagnostic for metabolic transformations on the C4–C5 portion of the molecule.

Figure 2A shows typical reconstructed ion chromatograms from GC/MS analysis of rat hepatocytes after incubation with GN. Under the GC conditions used, the Z and E isomers of GN eluted at 7.5 (P1) and 7.8 min (P2), respectively. Consistent with the rapid clearance observed in our kinetic experiments, only small amounts of the parent compound remained after the 1-h incubation. Incubation of GN with intact rat hepatocytes resulted in the detection of at least 11 metabolite peaks (G1–G11) not found in control incubations. Of the 11 new peaks found in rat hepatocyte extracts, four (G1, G2, G6, and G8) could be identified unequivocally by comparison to synthetic standards. An additional four peaks (G3, G4, G5, and G7) were tentatively assigned structures based on mass spectral similarity to synthetic standards or published spectra. The remaining three peaks presented spectral features similar to identified metabolites, but the structure for these peaks could not be assigned with confidence based on the available data.

Two prominent peaks not present in control incubations were detected at retention times of 8.37 min (G1) and 8.71 min (G2). The retention time and mass spectra of these metabolite peaks match those of synthetic Z- and E-6,7-epoxygeranyl nitrile (GNO; Fig. 3). The mass spectra of the Z and E isomers of GNO were quite similar, although some of the higher mass fragments are more prominent in the Z epoxide. Like the parent molecule, the molecular ion (\( m/z \) 165) is minimal or absent, although a small M-1 is sometimes detected. Some of the major ions in the spectra may be rationalized in terms of simple fragmentation, such as cleavage between C5 and C6, with opening of the epoxide ring (\( m/z \) 59) cleavage of the allylic bond at C5 to the epoxide (\( m/z \) 85) and loss of a methyl group (\( m/z \) 150). The fragment at \( m/z \) 94 common to both isomers probably represents fragmentation between C5 and C6.

Another apparent isomeric pair of monooxygenation products of GN was detected with retention times of 8.76 min (G3) and 9.21 min (G4). The EI mass spectra of these metabolites (Fig. 4) were essentially identical and lack the characteristic fragment at \( m/z \) 69, suggesting modification between C5 and C6. The molecular weight of this metabolite was confirmed to be 165 by CI-GC/MS, and the presence of an exchangeable proton was demonstrated by reaction with \( N\)-O-bis(trimethylsilyl)trifluoroacetamide (data not shown), suggesting a
hydroxylated metabolite. The spectra of G3 and G4 are dominated by the base peak at \( m/z \) 85, probably resulting from fragmentation between \( C_4 \) and \( C_5 \). Other significant peaks were observed at \( m/z \) 29 (\( \text{CHO}^- \)), 41 (\( \text{C}_3\text{H}_5^- \)), 55 (fragmentation at \( C_6-C_7 \)), and 67 (fragmentation of \( C_3-C_4 \) with migration of a proton). The spectrum obtained was nearly identical to that of artemisia alcohol (3,3,6-trimethyl-1,5-heptadien-4-ol), a hydrocarbon terpene with an analogous allylic hydroxyl group at \( C_4 \) (Fig. 4B). Based on these data, metabolites G3 and G4 are tentatively proposed to be isomers of 5-hydroxygeranyl nitrile (5-hydroxy-3,7-dimethyl-octa-2,6-dienenitrile).

Metabolites G5 and G6 had identical mass spectra and an apparent molecular weight of 165 (by CI-GC/MS), again suggesting an isomeric pair of monoxygenation products. The retention time and mass spectrum of metabolite G6 (Fig. 5C) matched those of synthetic (Z)-8-hydroxygeranyl nitrile (Fig. 5A). In this spectrum, the molecular ion at \( m/z \) 165 is barely detectable, although the M-1 ion typical of monoterpenes is present. The base peak at \( m/z \) 81 is probably due to cleavage at \( C_4-C_5 \), with retention of a proton by the nitrile-containing fragment. One possible source for the large fragment at \( m/z \) 43 is loss of the \( C_4 \) methyl group, followed by rearrangement and elimination of \( C_3\text{H}_5\text{O}^- \). This would also account for the fragment at \( m/z \) 108. Metabolite G5 was identified as (Z)-9-hydroxygeranyl nitrile (9-hydroxy-3,7-dimethyl-octa-2,6-dienenitrile) based on its relative abundance and its spectral similarity to metabolite G6. Metabolites G7 and G8 also had essentially identical mass spectra, which matched that of synthetic (E)-8-hydroxygeranyl nitrile (Fig. 5B). Furthermore, the retention time of G8 (Fig. 5D) matched that of the synthetic standard. Based on these data, metabolites G7 and G8 were identified as (E)-9-hydroxygeranyl nitrile and (E)-8-hydroxygeranyl nitrile, respectively. The fragmentation pattern of the E- and Z-isomer of 8-hydroxygeranyl nitrile was similar, although like GNO, many of the higher mass fragments were present at much lower abundance in the E-isomer.

Peaks G9, G10, and G11 were not found in control incubations and had essentially identical mass spectra, indicating another isomeric series (data not shown). All three of these metabolites lack fragments at \( m/z \) 69 and \( m/z \) 41 in the spectra suggests modification of the \( C_5-C_9 \) portion of the molecule. The spectra of these metabolites were similar in many respects to the (E)-C8/C9 alcohols, but definitive structures could not be assigned based on the current data.

The metabolite profile of GN in mouse hepatocytes showed some significant differences from the rat metabolite profile (Fig. 2B). Although both isomers of the putative 5-hydroxygeranyl nitrile (G3 and G4) were detected in mouse hepatocyte extracts, only the Z-isomer of
Artemisia alcohol (3,3,6-trimethyl-1,5-heptadien-4-ol) was obtained from the Wiley a 60-min incubation of rat hepatocytes with commercial GN. The spectrum of G1 was the more abundant isomer. Metabolites presence of the epoxide metabolites could not be assigned with confidence. A typical GC chromatogram from a rat hepatocyte incubation defined based on the available data. For this reason, all structure assignments proposed must be considered as tentative. LC/MS analysis of the aqueous phase of hepatocyte extracts after incubation with GN demonstrated the presence of three phase II metabolites common to all three species. The first of these, which eluted at 11.9 min (G17), had an apparent molecular weight of 472, which is consistent with a glutathione conjugate of epoxygeranyl nitrile. Because no epoxide formation was observed at C2–C3, G17 is presumed to result from conjugation of metabolites G1 and or G2. A minor peak having the same apparent molecular weight eluted at 12.3 min was observed only in rat hepatocytes, and may represent a regioisomer of G17. Based on steric considerations, these two metabolites may represent conjugation of GN epoxide at the C7 and C9 positions, respectively. Two additional phase II metabolites (G18 and G19), both with a molecular weight of 341 eluted at 14.2 and 14.6 min, respectively. This molecular weight is consistent with a glucuronide conjugate of hydroxylated GN. Furthermore, the MS/MS spectra of these metabolites contained fragments at m/z 175 and m/z 193, consistent with loss of a glucuronic acid moiety without and with the hydroxyl oxygen. Based on these data, G18 and G19 were tentatively identified as isomeric hydroxy-GN glucuronides. Although the position of glucuronic acid addition could not be definitively assigned based on the MS data, C8 and C9 seem likely based on the presence of the corresponding alcohols in hepatocyte extracts. In the aqueous residue from human hepatocyte extracts, a metabolite with a molecular weight of 355 was detected by LC/MS, eluting at 14.6 min (G20). This molecular weight is consistent with an acylglucuronide of 7-cyano-2,6-dimethylhepta-2,6-dienoic acid. Subsequent MS/MS analysis demonstrated fragments at m/z 134 (M-COO-glucuronic acid), m/z 175 (glucuronic acid), m/z 178 (7-cyano-2,6-dimethylhepta-2,6-dienoic acid aglycone), and m/z 193 (M-O-glucuronic acid). Based on these data, G20 was identified as a 7-cyano-2,6-dimethylhepta-2,6-dienoic acid glucuronide. Although the position of the carboxylate group could not be determined from the available data, it is presumed to be at C9 and/or C10 based on the phase I metabolites previously identified. An additional metabolite (G21) with a molecular weight of 341 was detected in hepatocytes from one hepatocyte donor (HL 3), suggesting another isomer of hydroxygeranyl nitrile glucuronide. The abundance of this metabolite was insufficient for MS/MS analysis, and the hydroxylolation site could not be defined based on the available data.

Biotransformation of CN in Rat, Mouse, and Human Hepatocytes. A typical GC chromatogram from a rat hepatocyte incubation with CN is shown in Fig. 6. Under the conditions used for GC/MS analysis, CN eluted at 7.45 min. The mass spectrum of CN is shown in Fig. 1B. In general, more extensive fragmentation was observed with CN and its metabolites compared with GN, consistent with the lower degree of unsaturation in CN. A significant molecular ion was observed, as well as a slightly more abundant M–H fragment. Similar to GN, the spectrum of CN has a base peak at m/z 69 and a strong fragment at m/z 41, which were diagnostic for transformations on the C7–C8 portion of the molecule. A significant M-CH3 fragment was observed at m/z 136, along with abundant fragments at 122 (M-29) and 108 (M-43) and 94 (M-57).

Only three phase I metabolites of CN could be identified unequivocally by comparison to synthetic standards (C1, C2, and C4). Two additional peaks were tentatively assigned structures based on mass.
spectral similarity to synthetic standards or published spectra (C3 and C7). Other peaks found in extracts of hepatocyte incubations appeared to be metabolites of CN, but no structures could be assigned based on the available spectral data.

Four phase I metabolites of CN were common to all species examined, and were analogous to the major metabolites of GN. (Fig. 7). Similar to the parent molecule, fragmentation was more extensive in CN metabolites compared with their GN analogs. 6,7-Epoxycitronellyl nitriles (C1, C2) were identified by comparison to the synthetic standards. Interestingly, only one stereoisomer of 6,7-epoxycitronellyl nitrile was observed in mouse hepatocytes, whereas both stereoisomers were observed in rat and human hepatocytes in approximately equal amounts (assuming similar ionization efficiencies). Metabolite C3 (Fig. 8) had a mass spectrum similar to metabolites G3 and G4 and apparent molecular weight of 167 by CI-GC/MS (data not shown). Thus, C3 was tentatively assigned the structure of 5-hydroxycitronellyl nitrile (5-hydroxy-3,7-dimethyl-oct-6-enenitrile). Unlike the corresponding GN metabolites (G3 and G4), C3 was observed in human hepatocytes. The other CN metabolite common to all three species was 8-hydroxy-CN (C4), which was identified by comparison to our synthetic standard (Fig. 7, B and D).

A CN metabolite unique to rat hepatocyte extracts was observed with a retention time of 10.24 min (C5). This metabolite seems to be an analog of GN metabolites G9–G11. As with its GN analogs, metabolite C5 could not be assigned a definitive structure based on its mass spectrum. In mouse, a novel metabolite (C6) eluted just after the CN epoxides (RT, 8.37 min). The EI mass spectrum of this metabolite lacked of fragments at m/z 41 and 69 characteristic of the terminal isobutylene moiety, suggesting oxidation on this portion of the molecule. The mass spectrum of this metabolite was significantly different from any of the synthetic CN metabolites and may be an analytical artifact. In human hepatocytes, a metabolite with a mass spectrum identical to synthetic 8-hydroxycitronellyl nitrile was detected at a slightly earlier retention time. This metabolite (C7) was tentatively

![Fig. 5. Electron impact mass spectra of synthetic 8-hydroxygeranyl nitrile isomers and corresponding rat metabolites. Authentic 8-hydroxygeranyl nitriles (A and B) were synthesized as described under Materials and Methods. Proposed fragmentation schemes are shown as insets. Rat metabolites G6 and G8 (C and D) were identified after a 60-min incubation of rat hepatocytes with commercial GN. The lower mass limit for analysis of the synthetic Z isomer was set at 35 amu, so the ions at m/z 29 and 31 are not seen in this spectrum.]
identified as 9-hydroxycitronellyl nitrile based on spectral similarity to the synthetic standard, elution order, and relative abundance.

Two phase II metabolites were tentatively identified in the aqueous residue of rat hepatocyte extracts after incubation with CN. Metabolite C8 eluted at a retention time of 12 min and had a molecular weight of 474, consistent with a glutathione conjugate of 6,7-epoxycitronellyl nitrile. This metabolite was also detected in human hepatocytes. A second phase II metabolite of CN was detected at 14.7 min (C9) with an apparent molecular weight of 343, consistent with an ether glucuronide of CN. MS/MS analysis of this metabolite demonstrated significant fragments at m/z 166 and m/z 175, consistent with fragmentation of the glucuronide linkage. Although the MS/MS data did not allow determination of the site of hydroxylation, the presence of 8-hydroxy-citronellyl nitrile (C4) in the organic extract suggests that this metabolite was a likely target for glucuronidation. Metabolite C9 was also detected in mouse hepatocyte incubations but not in human extracts. In human hepatocytes, a metabolite with an apparent molecular weight of 476 amu was detected by LC/MS (C10). This metabolite was tentatively identified as an acylglucuronide of CN. MS/MS analysis demonstrated significant fragments at m/z 175 and m/z 180, corresponding to the glucuronic acid and aglycone, respectively. Although the data did not allow unambiguous determination of the position of the acid moiety, the C9 position seems most likely based on steric considerations and on the presence of 8-hydroxycitronellyl nitrile in organic extracts.

Discussion

Although the general toxicity of acyclic monoterpene nitriles seems to be quite low, genotoxicity of GN constitutes a significant human health concern. Because metabolism seems to contribute to the genotoxicity of GN, the metabolic clearance and biotransformation of this compound were compared with those of the nongenotoxic analog CN in vitro. The facile metabolism of terpene nitriles observed in our clearance experiments is consistent with results obtained by Diliberto et al. (1988) with citral (3,7-dimethyl-2,6-octadienal), which was completely cleared from the plasma within 5 min after intravenous administration in rats. Likewise, metabolic clearance of limonene in rats is rapid, with a plasma half-life of approximately 12 min (Chen et al., 1998). Hepatocyte clearance of GN and CN was 2 to 5 times greater in mouse compared with rat. Similar species differences in kinetics have been observed for other nonpolar alkenes such as butadiene (Himmelstein et al., 1994) and isoprene (Peter et al., 1990). Not surprisingly, there was significant variability in hepatocyte clearance of GN and CN between the three human donors. Although this may merely represent the high variability in human drug-metabolizing enzyme expression, other factors such as gender differences or life style issues may also play a role. Given the similar in vitro kinetic parameters for GN and CN, it seems unlikely that differences in metabolic clearance of the parent compounds can account for their differential genotoxicity in mice.

As expected, GN and CN share many metabolic pathways. Proposed common pathways for biotransformation of monoterpene nitriles are shown in Fig. 9. As with other acyclic terpenes, oxidation of the terminal methyl group seems to be a major pathway for metabolism of terpene nitriles. Terminal hydroxylation products were not observed directly in mouse hepatocyte extracts by GC/MS in these experiments. However, isomeric glucuronides were tentatively identified in the aqueous residue by LC/MS, and subsequent experiments using mouse liver microsomes demonstrated formation of both 8- and 9-hydroxygeranyl nitrile (data not shown). The regionselectivity for oxidation at C8 has been observed with other monoterpene, such as citral (Diliberto et al., 1990), geraniol, and linalool (Chadha and Madyastha, 1984), and is probably driven by steric factors. However, although specificity for oxidation at C8 is reported to be absolute for citral and geraniol, evidence for oxidation of terpene nitriles at C9 was also observed in our experiments. A second significant pathway for biotransformation of GN and CN was epoxidation of the C6-C7 double bond. Epoxide formation has been reported with a variety of other terpenes, including pugelone (Madyastha and Raj, 1993), limonene (Poon et al., 1996), and cannabigerol (Harvey and Brown, 1990). However, for several close structural analogs of GN and CN, such as citral, geraniol, and linalool, no epoxide formation was observed (Chadha and Madyastha, 1984; Diliberto et al., 1990), possibly due to rapid competing reactions such as oxidation of the hydroxyl or aldehyde groups in these compounds. Interestingly, whereas rat and human hepatocytes converted both isomers of GN to the 6,7-epoxide, only the Z isomer was observed in mouse hepatocytes. In addition, in human hepatocytes, the Z epoxide was the predominant stereoisomer (data not shown). It is unclear from the current data whether this is the result of stereoselective epoxidation of Z-GN, or stereoselective removal of E-GNO by subsequent phase II metabolism.

Oxidation at the allylic C5 position was a minor pathway compared with epoxidation or hydroxylation of the terminal methyl groups. Allylic hydroxylation is a common pathway for alkenes, and has been observed at secondary allylic carbons in other terpenes such as trans-sobrorerol (Ventura et al., 1985) and pulegone (Thulasiram et al., 2001). Unlike oxidation products of the terminal isobutylene moiety, the E and Z isomers of the proposed 5-hydroxygeranyl nitrile had identical mass spectra (data not shown).

Some interesting mass spectral differences were noted for stereoisomeric metabolites of GN in which the transformation occurs on the terminal portion of the molecule (C7-C9). For 8 and 9-hydroxygeranyl nitriles and for 6,7-epoxygeranyl nitriles, the abundance of higher mass fragments is much greater in the Z isomer compared with the E isomer. This is particularly evident for the M-C9H14O1 ion series. Thus, the M-29, M-43, and M-57 ions are easily discernable in Z-8-hydroxygeranyl nitrile but absent in the E-isomer. Similar trends occurred for the epoxide metabolites. This phenomenon was not observed for the parent molecules or for G3 and G4, in which hydroxylation seems to occur at C5. The reason for these differences
is unknown. However, the Z isomers of the terminal oxidation products of GN can more easily assume a conformation in which the terminal oxygen can interact with the nitrile moiety, and one may speculate that this may somehow stabilize the higher mass fragments.

Diol formation was not observed in our experiments, suggesting that the epoxide metabolites of GN and CN may be relatively stable to enzymatic hydrolysis. This was rather unexpected, because diol metabolites have been observed for a number of other terpenes, such as limonene (Poon et al., 1996) and cannabigerol (Harvey and Brown, 1990). Attempts to identify diol metabolites after direct incubation of terpene nitrile epoxides with rat, mouse, or human liver microsomes were unsuccessful, and clearance of the epoxides in microsomal reaction mixtures without NADPH was negligible (data not shown). Highly hindered epoxides such as 1,1,2-trimethylstyrene oxide have been shown to be poor substrates for microsomal epoxide hydrolase (Oesch et al., 1971). On the other hand, it has been demonstrated that cytosolic epoxide hydrolase was active on hindered epoxides including a series of terpenoid epoxides, although the activity was quite low for 1,1-dimethyl-2-alkyl compounds (Mumby and Hammock, 1979). Thus, one may speculate that diol metabolites of GN and CN may have been formed by cytosolic epoxide hydrolase in our hepatocyte...
reactions but were not detected because of low abundance, decomposition in the GC/MS system, or poor chromatography.

The phase II metabolites detected in our studies were consistent with the phase I metabolites identified by GC/MS. The predominant phase II pathway for both GN and CN was glucuronidation of the monohydroxylated parent molecules, although conjugation of the 6,7-epoxides with glutathione was also observed in all species. Human hepatocytes catalyzed formation of an apparent acyl glucuronide that was not observed in rodent hepatocytes. Consistent with this is the observation that human and primate microsomes were significantly more efficient at formation of acylglucuronides from a series of nonsteroidal anti-inflammatory drugs compared with rodent microsomes (Magdalou et al., 1990).

The close structural similarity between GN and CN and the dependence of the in vitro clastogenicity of GN led us to hypothesize that differences in biotransformation of these two compounds may be responsible for their differential genotoxicity. Furthermore, it was hypothesized that biotransformation of the conjugated nitrile moiety of GN was likely to be responsible for the genotoxicity of this compound. However, we were unable to identify any metabolites associated with transformation of the conjugated nitrile functional group in the studies reported here. Furthermore, although definite structures could not be associated with several of the metabolites detected (G12-G16, C5-C6), all of these metabolites lacked the characteristic fragment at m/z 69, suggesting that metabolism had occurred at the terminal isobutylene rather than at the C2 double bond.

These data suggest that the α,β-unsaturated nitrile moiety in GN is resistant to oxidation. Epoxidation of simple α,β-unsaturated nitriles such as acrylonitrile is well documented (Guengerich et al., 1991; Ghanayem and Burka, 1996). Van Bladeren et al. (1981) demonstrated that substituted α,β-unsaturated nitriles, such as crotononitrile and cinnamionitrile, formed much less epoxide than the unsubstituted compound acrylonitrile (9 and 2%, respectively) when administered to rats, probably due to steric hindrance. These authors also found that 25 to 50% of the administered dose was excreted as mercapturates resulting from direct conjugation of the unsaturated nitrile with glutathione. Our in vitro biotransformation experiments did not provide any evidence for direct reaction of the conjugated nitrile moiety of GN with glutathione. Furthermore, little or no depletion of glutathione was observed when hepatocytes were incubated with GN at concentrations up to 1 mM, suggesting that neither GN nor its metabolites were strongly reactive with glutathione (data not shown). A similar lack of reactivity was observed for the terpene aldehyde citral (Diliberto et al., 1990). One possible explanation for the lack of metabolism of GN and similar compounds at the 2,3-double bond is steric hindrance by the bulky alkenyl and methyl substituents (Delbressine et al., 1981). Another factor that may contribute to the lack of reactivity of the 2,3-double bond may be rapid competing reactions, such as hydroxylation at C₈ and C₅, and epoxidation at C₆.

In summary, differences in the genotoxicity of GN and CN do not seem to be due to simple differences in hepatic clearance. Although we were not able to demonstrate metabolic differences between these
compounds that could be related to known genotoxic mechanisms, several metabolites remain to be identified. Furthermore, relevant metabolites may have escaped detection in due to low concentration, low volatility, poor ionization, or further metabolism. Thus, an explanation for differential genotoxicity of these two compounds may yet be found in differential biotransformation.

Acknowledgments. We would like to thank Dr. Xing Han for conducting NMR analysis of synthetic standards. We would also like to thank Brian Shertz and Robert Mingoia for invaluable technical assistance in the conduct of these studies.

References


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