DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
DMD Fast Forward. Published on March 15, 2006 as doi:10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.

DMD #5496

**Comparative Metabolism of Geranyl Nitrile and Citronellyl Nitrile in Mouse, Rat and Human Hepatocytes**

Raymond A. Kemper, Diane L. Nabb, Shawn A. Gannon, Timothy A. Snow and Anne Marie Api

DuPont Haskell Laboratory for Health and Environmental Sciences, Newark DE (RAK, DLN, SAG, TAS) and Research Institute for Fragrance Materials, Woodcliff NJ (AMA).

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024



Copyright 2006 by the American Society for Pharmacology and Experimental Therapeutics.

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



Running Title: Metabolism of Terpene Nitriles

Correspondence: Raymond A, Kemper, Ph.D.

Tocixology and Safety Assessment, Boehringer-Ingelheim Pharmaceuticals Inc.

175 Briar Ridge Rd, Fairfield CT 06877

Email: rkemper@rdg.boehringer-ingelheim.com

Phone: (203) 798-5515
Fax: (203) 798-5797
Text = 37 pages
Tables = 1
Figures = 9
References = 28
Abstract = 230 words
Introduction = 541 words
Discussion = 1494 words

Nonstandard Abbreviations:

Geranyl Nitrile – GN, Citronellyl Nitrile – CN, 6,7-Epoxygeranyl Nitrile – GNO, 6,7-

Epoxycitronellyl Nitrile – CNO

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



**ABSTRACT**

Geranyl nitrile (GN) and citronellyl nitrile (CN) are fragrance components used in consumer and personal care products. Differences in the clastogenicity of these two terpenes are postulated to result from differential biotransformation, presumably involving the conjugated nitrile moiety. The metabolic clearance and biotransformation of GN and CN were compared in primary hepatocytes from mice, rats and humans. For determination of intrinsic clearance, GN and CN were incubated with hepatocytes in sealed vials and the headspace was sampled periodically by solid phase microextraction and analyzed by GC/MS. For metabolite identification, GN and CN were incubated with hepatocytes from each species for 60 min and reaction mixtures were extracted and analyzed by mass spectroscopy. Both GN and CN were rapidly metabolized in hepatocytes from all species ($T_{1/2}$ 0.7-11.6 min). Within a species, intrinsic clearance was similar for both compounds, and increased in the order human < rat << mouse. Major common pathways for biotransformation of GN and CN involved 1) epoxidation of the 6-alkenyl moiety followed by conjugation with GSH, 2) hydroxylation of the terminal methyl group(s) followed by direct conjugation with glucuronic acid in rodents or further oxidation to the corresponding acid in human cells and 3) hydroxylation of the allylic $C_5$ position. No evidence for either phase I or phase II metabolism of the conjugated nitrile moiety was obtained. Thus the presumed metabolic basis for differences in genotoxicity remains elusive.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



## INTRODUCTION

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 (Figure 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 1995/1996, 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 the various potential routes of human exposure has not been assessed.

The acute toxicity of both nitriles is low, with oral $LD_{50}$ values in rats of approximately 3 g/kg for both compounds and dermal $LD_{50}$ values in rabbits of approximately 4 g/kg (RIFM 1974a, 1974b). The acute toxicity of geranyl nitrile is also low by the inhalation route, with a 4-hour $LC_{50}$ greater than 5.2 mg/L (BASF 1989). In a 28-day sub-chronic study, decreased body weight, minor changes in clinical chemistry and urinalysis parameters and increased serum alpha-2μ globulin were observed in rats given either 150 or 450 mg/kg/d, for 28 days (BASF, 2003). The NOEL for this study was 50 mg/kg/d. Long-term toxicity data are not currently available for either compound.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



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; BASF 2003a). These effects were not observed with CN, suggesting a role for the conjugated nitrile moiety in the mechanism of genotoxicity. Furthermore, the dependence of the *in vitro* clastogenicity of GN on the presence of an NADPH-fortified S-9 fraction suggests that metabolic activation of the parent compound may be a prerequisite for genotoxicity.

Almost no information exists on the metabolism of terpene nitriles. A biomarker study in rats indicated that only minute amounts of cyanide were released from either CN or GN following dermal application, 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.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



## 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 consumption. 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 prior to 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%.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



*Clearance Kinetics*

Hepatocytes were suspended in L-15 medium at a concentration of 2.5 x $10^6$ 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 10s by solid phase microextraction (SPME) using a 1 cm x 100 µm nonbonded polydimethylsiloxane SPME fiber (Sigma-Aldrich, St. Louis, MO). Headspace samples were analyzed by GC/MS using selected ion monitoring (SIM) 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 (SIM). 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 (Figure 1). Analyses were conducted using an HP6890 GC coupled to 5970 GC/MSD (Agilent Technologies, Wilmington, DE) equipped with a 30 m x 0.25mm x 1 µm DB-5MS column (J&W, Agilent Technologies, Wilmington DE). The following chromatographic parameters were used for quantitative

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



analysis of GN and CN: Injection port temperature, 280 °C; Desorption time, 30s, 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 x $10^6$ 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 prior to 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 mmHg at 25 °C for GN vs. 76 mmHg for ethyl acetate). Moreover, all of the synthetic oxidative



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



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 29-300 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 (GC-CI-ToF). Analyses were carried out using a Micromass GC-ToF (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., Manchester, UK). Separation of metabolites was accomplished using a Zorbax SB-$C_{18}$ RP, 2.1 x 150 mm, 5µm particle size column (Agilent Technologies, Wilmington DE). 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 0.3mL/min. The HPLC was programmed with a linear gradient starting at 3% B increasing to 100% B over 30 minutes, holding 5 minutes returning to 3% B at 35.1 minutes and holding for 5 minutes.



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



*NMR Analysis*

$^{1}$H-NMR was performed on a 360-MHz Bruker AMX spectrometer (Bruker BioSpin, Bremmen, Germany) at 23 °C with a dual $^{1}$H/$^{19}$F probe. Samples were measured in $CDCl_3$. 1D 90°-pulse spectra were collected with 5-kHz sweep width, 10 pulse delay, 16 K data points, and 4 scans. 2D COSY spectra were recorded with 512 complex $t_1$ increments, 8192 $t_2$ points, and 16 scans for each free induction decay (FID). Chemical shifts were referenced to TMS .

*Synthesis of Selected Metabolites of GN and CN*

Several proposed metabolites of GN and CN were synthesized retrospectively to assist in identification of unknowns in hepatocyte biotransformation experiments. Epoxide metabolites of CN and GN were synthesized by reaction of the parent compounds with *m*-chloroperoxybenzoic acid (m-CPBA). Briefly, 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 hrs 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 cold 0.1N 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 resulting products were analyzed by GC/MS and $^{1}$H-NMR. **Z- 5-(3,3-Dimethyl-oxiranyl)-3-methyl-pent-2-enenitrile (Z-GNO)**: δ1.294 (s, 3 H, $CH_3$), 1.335 (s, 3 H, $CH_3$), 1.75 (m, 2 H, $CH_2$), 1.954 (s, 3H, $CH_3$), 2.567 (t, J = 7.5 Hz, 2 H, $CH_2$), 2.760 (t, J = 7.5 Hz, 1 H, CH), and 5.170 (s, 1 H, CH). **E- 5-(3,3-Dimethyl-oxiranyl)-3-methyl-**



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



**pent-2-enenitrile (E-GNO)**: δ1.277 (s, 3 H, $CH_3$), 1.320 (s, 3 H, $CH_3$), 1.70 (m, 2 H, $CH_2$), 2.087 (s, 3H, $CH_3$), 2.350 (m, 2 H, $CH_2$), 2.695 (t, J = 7.5 Hz, 1 H, CH), and 5.177 (s, 1 H, CH). **5-(3,3-Dimethyl-oxiranyl)-3-methyl-pentanenitrile (CNO)**: δ1.077 (m, 3 H, $CH_3$), 1.250 (s, 3 H, $CH_3$), 1.280 (s, 3 H, $CH_3$), 1.530 (m, 4 H, 2-$CH_2$), 1.920 (m, 1 H, CH), 2.310 (m, 2 H, $CH_2$), and 2.676 (t, J = 4.8 Hz, 1 H, CH).

Metabolites resulting from oxidation of the $C_8$ methyl group were synthesized as described by Diliberto et al., (1990) for preparation of citral metabolites. Briefly, GN or CN (24 mmol) was added to a mixture of 90% t-butylperoxide (12 mL) in dichloromethane, containing selenium dioxide (60 mg) and salicylic acid (420 mg). The mixture was stirred at room temperature for 24 hrs. The crude reaction mixture was diluted with diethyl ether (50 mL) and washed 4 times with 10% sodium hydroxide (25 mL). The ether layer was then washed 4 times with HPLC grade water, followed by saturated sodium chloride solution, dried over sodium sulfate and concentrated under vacuum. The crude product was dissolved in hexane and subjected to flash chromatography on silica gel, using hexane: ethyl acetate (7:3) as the elution solvent. The final products, 8-hydroxy-3,7-demethylocta-2,6-diene nitrile (8-hydroxyGN)and 8-hydroxy-3,7-dimethyloct-6-ene nitrile (8-hydroxyCN) were concentrated to a constant weight under vacuum and stored at –20 C. Approximately 200 mg of each alcohol was recovered from this procedure. In all cases, the product was a clear to pale yellow oil. The structure of 8-hydroxy-GN was confirmed by NMR and GC/MS. 8-Hydroxy-CN was analyzed by GC/MS. **(Z)- 8-Hydroxy-3,7-dimethyl-octa-2,6-dienenitrile (8-HO-GN)**: δ1.687 (s, 3 H, $CH_3$), 1.932 (d, J = 1.5 Hz, 3 H, $CH_3$), 2.285 (q, J = 7.5 Hz, 2 H,

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



$CH_2$), 2.471 (t, J = 7.5 Hz, 2 H, $CH_2$), 4.016 (s, 2 H, $CH_2OH$), 5.128 (s, 1 H, CH), and 5.1430 (d, J = 1.3 Hz, 1 H, CH).

The $C_8$ aldehyde, 8-oxo-3,7-dimethylocta-2,6-diene nitrile (8-oxoGN) , was synthesized by treatment of 8-hydroxyGN (100 mg) with 1 g of activated $MnO_2$ in 5 mL of diethyl ether. The mixture was stirred for 18 hrs at room temperature. At the conclusion of the reaction, the mixture was centrifuged to remove $MnO_2$ and the supernatant was concentrated under vacuum to yield approximately 50 mg of the aldehyde with a purity of approximately 95%. (**Z)-3,7-Dimethyl-8-oxo-octa-2,6-dienenitrile (8-oxo-GN)**: δ1.778 (s, 3 H, $CH_3$), 1.963 (s, 3 H, $CH_3$), 2.618 (m, 2 H, 2-$CH_2$), 5.202 (s, 1 H, CH), 6.462 (t, J = 1.3 Hz, 1 H, CH), and 9.432 (s, 1 H, CHO).

For synthesis of 7-cyano-2,6-dimethylhepta-2,6-dienoic acid , 35 mg of 8-oxogeranylnitrile was dissolved in 1 mL of 50% aqueous dimethylsulfoxide containing 16 mg of $KH_2PO_4$. $NaClO_2$ (50 mg/mL, 1.5 mL) was added drop wise, and the mixture was stirred overnight at room temperature (Diliberto et al., 1990). The mixture was made alkaline by addition of 3 mL of 5% sodium bicarbonate and extracted 3 times with dichloromethane (10 mL). The aqueous phase was acidified with 10N HCl and the product acids were extracted into diethyl ether. The ethereal extract was washed 3 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**: δ1.886 (s, 3 H, $CH_3$), 1.954 (d, J = 1.5 Hz, 3 H, $CH_3$), 2.457 (q, J = 7.3 Hz, 2 H, 2-$CH_2$), 2.573 (t, J = 7 Hz, 2 H, $CH_2$), 5.187 (s, 1 H, CH), and 6.873 (t, J = 1.3 Hz, 1 H, CH).

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



*Data Analysis*

Non-compartmental 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 ($CL_z$) by the number of cells in the reaction mixture.

(1) $CL_H$ (mL/min/$10^6$ cells) = $CL_z$/2.5 (x $10^6$)

Values were then scaled to estimate whole animal intrinsic clearance ($CL_I$) assuming a liver weight equivalent to 5% of body weight (50g/kg) and a hepatocellularity of 1.28 x $10^8$ cells/g liver for rodents (Seglen, 1976). The corresponding values for humans were a liver weight equivalent to 2.5% of body weight (25g/kg) and a hepatocellularity of 1.37 x $10^8$ cells/g liver (Arias et al., 1982). Equation 2 illustrates the calculation for rodents

(2) $CL_I$ (mL/min/kg) = $CL_H$ • (128 [x $10^6$] cells/g liver) • (50 g liver/kg body weight)

All data are presented as means ± SD 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



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



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 co-eluted 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 to rat hepatocytes). Two of the three donors (HL1 and HL3) metabolized GN and CN much more slowly than rodents, while the third donor (HL2) metabolized the compounds at rates comparable to 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, while phase II metabolites were identified in the aqueous phase by LC/MS.



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



The electron impact (EI) mass spectrum of GN (Figure 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 $C_4$-$C_5$ bond, and the peak at m/z 41 represents $C_3H_5^+$, 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 $C_5$-$C_9$ portion of the molecule.

Figure 2A shows typical reconstructed ion chromatograms from GC/MS analysis of rat hepatocytes following 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 one-hour 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 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



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



metabolite peaks match those of synthetic *Z*- and *E*-6,7-epoxygeranyl nitrile (GNO, Figure 3). The mass spectra of the Z and E isomers of GNO were quite similar; though 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, though 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 $C_6$ and $C_7$ with opening of the epoxide ring (m/z 59) cleavage of the allylic bond at $C_4$ 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 likely represents fragmentation between $C_5$ and $C_6$.

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 (Figure 4) were essentially identical and lack the characteristic fragment at m/z 69, suggesting modification between $C_5$ and $C_9$. 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 BSTFA (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 ($CHO^+$), 41 ($C_3H_5^+$), 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 $C_4$ (Figure 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).

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



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 monooxygenation products. The retention time and mass spectrum of metabolite **G6** (Figure 5C) matched those of synthetic (*Z*)-8-hydroxygeranyl nitrile (Figure 5A). In this spectrum, the molecular ion at m/z 165 is barely detectable, though the M-1 ion typical of monoerpenes 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 $C_9$ methyl group, followed by rearrangement and elimination of $C_2H_3O^{+\cdot}$. 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 (Figure 5B). Furthermore, the retention time of **G8** (Figure 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, though like GNO, many of the higher mass fragments were present at much lower abundances 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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



similar in many respects to the (*E*)-$C_8$/$C_9$ 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 (Figure 2B). Although both isomers of the putative 5-hydroxygeranyl nitrile (**G3** and **G4**) were detected in mouse hepatocyte extracts, only the *Z*-isomer of 6,7-epoxygeranyl nitrile (**G2**) was detected, and neither 8- nor 9-hydroxygeranyl nitriles (**G5**-**G8**) were detected. Furthermore, mouse hepatocyte extracts contained several phase I metabolites not found in rat hepatocytes (**G12**-**G16**). All of these metabolites lacked the characteristic ion pair at m/z 41 and m/z 69, indicating modification between $C_5$ and $C_9$. In general, the spectra of **G12** and **G13** were similar to that of synthetic 8-oxogeranyl nitrile, while those of **G14**-**G16** contain fragments consistent with carboxylic acids. Synthetic 7-cyano-2,6-dimethylhepta-2,6-dienoic acid did not chromatograph well by GC, so a direct comparison with unknown **G14**-**G16** was not possible. Thus, although some limited structural inferences could be made concerning these metabolites, structures for **G12**-**G16** could not be assigned with confidence.

GC/MS analysis of human hepatocyte extracts demonstrated the presence of the epoxide metabolites **G1** and **G2**. As with the mouse, **G1** was the more abundant isomer. Metabolites **G8** (8-hydroxygeranyl nitrile) and **G11** (unknown) were also detected in human hepatocyte extracts by GC/MS. Unlike both mouse and rat, neither **G3** nor **G4** were found in human hepatocyte extracts. No unique phase I metabolites were detected in human hepatocytes.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



Since authentic standards were not available for phase II metabolites of GN, identification is based solely on mass spectral data. For this reason, all structure assignments proposed must be considered as tentative. LC/MS analysis of the aqueous phase of hepatocyte extracts following incubation with GN demonstrated the presence of 3 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. Since no epoxide formation was observed at $C_2$-$C_3$, **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 $C_7$ and $C_6$ 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. Further, 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, $C_8$ and $C_9$ appear 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 minutes (**G20**). This molecular weight is consistent with an acylglucuronide of 7-cyano-2,6-dimethylhepta-2,6-dienoic acid . Subsequent MS/MS

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



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 $C_8$ and/or $C_9$ 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 hydroxylation 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 Figure 6. Under the conditions used for GC/MS analysis, CN eluted at 7.45 minutes. The mass spectrum of CN is shown in Figure 1B. In general, more extensive fragmentation was observed with CN and its metabolites compared to 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 $C_5$-$C_9$ portion of the molecule. A significant M-$CH_3$ fragment was observed at m/z 136, along with abundant fragments at 122 (M-29) and 108 (M-43) and 94 (M-57).

Only 3 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



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



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. (Figure 7). Similar to the parent molecule, fragmentation was more extensive in CN metabolites compared to their GN analogues. 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, while both stereoisomers were observed in rat and human hepatocytes in approximately equal amounts (assuming similar ionization efficiencies). Metabolite **C3** (Figure 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 (Figure 7 panels B and D).

A CN metabolite unique to rat hepatocyte extracts was observed with a retention time of 10.24 min (**C5**). This metabolite appears to be an analogue of GN metabolites **G9**-**G11**. As with its GN analogues, 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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



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 identified as 9-hydroxycitronellyl nitrile based on spectral similarity to the synthetic standard, elution order and relative abundance.

Two phase II metabolites weretentatively identified in the aqueous residue of rat hepatocyte extracts following 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. While 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 fragements 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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



of the acid moiety, the $C_8$ 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 appears to be quite low, genotoxicity of GN constitutes a significant human health concern. Since metabolism appears to contribute to the genotoxicity of GN, the metabolic clearance and biotransformation of this compound were compared to those of the non-genotoxic analogue 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 minutes following intravenous administration in rats. Similarly, 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 2-5 times greater in mouse compared to rat. Similar species differences in kinetics have been observed for other non-polar 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. While 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 appears unlikely that differences in metabolic clearance of the parent compounds can account for their differential genotoxicity in mice.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



As expected, GN and CN share many metabolic pathways. Proposed common pathways for biotransformation of monoterpene nitriles are shown in Figure 9. As with other acyclic terpenes, oxidation of the terminal methyl group appears 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 regioselectivity for oxidation at $C_8$ has been observed with other monoterpenes, such as citral (Diliberto et al., 1990), geraniol and linalool (Chadha and Madyastha, 1984), and is probably driven by steric factors. However, while specificity for oxidation at $C_8$ is reported to be absolute for citral and geraniol, evidence for oxidation of terpene nitriles at $C_9$ was also observed in our experiments. A second significant pathway for biotransformation of GN and CN was epoxidation of the $C_6$-$C_7$ 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 analogues 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, while rat and human hepatocytes converted both isomers of GN to the 6,7-epoxide, only the *Z* isomer was observed in mouse hepatocytes. Additionally, in human hepatocytes, the Z-epoxide was the predominant stereoisomer (data not shown). It is unclear from the current data whether this is the

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



result of stereoselective epoxidation of *Z*-GN, or stereoselective removal of *E*-GNO by subsequent phase II metabolism.

Oxidation at the allylic $C_5$ position was a minor pathway compared to 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-sobrerol (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 the mass spectral differences were noted for stereoisomeric metabolites of GN in which the transformation occurs on the terminal portion of the molecule ($C_7$-$C_9$). 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 to the E isomer. This is particularly evident for the M-$C_nH_{2n-1}$ 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 occur for the epoxide metabolites. This phenomenon was not observed for the parent molecules or for **G3** and **G4**, in which hydroxylation appears to occur at $C_5$. 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 he epoxide metabolites of GN and CN may be relatively stable to enzymatic hydrolysis. This was rather unexpected, since diol metabolites have been observed for a number of other

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



terpenes, such as limonene (Poon et al., 1996) and cannabigerol (Harvey and Brown, 1990). Attempts to identify diol metabolites following 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, though 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 due to 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 molecues, though 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 non-steroidal anti-inflammatory drugs compared to 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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



these two compounds may be responsible for their differential genotoxicity. Further, 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 $C_2$ 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 crotonitrile and cinnamonitrile 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 25-50% 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 GSH 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 GSH (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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



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 $C_8$ and $C_5$ and epoxidation at $C_6$.

In summary, differences in the genetoxicity of GN and CN do not appear 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.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



**Acknowledgements**

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

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



**References**

Arias IM, Popper H, Schachter D and Shafritz DA (1982) The *Liver: Biology and Pathobiology*. Raven Press, New York.

BASF (1989) Acute toxicity studies on geranyl nitrile and citronellyl nitrile (unpublished report.

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.

BASF (2003a) Cytogenetic study *in vivo* with geranyl nitrile in the mouse micronucleus test, single oral administration. Unpublished report.

Brown R P, Delp M D, Lindstedt S L, Rhomber, L R, and Beliles R P (1997) Physiological parameter values for physiologically based pharmacokinetic models. Toxicol. Ind. Health **13:** 407–484.

Budzikiewicz H, Djerassi C and Williams DH (1967) Lower Terpenes, in: *Mass Spectrometry of Organic Compounds*, Holden-Day Inc, San Fransisco, pp 141-164.

Chadha A and Madyastha M (1984) Metabolism of geraniol and linalool in the rat and effects on liver and lung microsomal enzymes. *Xenobiotica*, **14(5):** 365-374.

Chen H, Chan KK and, Budd T (1998) Pharmacokinetics of d-limonene in the rat by GC-MS assay. *J Pharm Biomed Analysis,* **17(4-5):** 631-640.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



Delbrissine, LPC, Seutter-Berlage F, and Seutter E (1981) Identification of urinary mercapturic acids formed from acrylate, methacrylate and crotonate in the rat. Xenobiotica, 11(4): 241-247.

Diliberto JJ, Srinivas P, Overstreet D, Usha G, Burka LT and Birnbaum LS (1990) Metabolism of citral, an α,β-unsaturated aldehyde, in male F344 rats. *Drug Metab Disp*, **18(6):** 866-875.

Diliberto JJ, Usha G and Birnbaum LS (1988) Disposition of citral in male Fisher rats. Drug Metab Disp, 16(5): 721-727.

Ghanayem BI and Burka LT (1996) Excretion and identification of methacrylonitrile metabolites in the bile of male Fisher F344 rats. *Drug Metab. Disp.* **24:** 390-394.

Guengerich FP, Kim DH and Iwasaki M (1991) The role of human P450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.* **4:** 168-179.

Harvey DJ and Brown NK (1990) In vitro metabolism of cannabigerol in several mammalian species. *Biomed Environ Mass Spec*. **19:** 545-553.

Himmelstein MW, Turner MJ, Asgharian B and Bond JA (1994) Comparison of blood concentrations of 1,3-butadiene and butadiene epoxides in mice and rats exposed to 1,3-butadiene by inhalation. *Carcinogenesis* **15(8):** 1479-1486.

Houston JB (1994) Utility of in vitro drug metabolism data in predicting *in vivo* metabolic clearance. Biochem Pharmacol, **47(9):** 1469-1479.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



Madyastha KM and Raj CP (1993) Studies on the metabolism of the monoterpene ketone, R-(+)-pulegone – a hepatotoxins in rat: isolation and characterization of new metabolites. *Xenobiotica*, **23(5):** 509-518.

Magdalou J, Chajes V, Lafaurie C and Siest G. (1990) Glucuronidation of 2-arylpropionic acids pirprofen, flurbiprofen and ibuprofen by liver microsomes. *Drug Metab Disp.* **18(5):** 692-697.

Mumby S and Hammock BD (1979) Substrate selectivity and stereochemistry of enzymatic epoxide hydration in the soluble fraction of mouse liver. *Pesticide Biochem Physiol*, **11:** 275-284.

Oesch F, Kaubisch N, Jerina DM and Daly JW (1971) Hepatic epoxide hydrolase. Structure-activity relationships for substrates and inhibitors. *Biochemistry*, **26(10):** 4858-4866.

Peter H, Wiegand H-J, Filser, JG, Bolt HM and Laib R J (1990**)** Inhalation pharmacokinetics of isoprene in rats and mice Environ Health Persp, **86:** 89-92.

Potter J, Smith RL and Api AM (2001) An assessment of the release of cyanide from the fragrance materials bezyl cyanide, geranyl nitrile and citronellyl nitrile applied dermally to the rat. *Food Chem Toxicol* **39:** 147-151.

RIFM (Research Institute for Fragrance Materials, Inc.) (1974a) Report on acute toxicity studies. RIFM report No 1778.

RIFM (Research Institute for Fragrance Materials, Inc.) (1974b) Report on acute oral and dermal toxicity studies. RIFM report No 2028.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



Seglen PO (1976) Preparation of isolated rat liver cells, in: *Methods in Cell Biology*, Vol. 19 (DM Prescott, Ed.) Academic Press, New York, pp 29-83.

Thulasiram HV, Bhat VB and Madyastha MK. (2001) Effect of ring size in R-(=)-pulegone-mediated hepatotoxicity: Studies on the metabolism of R-(+)-4-methy-2-(1-methylidene)-cyclopentanoone and DL-Camphorone in rats. *Drug Metab Disp* **29(6)**: 821-829.

Ventura P, Schiave M and Serafini, S. (1985) Further studies of trans-sobrerol metabolism: rat, dog and human urine. *Xenobiotica* **15(4):** 317-325.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



Footnotes

This research was supported by the Research Institute for Fragrance Materials (RIFM), Woodcliff Lake, NJ, USA).

This work was presented in part at the 44th annual meeting of the Society of Toxicology, New Orleans, LA, March 6-10th, 2005.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



Figure Legends

Figure 1. *Structures and electron impact ionization mass spectra of geranyl nitrile (A) and citronellyl nitrile (B).* Test compounds were diluted in acetonitrile and analyzed by GC/MS as described under Materials and Methods.

Figure 2. *Representative chromatograms from GC/MS analysis of rat (A) and mouse (B) hepatocyte extracts following incubation with geranyl nitrile.* Geranyl nitrile (250 μM) was incubated with hepatocytes (5 x $10^6$ cell/mL in L-15 medium) in septum-cap vials for 60 min at 37 °C. Incubation mixtures were extracted with ethyl acetate, concentrated and analyzed by GC/MS. Because of high background noise in the total ion chromatogram, reconstructed ion chromatograms for m/z 132 (monooxidized parent-methyl - $H_2O$) and m/z 85 (2-methylbutyl-2-ene + 16, inset) are presented for clarity in panel A. Panel B (mouse) illustrates a typical total ion chromatogram. No unique metabolites were observed in human hepatocyte extracts (chromatogram not shown)

Figure 3. *Electron impact mass spectra of synthetic 6,7-epoxygeranyl nitrile isomers and corresponding rat metabolites.* Authentic 6,7-epoxygeranyl nitriles (Panels A and B) were synthesized as described under materials and methods. Proposed fragmentation schemes are shown as insets. Rat metabolites **G1** and **G2** (Panels C and D) were identified in following a 60 minute incubation of rat hepatocytes with commercial GN.

Figure 4. *Electron impact mass spectra of rat metabolite **G3**(A) and Artemisia alcohol (B).* . Rat metabolites **G3** and **G4** (not shown) were tentatively identified following a 60 minute incubation of rat hepatocytes with commercial GN. The spectrum of



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



Artemisia alcohol (3,3,6-trimethyl-1,5-heptadien-4-ol) was obtained from the Wiley mass spectral database using HP Chemstation software.

Figure 5 *Electron impact mass spectra of synthetic 8-hydroxygeranyl nitrile isomers and corresponding rat metabolites.* Authentic 8-hydroxygeranyl nitriles (Panels A and B) were synthesized as described under materials and methods. Proposed fragmentation schemes are shown as insets. Rat metabolites **G6** and **G8** (Panels C and D) were identified following a 60 minute 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.

Figure 6. *Representative total ion chromatogram from GC/MS analysis of rat hepatocyte extracts following incubation with citronellyl nitrile.* Citronellyl nitrile (250 µM) was incubated with hepatocytes (5 x $10^6$ cell/mL in L-15 medium) in septum-cap vials for 60 min at 37 °C. Incubation mixtures were extracted with ethyl acetate, concentrated and analyzed by GC/MS as described under Materials and Method.

Figure 7. *Electron impact mass spectra of synthetic 6,7-epoxycitronellyl nitrile and 8-hydroxycitronellyl nitrile and corresponding rat metabolites.* Authentic standards (Panels A and B) were synthesized as described under Materials and Methods. Proposed fragmentation schemes are shown as insets. Rat metabolites **G6** and **G8** (Panels C and D) were identified following a 60 minute incubation of rat hepatocytes with commercial GN

Figure 8. *Electron impact mass spectrum of rat metabolite **C3***. The metabolite was observed following a 60 minute incubation of rat hepatocytes with commercial

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



citronellyl nitrile. The proposed structure and fragmentation rationale for metabolite **C3** are shown in the inset.

Figure 9. *Common metabolic pathways for terpene nitriles in rat, mouse and human hepatocytes.* The hashed bond at $C_2$-$C_3$ is intended to represent both geranyl and citronellyl nitrile. Metabolites shown were observed in all three species, with the exception of 5-hydroxygeranyl nitrile, which was not found with human hepatocytes. With geranyl nitrile, isomeric (E,Z) pairs of metabolites were frequently observed. For clarity, only the E-isomer is shown. Intermediates in brackets are inferred based on the presence of downstream metabolites. Glutathione conjugation may also occur at $C_6$ (not shown). CYP – cytochromes P-450, ADH – alcohol dehydrogenase, ALDH – aldehyde dehydrogenase, GSH – glutathione, GST – glutathione S-transferase, UDPGT – UDP-glucuronyltransferase.

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.



TABLE 1.

*Kinetic parameters for metabolism of geranyl nitrile and citronellyl nitrile by rat, mouse and human hepatocytes.*

Test compounds (25 µM) were incubated with hepatocytes (2.5 x $10^6$ cells/mL) from the species indicated. The headspace vapor was sampled periodically and analyzed for test compounds by GC/MS as described under Materials and Methods. Data are expressed as mean ± SD.

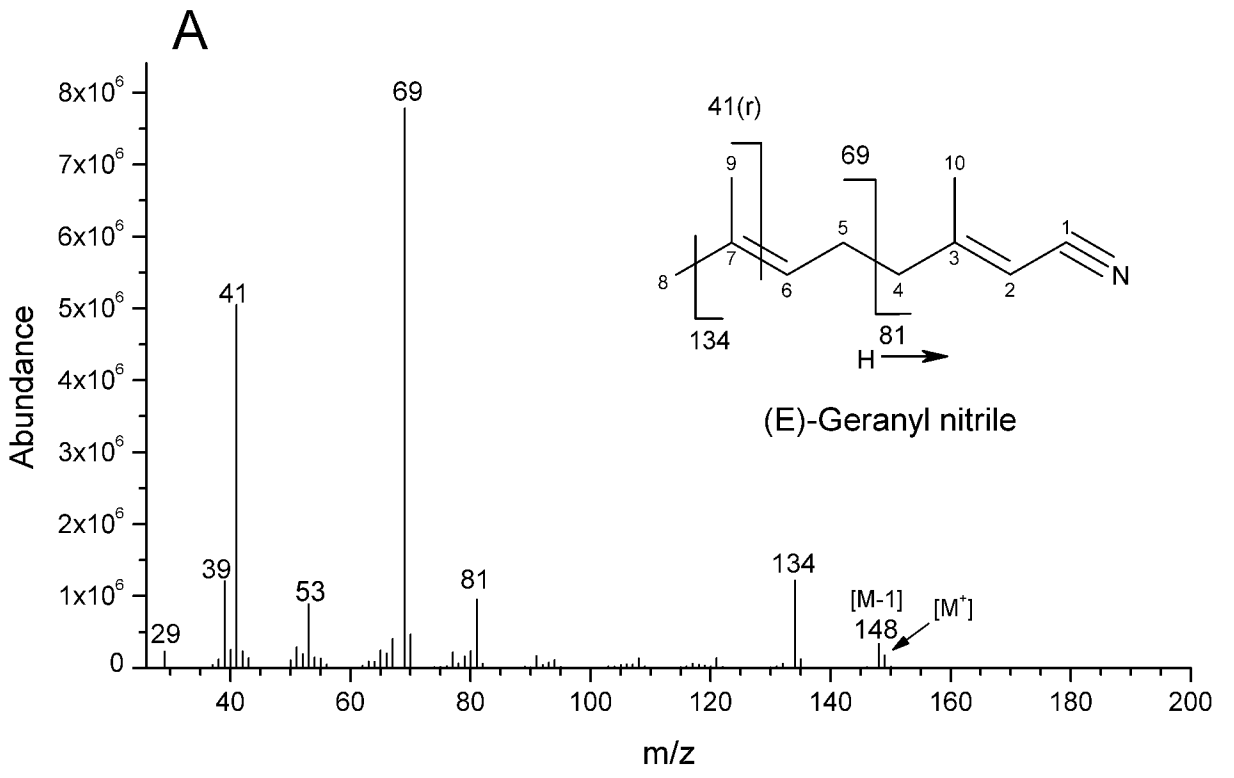
| **Geranyl Nitrile** | **$\lambda_z$ ($min^{-1}$)[a]** | **$T_{1/2}$ (min)** | **$CL_H$ (mL/min*$10^6$ cells)** | **$CL_I$ (mL/min/kg)** |
|---|---|---|---|---|
| **Rat** | 0.22 ± 0.02 | 3.20 ± 0.22 | 0.09 ± 0.04 | 565 ± 274 |
| **Mouse** | 1.00 ± 0.18 | 0.71 ± 0.13 | 0.47 ± 0.09 | 3009 ± 277 |
| **Human** | | | | |
| HL1 | 0.34 | 2.06 | 0.15 | 519 |
| HL2 | 0.06 | 10.92 | 0.027 | 93 |
| HL3 | 0.05 | 15.28 | 0.02 | 82 |

| **Citronellyl Nitrile** | **$\lambda_z$ ($min^{-1}$)[a]** | **$T_{1/2}$ (min)** | **$CL_H$ (mL/min*$10^6$ cells)** | **$CL_I$ (mL/min/kg)** |
|---|---|---|---|---|
| **Rat** | 0.24 ± 0.00 | 2.89 ± 0.05 | 0.14 ± 0.02 | 926 ± 131 |
| **Mouse** | 0.55 ± 0.09 | 1.28 ± 0.23 | 0.27 ± 0.09 | 1749 ± 563 |
| **Human** | | | | |
| HL1 | 0.27 | 2.62 | 0.22 | 758 |
| HL2 | 0.05 | 13.08 | 0.02 | 83 |
| HL3 | 0.04 | 19.19 | 0.03 | 92 |

[a] $\lambda_z$ – elimination rate constant, $T_{1/2}$ – elimination half-life, $CL_H$ – hepatocyte intrinsic clearance, $CL_I$ – predicted in vivo intrinsic clearance



Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024


A
Abundance
m/z
69
41
39
53
81
134
29
148
[M-1]
[M+]
41(r)
69
134
81
H
(E)-Geranyl nitrile

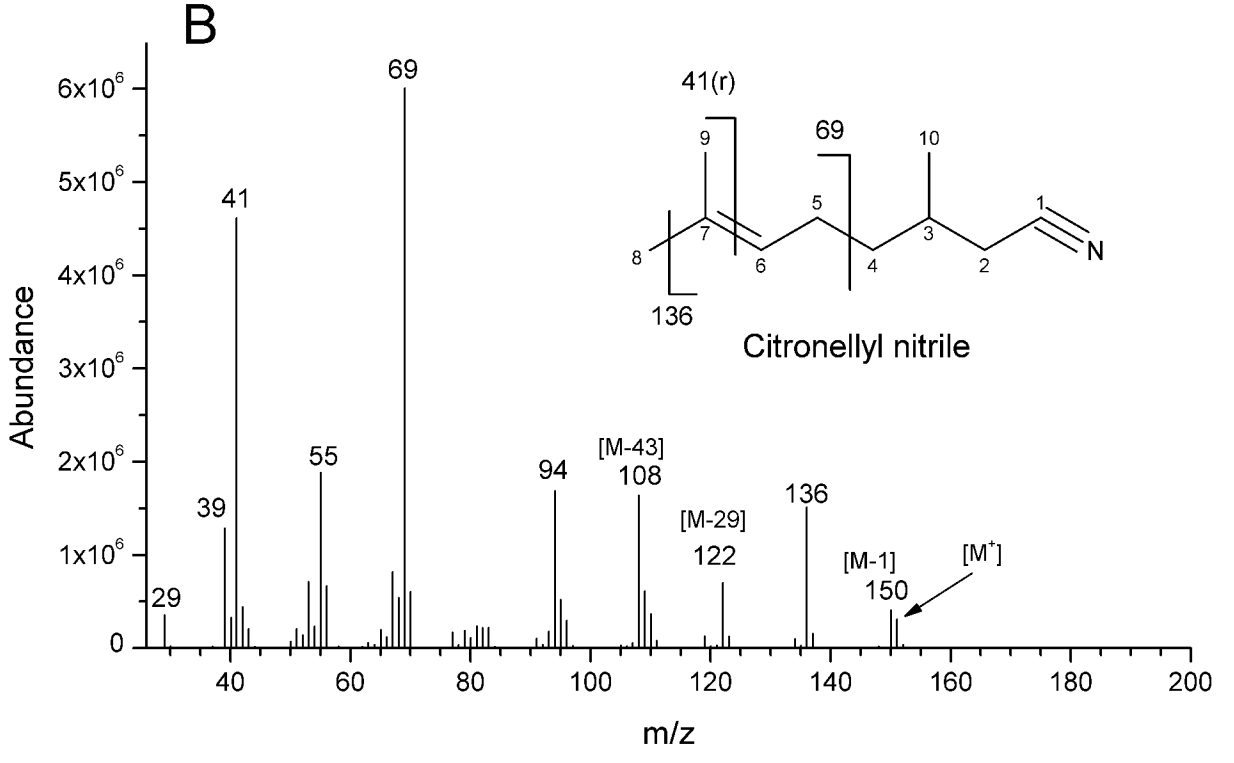
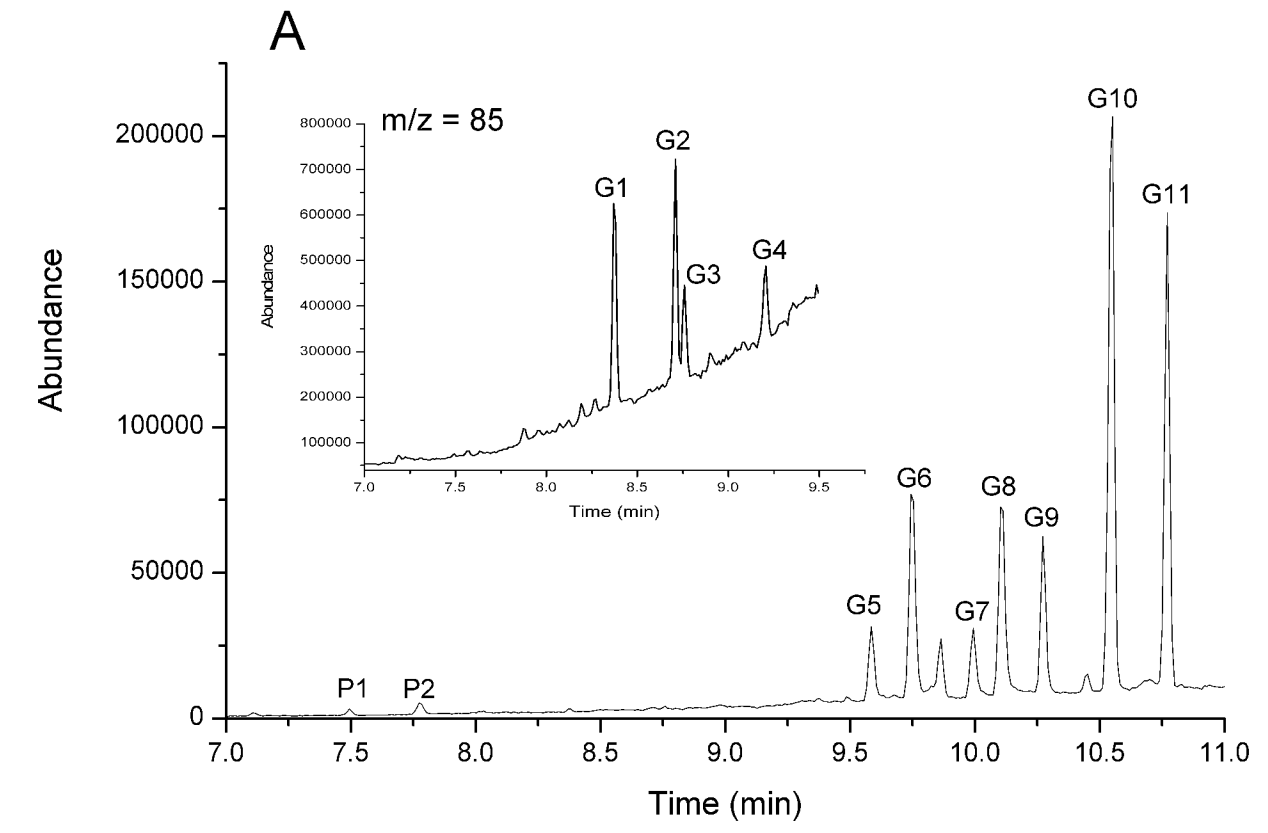

B
Abundance
m/z
69
41
39
55
94
[M-43]
108
[M-29]
122
136
29
[M-1]
150
[M+]
41(r)
69
136
Citronellyl nitrile

Figure 1


A
m/z = 85
Abundance
Time (min)
G1
G2
G3
G4
G5
G6
G7
G8
G9
G10
G11
P1
P2

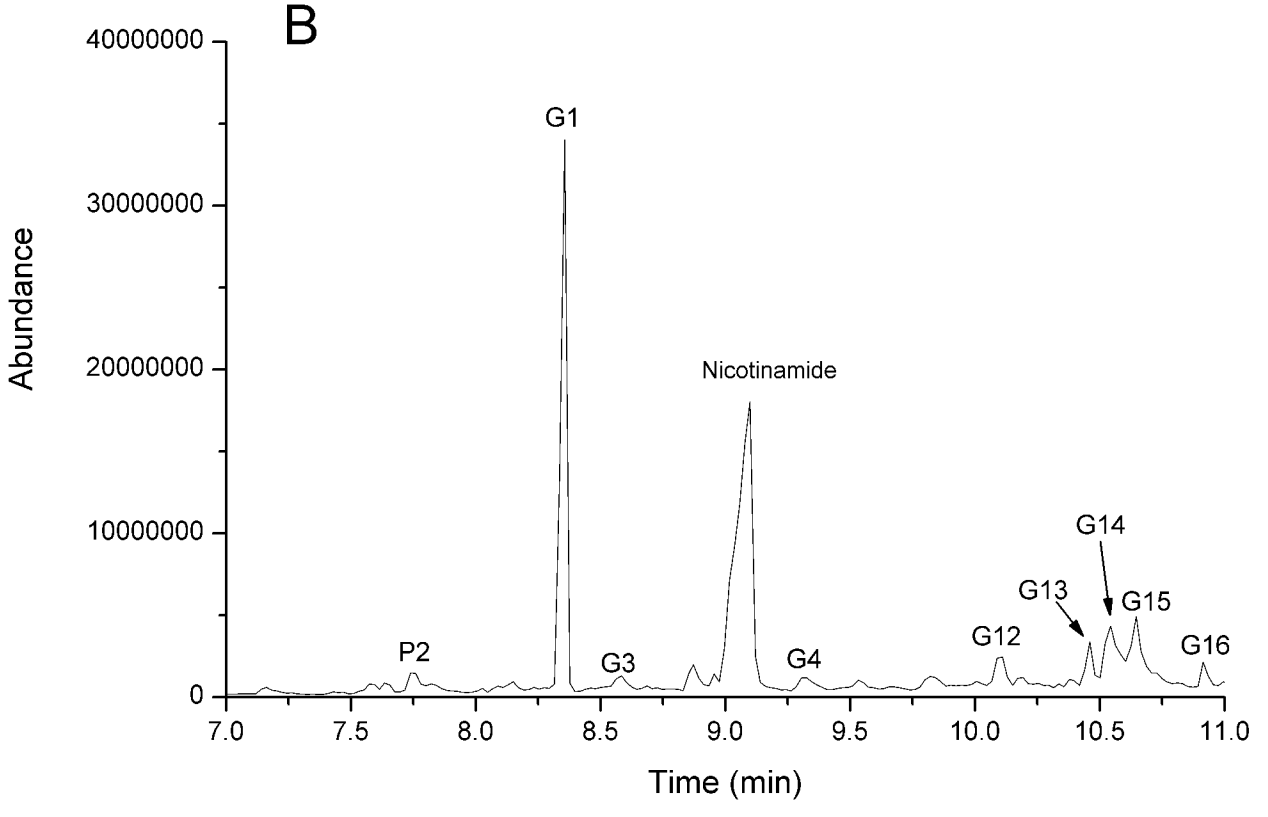
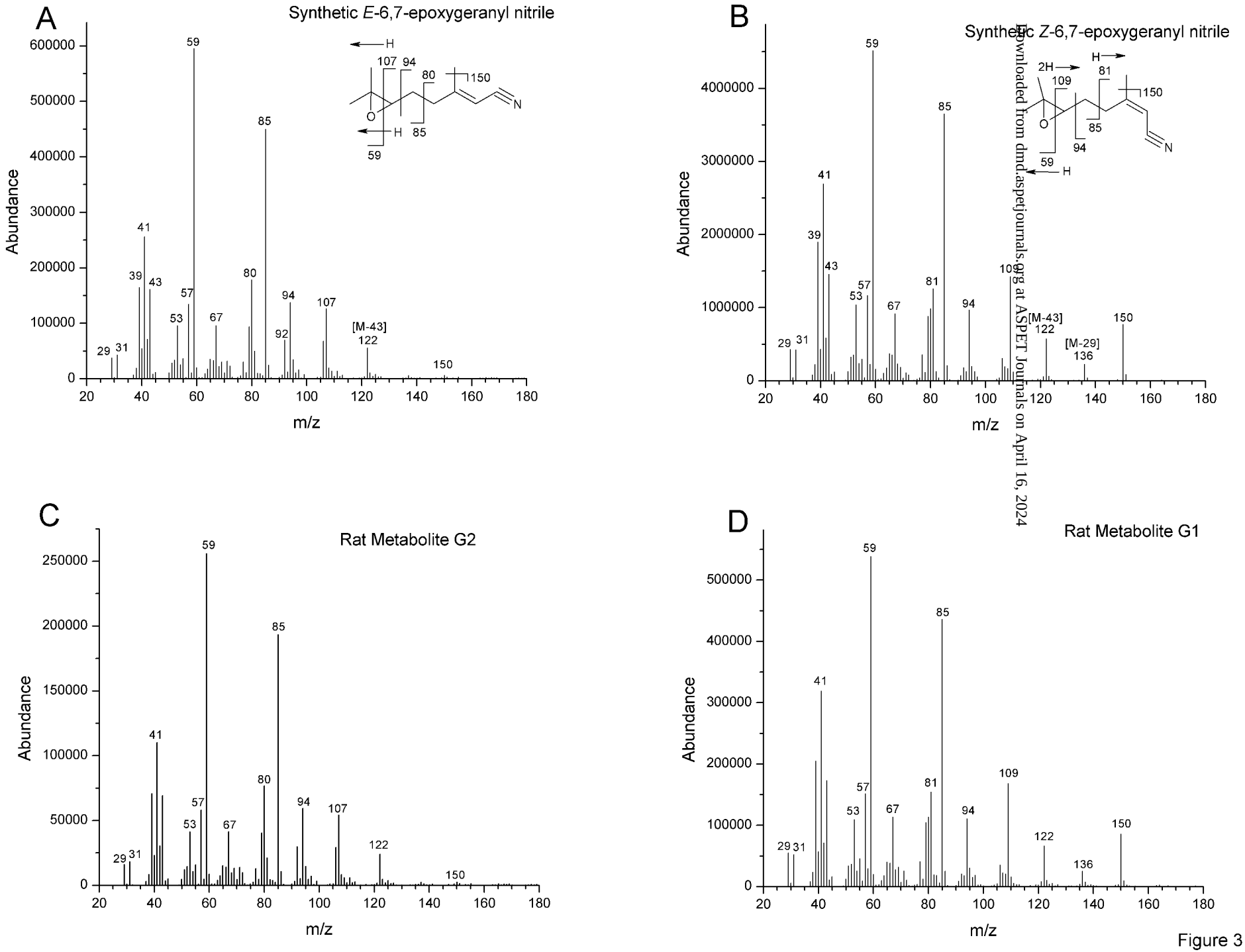
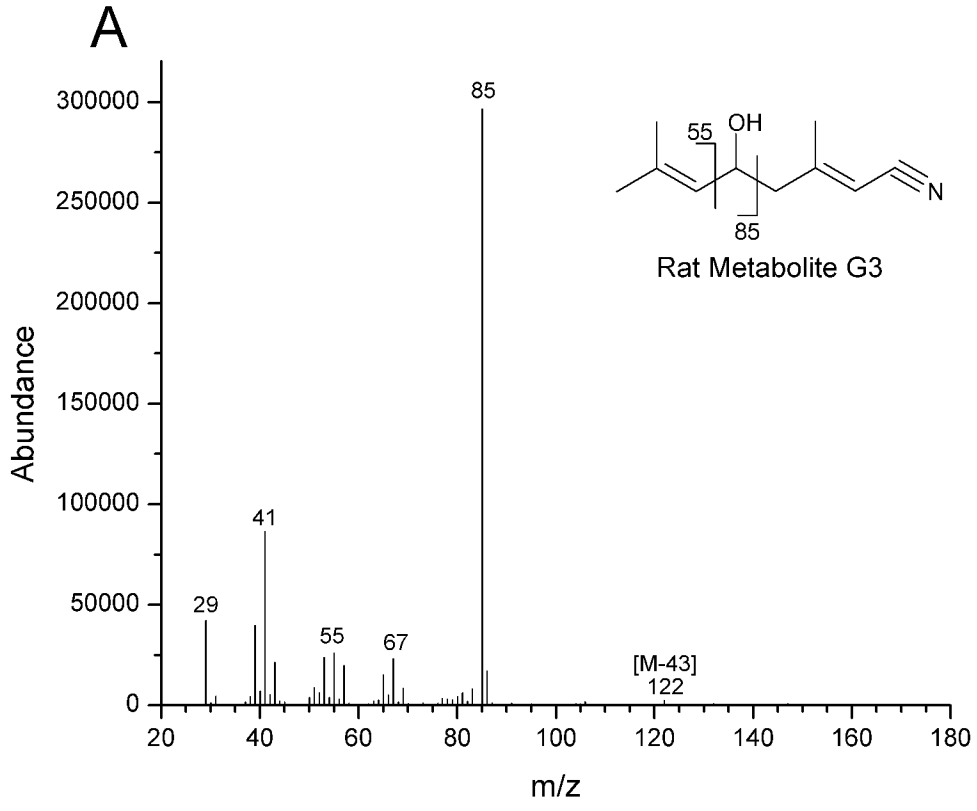

B
Abundance
Time (min)
G1
Nicotinamide
P2
G3
G4
G12
G13
G14
G15
G16

Figure 2




Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 3


A
Abundance
m/z
85
41
29
55
67
[M-43]
122
OH
55
85
N
Rat Metabolite G3

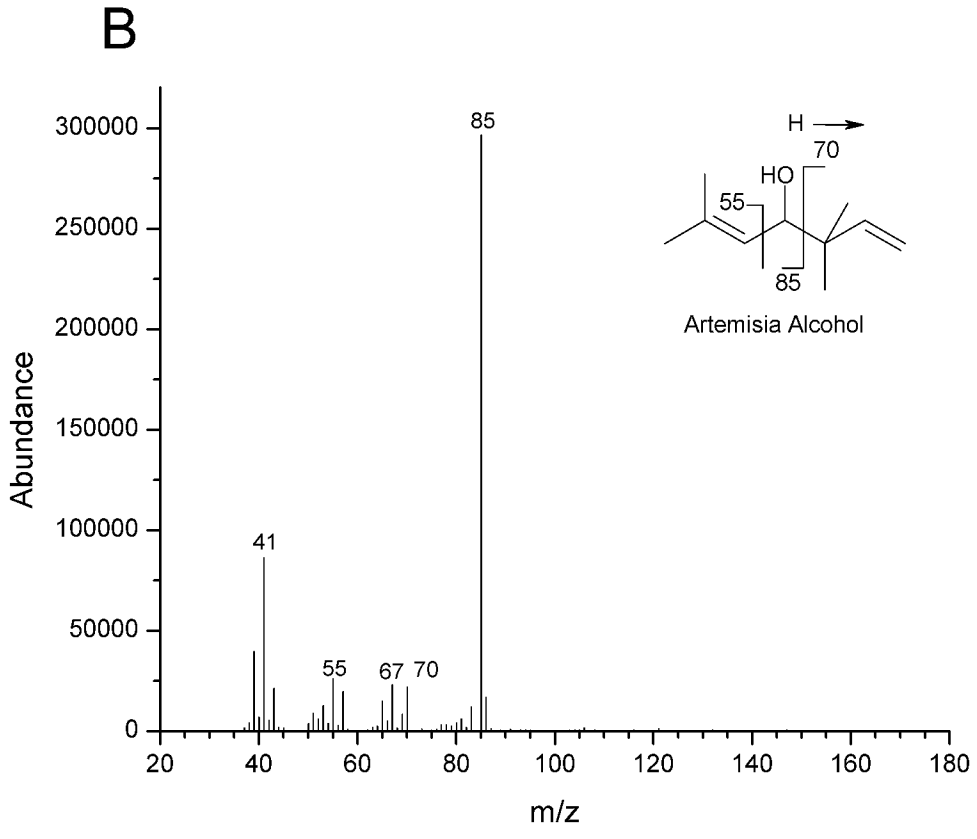
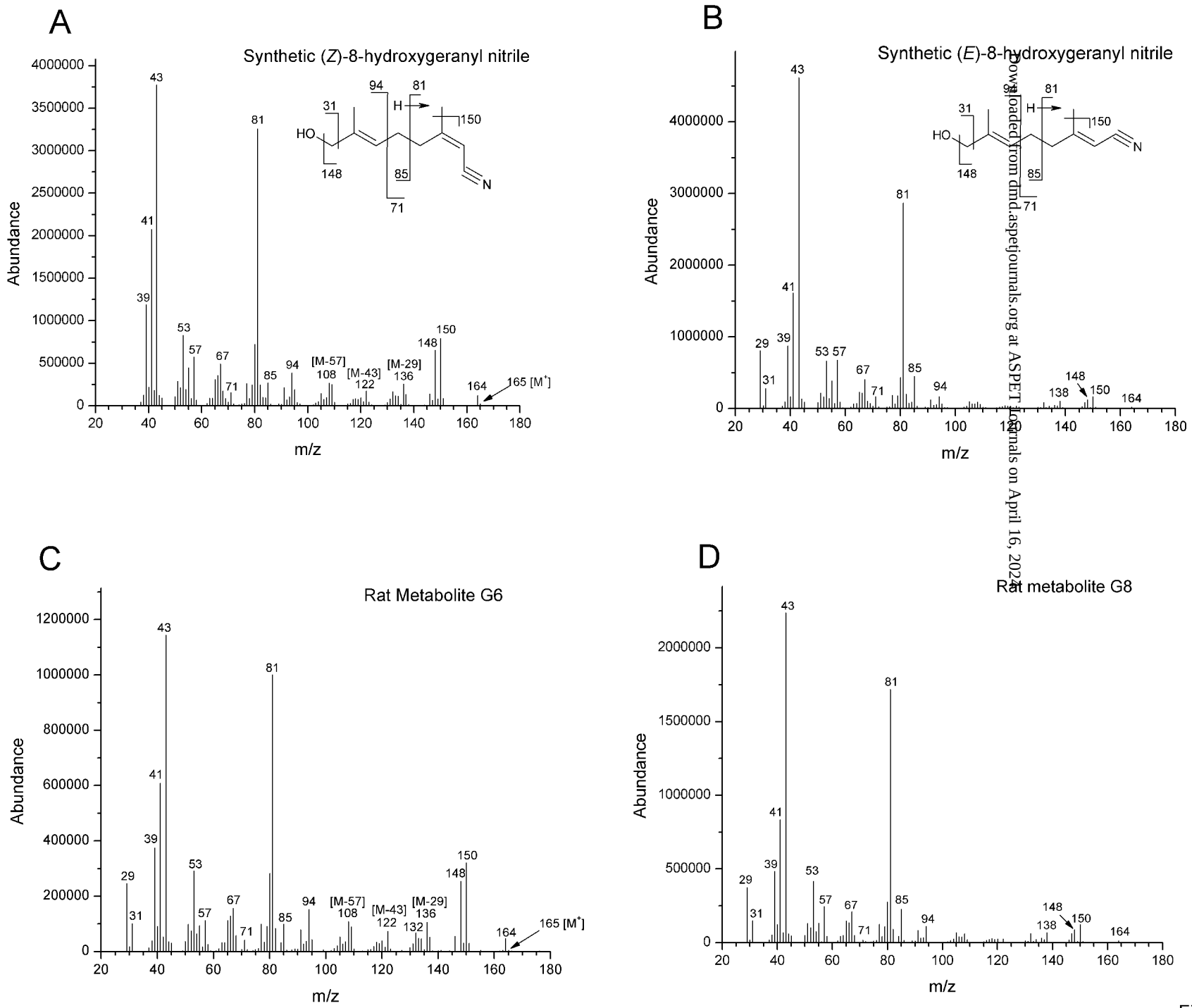
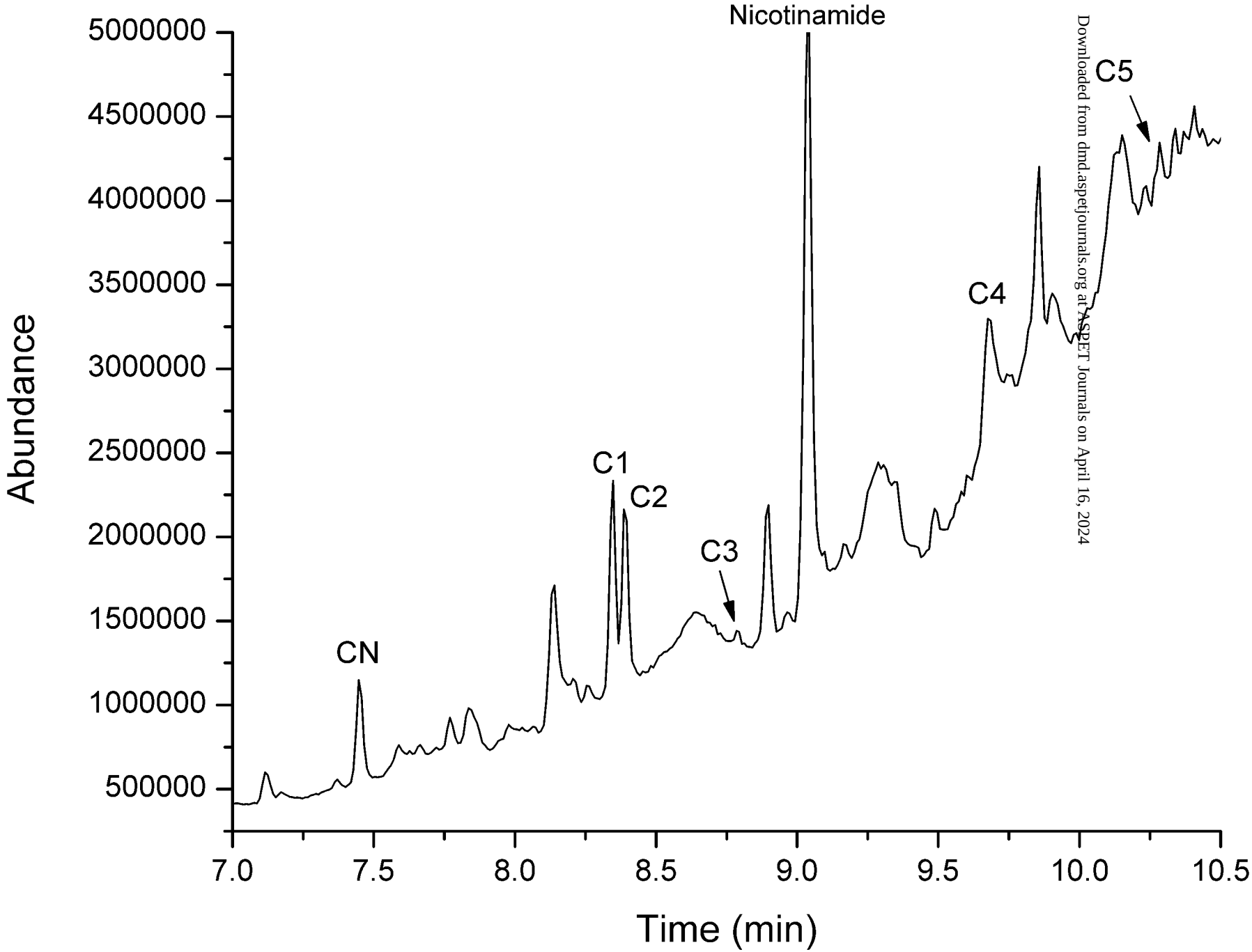

B
Abundance
m/z
85
41
55
67
70
H
70
HO
55
85
Artemisia Alcohol

Figure 4



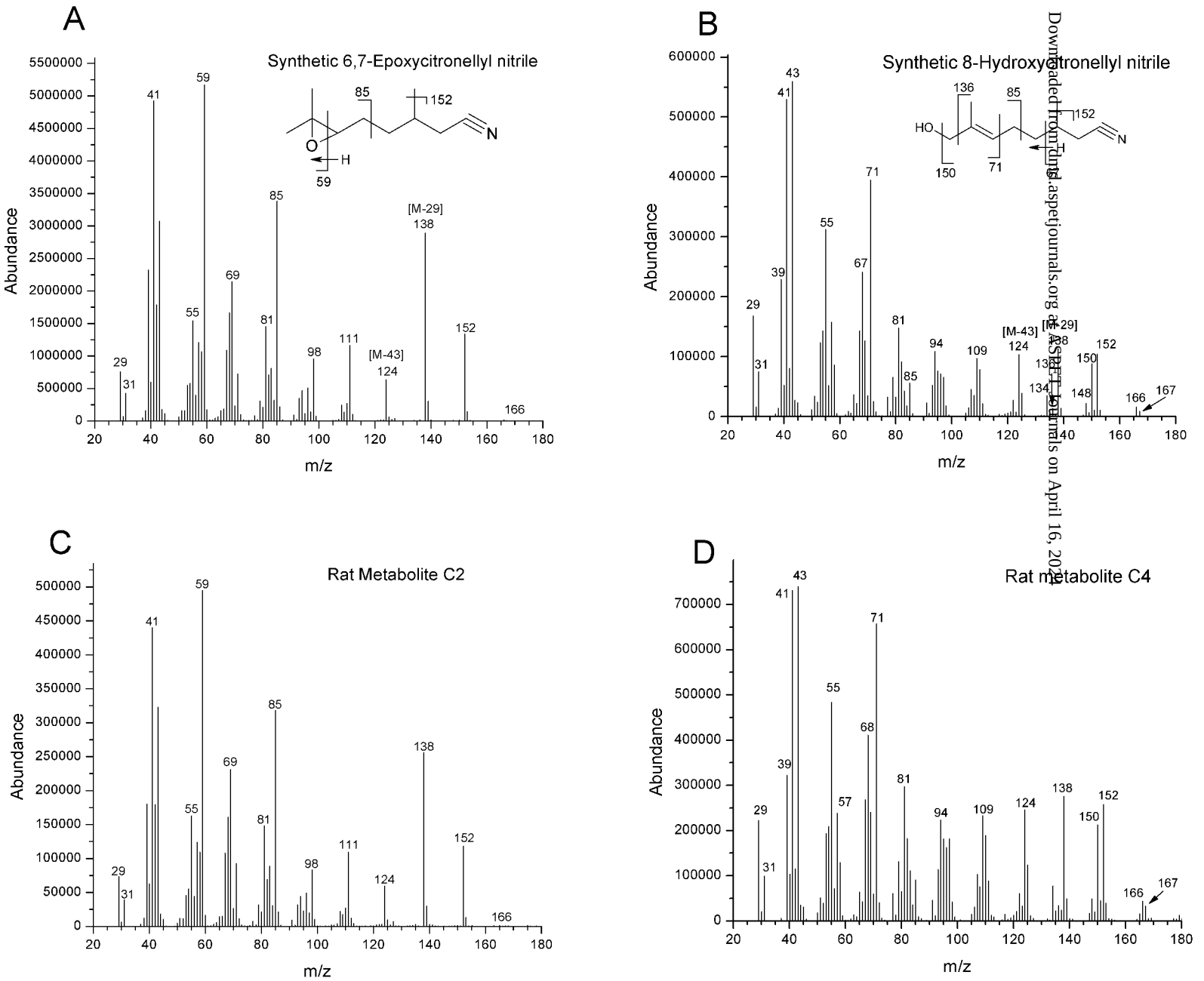

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 5



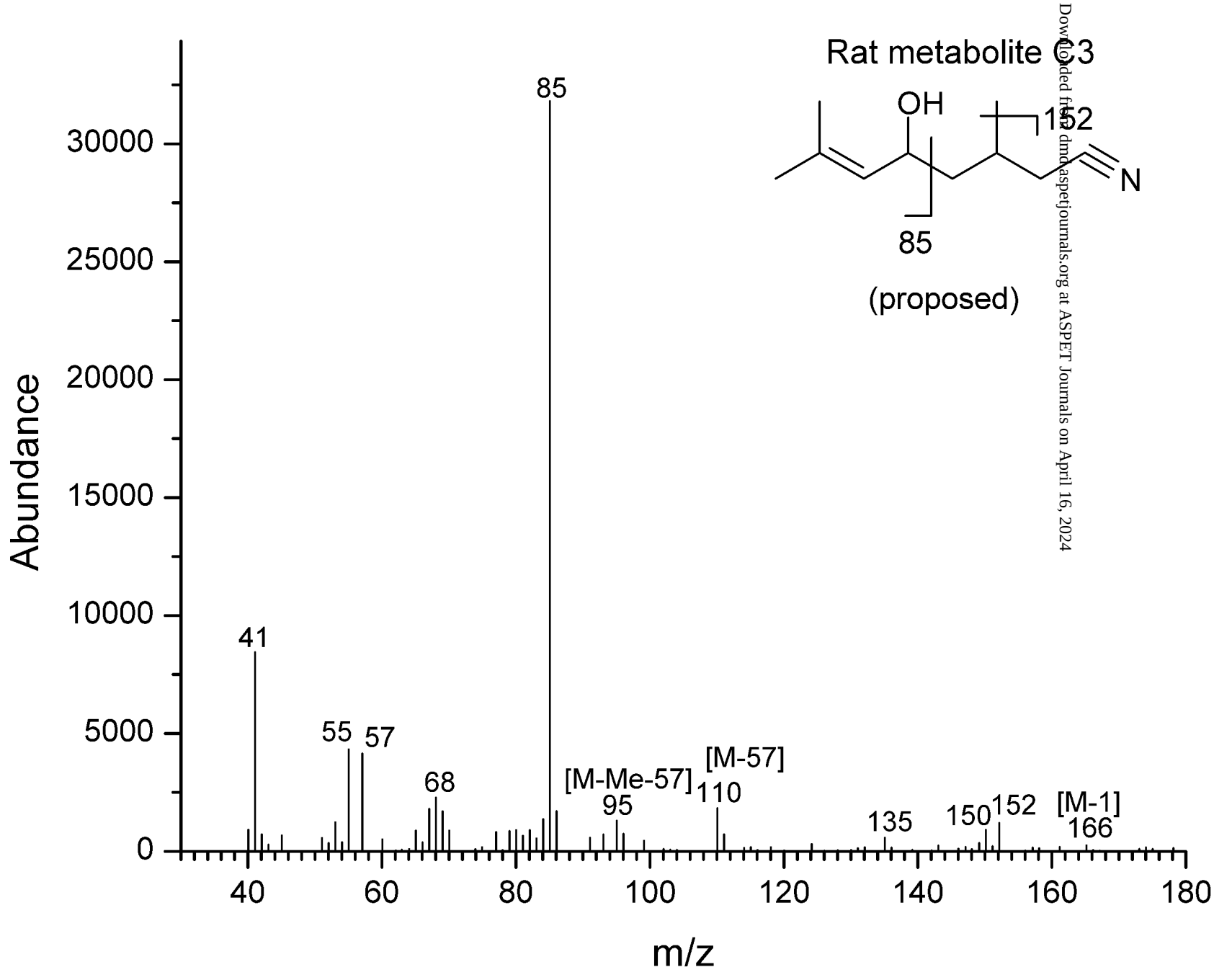

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 6




Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2021

Figure 7




Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

Figure 8

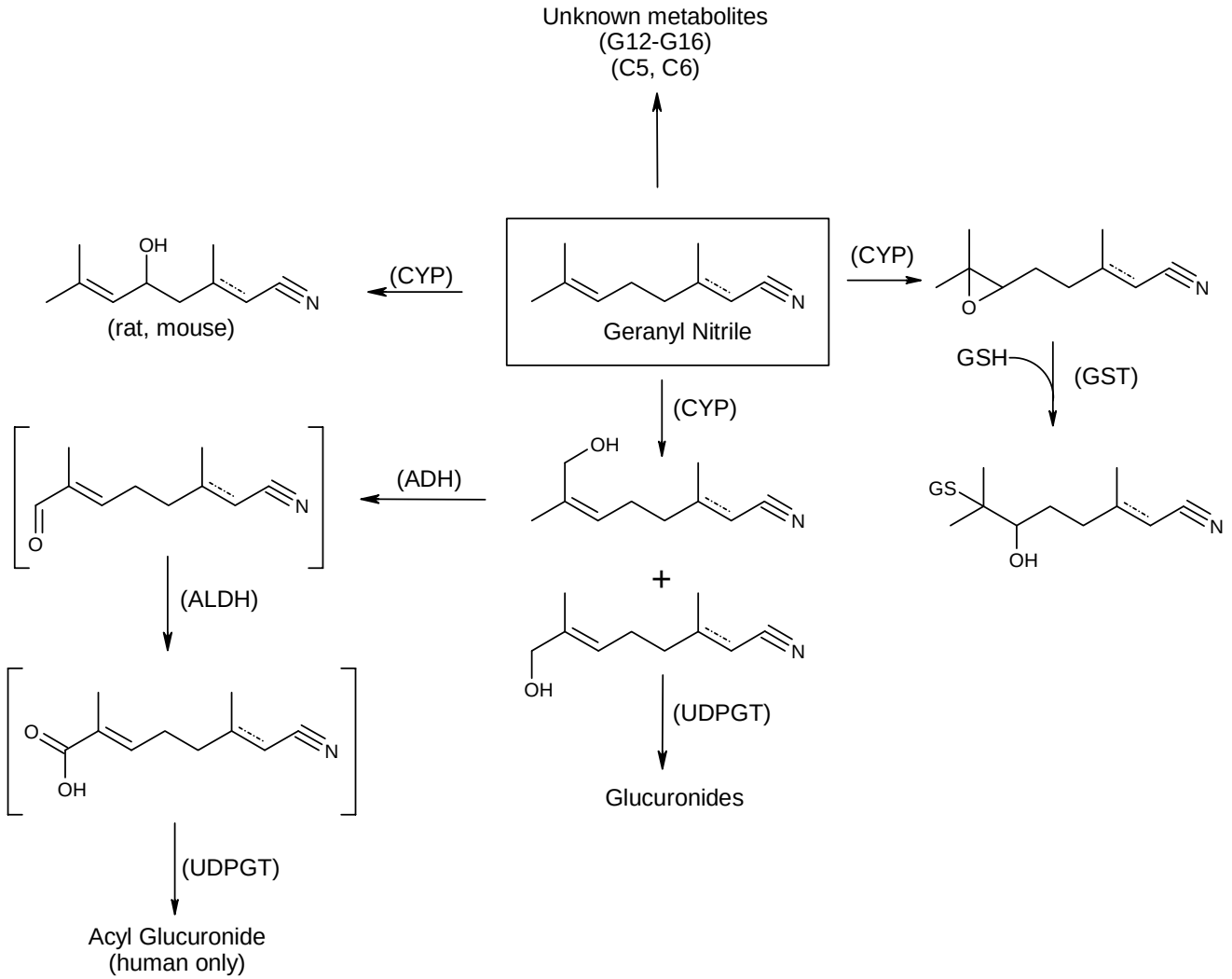



Figure 9

Downloaded from dmd.aspetjournals.org at ASPET Journals on April 16, 2024

DMD Fast Forward. Published on March 15, 2006 as DOI: 10.1124/dmd.105.005496
This article has not been copyedited and formatted. The final version may differ from this version.