Identification and Synthesis of a Unique Disulfide Dimeric Metabolite of Primisulfuron-methyl in the Mouse

Thomas M. Capps,* Nina E. Heard, Dana P. Simmons, and Connie L. Connor

Metabolism Department, Ciba-Geigy Corporation, Ciba Plant Protection, P.O. Box 18300, Greensboro, North Carolina 27419

The major metabolic pathway for primisulfuron-methyl in animals involves hydroxylation of the pyrimidine ring followed by conjugation. This investigation found a unique metabolite in mice fed at high feeding levels (>5000 ppm) of the compound. The unique metabolite is identified as a disulfide dimer of the parent compound, and its synthesis is reported.

Primisulfuron-methyl (CGA-136872), the active ingredient in Beacon herbicide, has been developed by Ciba for selective control of undesirable grasses in corn. The major metabolic pathway for detoxification of CGA-136872 in corn plants involves phenyl ring hydroxylation followed by hydrolysis and/or glucose conjugation. In rats and large animals, the major pathway involves hydroxylation of the pyrimidine ring followed by hydrolysis and/or glucuronic acid conjugation.

Previous studies (Capps and Cassidy, 1986; Capps and Worsham, 1993; MacKenzie, 1987) were conducted to investigate the metabolism of CGA-136872 in rats and mice treated subchronically for 28 days at feeding levels ranging from 100 to 20 000 ppm of unlabeled CGA-136872 in the feed. After 28 days, the animals were given an oral pulse (5 ppm) of [^{14}C]-CGA-136872. In the study with mice, a new metabolite was present in the feces at feeding levels >5000 ppm. This metabolite was not present at the lower feeding levels in mice (<5000 ppm) and was not observed at any feeding level in the rat.

This investigation reports on (1) the isolation and conclusive identification of the new metabolite D, present in mouse feces at the high feeding levels of CGA-136872 (>5000 ppm), and (2) the synthesis of this metabolite.

MATERIALS AND METHODS

Materials. CGA-136872, methyl 2-[[[[[4,6-bis(difluoromethoxy)-2-pyrimidinyl]amino]carbonyl]amino]sulfonyl]benzoate, labeled (1) uniformly in the phenyl ring (U-ring-^{14}C) and (2) at the 2-position in the pyrimidine ring (2-^{14}C) was prepared by the Chemical Synthesis Group, Metabolism Department, Ciba-Geigy Corp., with specific activities of 29.3 (ϕ-label) and 30.4 μCi/mg (Δ-label). Other metabolite standards utilized for cochromatography were also prepared by the Chemical Synthesis Group.

Test System. Forty male Crl:CD-1 (ICR)BR mice were fed a diet containing 10 000 ppm of CGA-136872 technical for 34 days. On days 28, 30, and 32, 20 of the mice were administered oral gavage doses of ~0.025 mg of Δ-[^{14}C]-CGA-136872 (group 1) and 20 of the mice were administered oral gavage doses of ~0.025 mg of ϕ-[^{14}C]-CGA-136872 (group 2). Each animal received a nominal dose of 0.025 mg of [^{14}C]-CGA-136872 in a volume of 0.25 mL (0.25 N $NaHCO_3$ was the carrier). Both groups continued to be fed the unlabeled CGA-136872 in the feed until termination of the in-life portion (48 h after the last ^{14}C dose).

Urine and feces samples were collected at 0–8, 8–24, and 24–48 h postdose after each of the three radiolabeled doses. Urine and feces samples were pooled according to group, dose day, and collection interval, resulting in a total of 18 composite samples for each matrix. All of the composite fecal samples were then homogenized with approximately twice their weight of water.

Feces Extraction Method. Pooled, homogenized feces were extracted three times by blending with a 9:1 solution of acetonitrile/water (containing 0.5% H_3PO_4) and vacuum filtered. The combined filtrate was transferred to a round-bottom flask, and acetonitrile was removed under vacuum. The pH of remaining aqueous sample was checked and adjusted (when necessary) to 2.0 with 1 N HCl and then partitioned three times with chloroform (1:1 v/v). The chloroform was removed and the sample reconstituted in acetonitrile (5–15 mL). Duplicate aliquots (10–100 μL) of aqueous and organic soluble fractions were counted. Duplicate aliquots (~0.025 g) of air-dried residue were combusted in a Harvey oxidizer; $^{14}CO_2$ was collected in Oxosol C14 (National Diagnostics) and counted. The ^{14}C distribution among the aqueous and organic soluble fractions and nonextractable residue was then calculated.

HPLC Screening and Purification of Fecal Extracts. HPLC analyses were performed using the following instruments and settings: a Perkin-Elmer Series 400 liquid chromatograph and LC-95 UV detector (at 254 nm) equipped with a Raytest Ramona LS flow detector (600-μL glass cell) for ^{14}C detection, an ISCO Foxy fraction collector, a Waters Intelligent Sample Processor 712 (WISP), and a Soltec strip chart recorder at 30 cm/h recording speed. Screening of the fecal extracts was performed using system 1 as follows: a Whatman Partisil 5-C8-RAC column [9.4 mm (i.d.) × 10 cm] at a flow rate of 1.0 mL/min with solvent A being 0.5% phosphoric acid and solvent B being CH_3CN and linear gradient conditions of 100% A to 100% B in 40 min. The isolation of metabolite D and HPLC cochromatography of other fecal metabolites with standards was performed using system 2 as follows: a Whatman Partisil 10 ODS-2 analytical column [4.6 mm (i.d.) × 25 cm] at a flow rate of 1.0 mL/min with solvents A and B as listed above and a linear gradient of 100% A to 80% B in 40 min followed by a 5-minute hold in conditions.

The various metabolites were monitored by UV detection coupled with ^{14}C flow detection and quantitated by liquid scintillation counting. Metabolite D was the least polar metabolite, eluting last off the HPLC column (retention time ~37 min).

Metabolite D Isolation and Collection. A Waters Intelligent Sample Processor (WISP) was used to control multiple HPLC injections of ~15 000 dpm of fecal extract, using system B parameters. Fractions were collected at 1-min intervals beginning at the first ^{14}C peak elution and continuing through elution of the last ^{14}C peak.

Sample Preparation with Waters C_{18} Sep-Pak Cartridge. Fractions containing metabolite D (from HPLC) were combined and transferred to a round-bottom flask, and the acetonitrile was removed via rotary evaporation. The remaining aqueous residue was reduced to ≤5 mL volume under a stream of nitrogen. A Waters C_{18} Sep-Pak was conditioned with ~2 mL of methanol and then ~5 mL of H_2O. The aqueous sample was applied and washed with ~4 mL of H_2O and then ~4 mL of a solution of 80% H_2O/20% MeOH, and sample was eluted in ~2 mL of methanol. The methanol was then evaporated from the sample under a stream of nitrogen.

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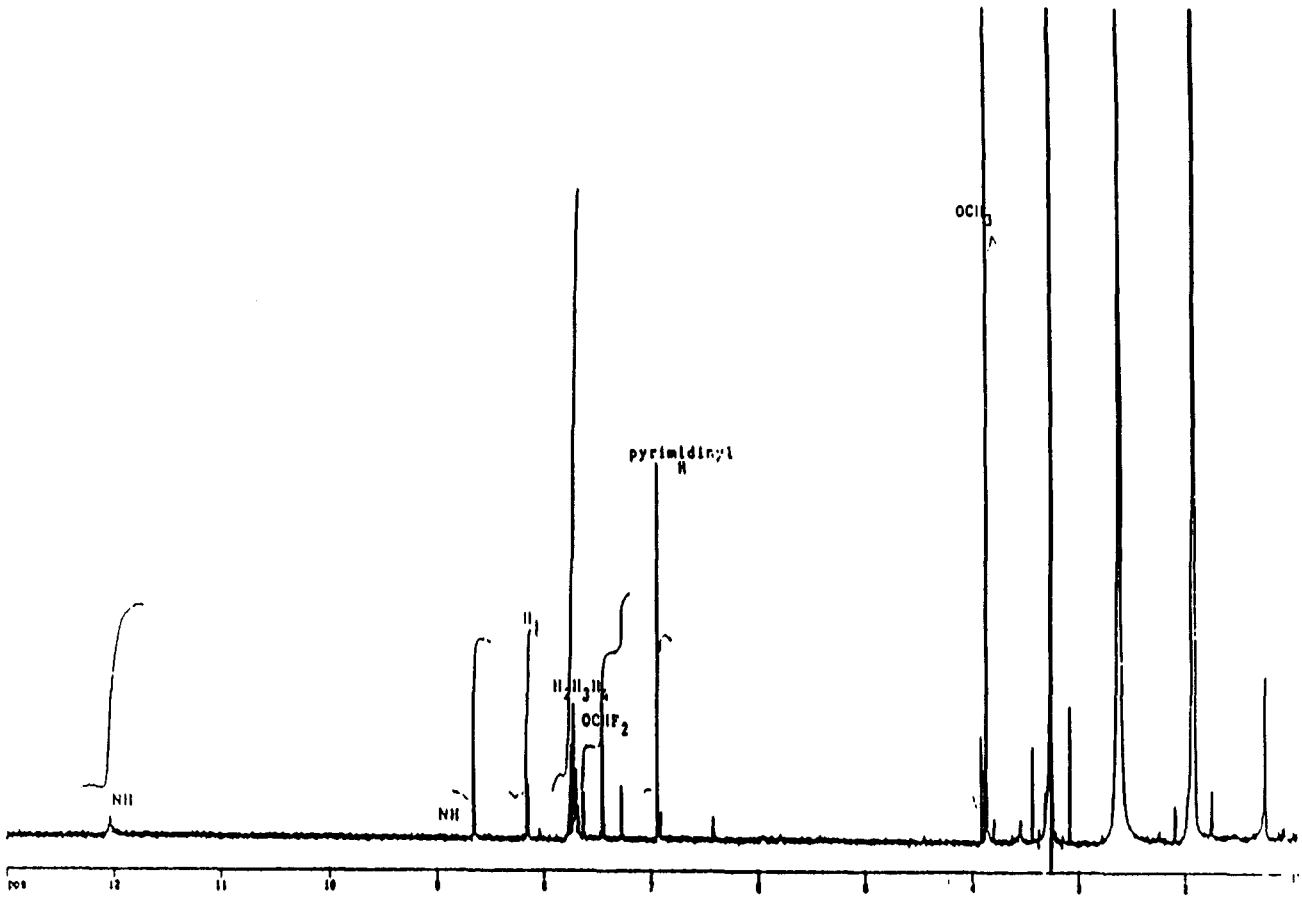


Figure 1. Proton NMR spectrum of metabolite D.

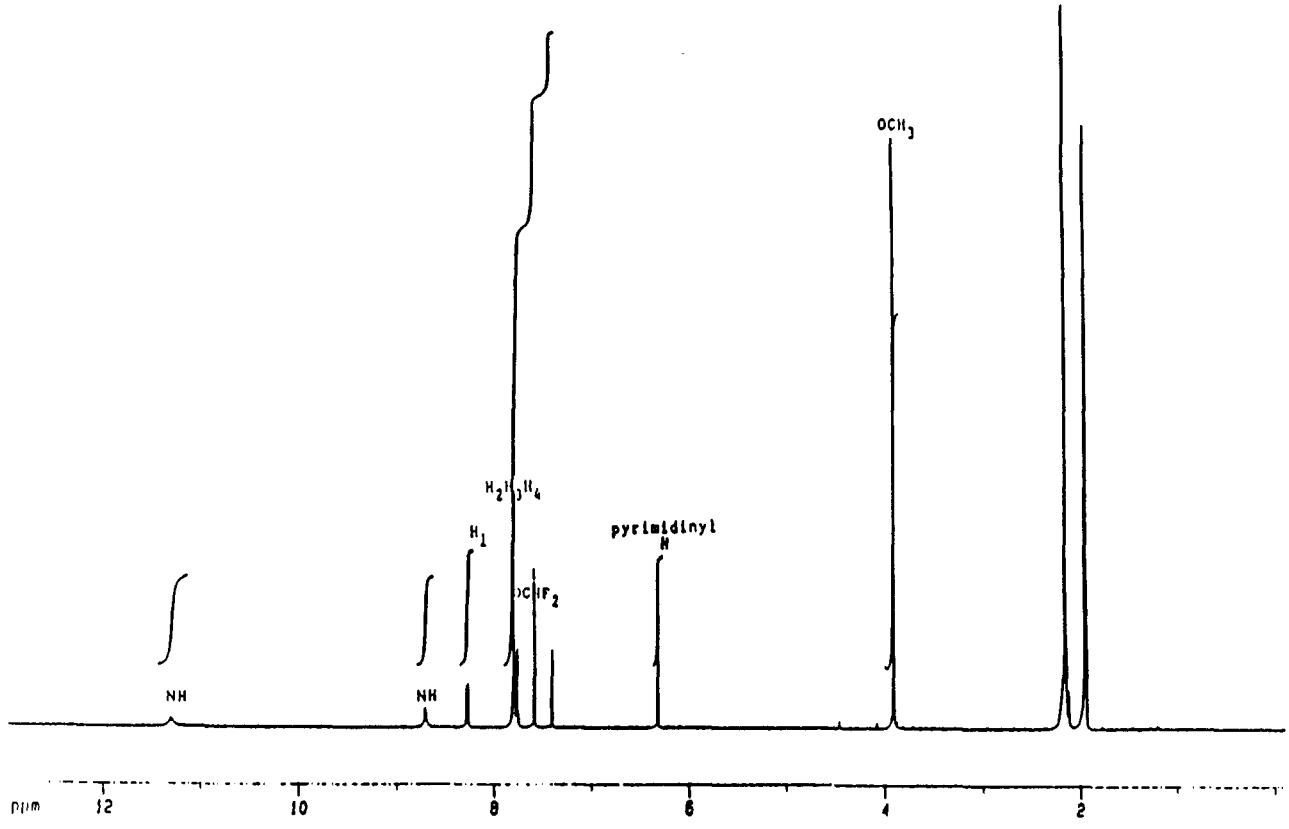
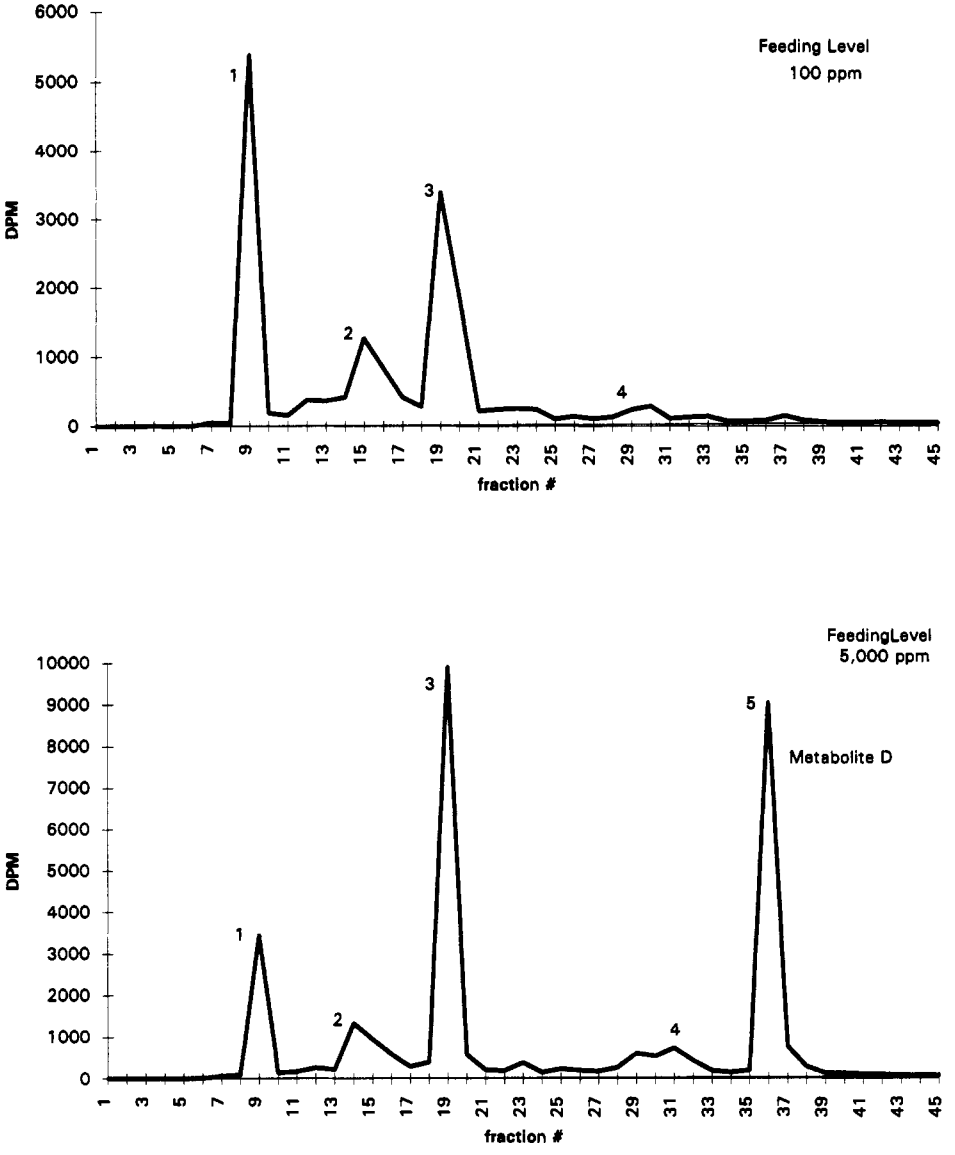


Figure 2. Proton NMR spectrum of CGA-136872.

Nuclear Magnetic Resonance. Proton NMR spectra of metabolite D and parent standard were obtained on a Bruker AMX-400 NMR spectrometer equipped with a QNP detection probe set at 400.13 MHz. Spectra were acquired at room temperature in acetonitrile-d_3. Chemical shifts were relative to acetonitrile at 1.93 ppm.



1. Saccharin
2. CGA-120844 2-Carboxymethyl sulfonamide
3. CGA-239769 Methyl 2-[[[[[4,6-bis(difluoromethoxy)-5-hydroxy-2-pyrimidinyl]-amino]carbonyl] amino]sulfonyl]benzoate
4. CGA-136872
5. Metabolite D

Figure 3. Metabolite profiles for male mice dosed orally with [^{14}C]-CGA-136872.

Metabolite D. Nuclear magnetic resonance spectra (^{1}H NMR) of metabolite D (Figure 1) showed resonances at δ 12.08 (s, 1H, NH), 8.64 (s, 1H, NH), 8.15 (m, 1H, Ar H), 7.72 (m, 3H, Ar H), 7.45 (t, 1H, J = 70 Hz, $OCHF_2$), 6.95 (s, 1H, pyrimidinyl), and 3.86 (s, 3H, OCH_3).

Beacon (CGA-136872) Standard. Nuclear magnetic resonance spectra (^{1}H NMR) of CGA-136872 (Figure 2) showed resonances at δ 11.28 (s, 1H, NH), 8.68 (s, 1H, NH), 8.26 (m, 1H, Ar H), 7.81 (m, 3H, Ar H), 7.56 (t, 1H, J = 71.2 Hz, $OCHF_2$), 6.29 (s, 1H, pyrimidinyl), and 3.91 (s, 3H, OCH_3).

Mass Spectrometry. Fast atom bombardment (FAB) mass spectra were acquired using a VG-70-250SQ spectrometer controlled by an integrated 11-250J data system. A FAB mass spectrum of metabolite D was obtained in a magic bullet matrix (5:1 dithiothreitol/dithioerythritol with addition of 1% trifluoroacetic acid). The primary beam was Xe at 8 kV and 1 MA. Thermospray LC/MS was acquired on a Vestec 201 by direct injection in both positive and negative modes. Mobile phase was a 50:50 solution of 0.1 N NH_4OAc/MeOH. Flow rate was 0.8 mL/min. Vaporizer temperature was 180 °C, tip heater temperature was 280 °C, and source temperature was 230 °C.

Accurate mass measurement via high-resolution mass spectrometry was performed at Research Triangle Institute in Research Triangle Park, NC. Analysis was performed using a VG-ZAB-E with 11-250J data system and a matrix of 30:70 thioglycerol/glycerol. Mass was measured in MCA mode using CSI as reference. Several scans were acquired on the reference, and then the acquisition was suspended. The sample was loaded and inserted, and the acquisition was resumed and acquired until adequate sample signal was obtained.

RESULTS AND DISCUSSION

In previous studies with mice by MacKenzie (1987), HPLC analysis had revealed the presence of a new metabolite D in feces at high feeding levels (>5000 ppm)

Scheme I

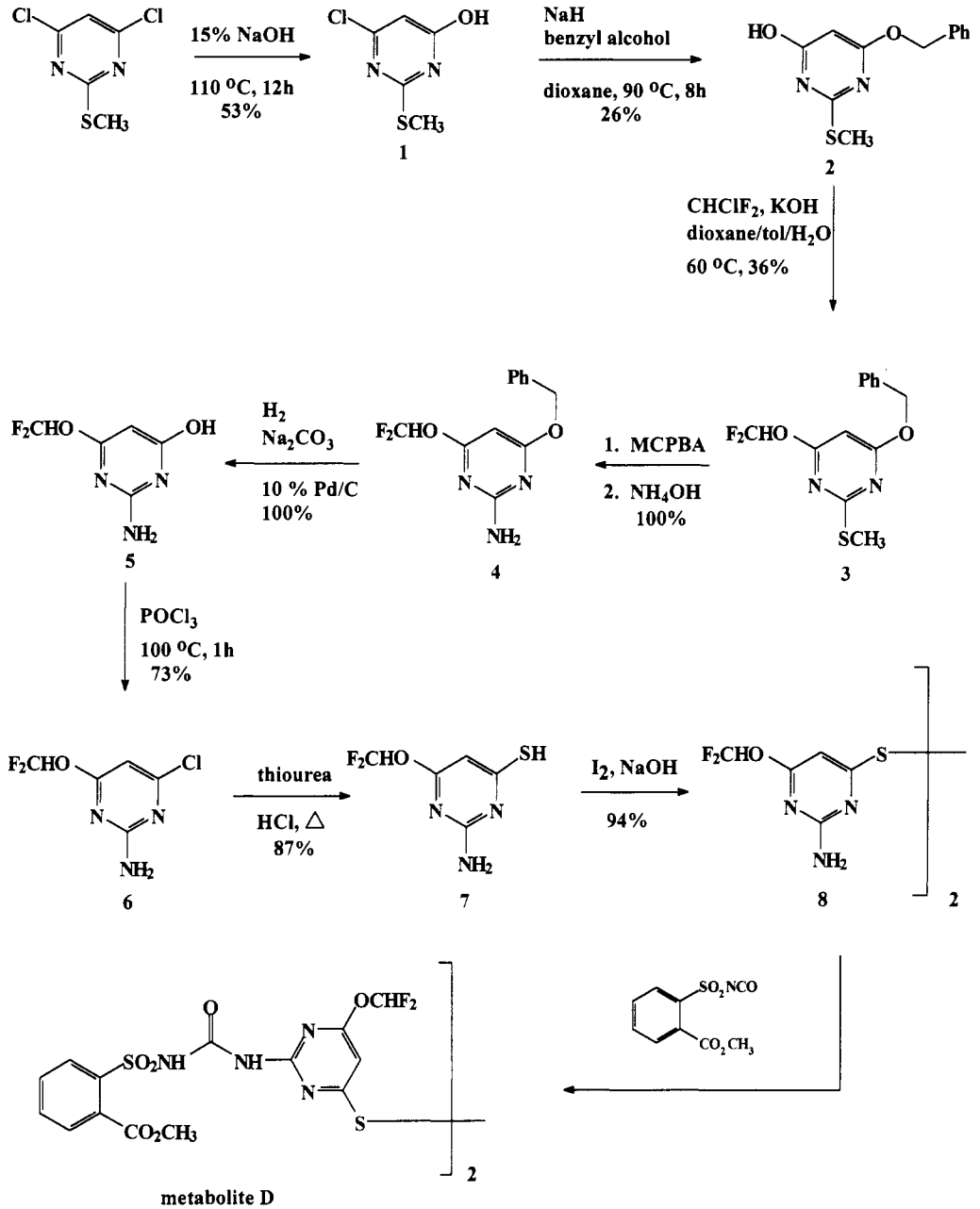


Table I

peak	formula	calcd mass	obsd mass	deviation (mmu)
435	$C_{14}H_{13}O_6N_4S_2F_2$	435.0244	435.0226	4.1 ppm (−1.8)
867	$C_{28}H_{23}O_{12}N_8S_4F_4$	867.0254	867.0242	1.4 ppm (−1.2)

of CGA-136872 (Figure 3). The HPLC profile also showed the presence of saccharin, CGA-120844, and CGA-239769, which were identified in a previous rat study. In this study mice were fed for 28 days a diet containing 10 000 ppm of CGA-136872. After 28, 30, and 32 days, the animals were given a pulse (0.025 mg) of [^{14}C]-CGA-136872 and urine and feces were collected. Analysis of fecal extracts by HPLC at the high feeding level showed the presence of the new metabolite D. Metabolite D was purified by HPLC followed by analysis via ^{1}H NMR, mass spectrometry, and accurate mass measurements.

^{1}H NMR spectra of parent and metabolite D (Figures 1 and 2) were similar in most aspects with one major difference being the downfield shift of the metabolite pyrimidinyl ring proton from δ 6.29 to δ 6.95. The other major difference involved the integration of the metabolite D $OCHF_2$ resonance, δ 7.45, which indicated the presence of only one proton in the metabolite vs two protons in the parent compound, δ 7.56. These data suggest one less difluoromethoxy group on metabolite D than on the parent compound.

FAB analysis of metabolite D (in a magic bullet matrix) produced a spectrum in which the matrix peaks were almost completely suppressed. The base peak at m/z 867 was assigned as the protonated molecular ion, $(M + H)^+$, and was supported by the observation of a signal at 889 $[(M + Na)^+]$. A major fragment was observed at m/z 435 and suggested the possibility of a dimer.

TSP LC/MS showed major positive ions at 385, 233, and 194 and major negative ions at 443, 383, and 192—consistent with the structure of the proposed dimer as its thermal decomposition products.

Accurate mass measurements performed at Research Triangle Institute in Research Triangle Park, NC, showed the mass measurements given in Table I for the two major peaks—results consistent with the elemental formulas of

the protonated molecular ions of the disulfide dimer and the corresponding thiol monomer.

On the basis of the cumulative evidence of mass spectral data, exact mass measurement, and nuclear magnetic resonance spectra data, the unknown mouse fecal metabolite D was postulated to be a disulfide dimer of the parent molecule [CA Index name: benzoic acid, 2,2'-[dithiobis[[[[6-(difluoromethoxy)-4,2-pyrimidinediyl]imino]carbonyl]imino]sulfonyl]bis-, dimethyl ester] with a molecular formula of $C_{28}H_{22}O_{12}N_8S_4F_4$ and a molecular weight of 866.8.

The structure of metabolite D was confirmed to be the disulfide dimer by synthesis of the postulated metabolite. The mass spectral and NMR data were in complete agreement with those obtained from the synthetic standard.

SYNTHESIS

The desired metabolite D, 2,2'-[dithiobis[[[[6-(difluoromethoxy)-4,2-pyrimidinediyl]imino]carbonyl]imino]sulfonylbenzoic acid] methyl ester was prepared in nine steps (Scheme I) from commercially available 4,6-dichloro-2-(methylthio)-pyrimidine. This starting material was treated with sodium hydroxide at 110 °C to afford hydroxypyrimidine 1 (Koppel et al., 1961), which was not characterized but used immediately in the next step of the sequence. Addition of sodium benzylalkoxide afforded the corresponding benzyloxypyrimidine 2 in an overall yield of 14% from the dichloro compound. This compound was reacted with Freon 22 in the presence of base and benzyltrimethylammonium chloride to give 6-(benzyloxy)-4-(difluoromethoxy)-2-(methylthio)pyrimidine (3) in a 36% yield. The thiomethyl substituent was oxidized to the corresponding sulfone with MCPBA and the sulfone then displaced with ammonium hydroxide to give 2-aminopyrimidine 4 (100%). Quantitative removal of the benzyloxy substituent was accomplished via catalytic hydrogenolysis to afford the hydroxyaminopyrimidine 5. This material was chlorinated using phosphorous oxychloride to afford the corresponding chloropyrimidine 6, previously prepared by Meyer (1983), in 73% yield. Conversion of this material to 6-mercaptopyrimidine 7 was accomplished in 87% yield by treatment with thiourea in hydrochloric acid. When treated with iodine and base, this material dimerized to the disulfide 8. Addition of 2-carboxymethylsulfonyl isocyanate to this compound in refluxing dichloromethane provided the desired metabolite D in 74% yield from 7.

In conclusion, the approach to metabolite D presented here allows for its preparation on moderate scale from commercially available starting materials. The overall unoptimized yield for the nine-step synthetic sequence is 3.75%.

EXPERIMENTAL PROCEDURES

General. Unless noted otherwise, reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were uncorrected. Infrared spectra were recorded on a Mattson Instruments Galaxy 2020 FT IR. ^1H, ^{13}C, and ^{19}F NMR were recorded at 400, 100.6, and 376.5 MHz, respectively, on a Bruker AMX-400 NMR spectrometer. Chemical shifts (δ) are expressed in parts per million downfield from internal tetramethylsilane; coupling constants (J) are expressed in hertz. Mass spectra were measured on a Finnigan TSQ 700 instrument using Xe bombardment at 6 kV and 1 mA. Pseudomolecular ions and important fragments are reported in m/z (percent base peak). Elemental analyses were performed by Galbriath Laboratories, Knoxville, TN.

2,2'-[Dithiobis[[[[6-(difluoromethoxy)-4,2-pyrimidinediyl]-imino]carbonyl]imino]sulfonylbenzoic acid] Methyl Ester (Metabolite D). *Analytical Data*: mp 155–158 °C; IR (KBr) 3418–3100, 1732, 1579, 1506, 1454, 1363, 1317, 1060, 758 cm^{-1}; ^1H NMR (CD_3CN) δ 12.0 (bs, 2H), 8.6 (s, 2H), 8.17 (d, J = 8.1, 2H), 7.75 (m, 6H), 7.46 (t, J = 71.2, 2H), 6.93 (s, 2H), 3.92 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 170.6, 170.5, 167.8, 165.8 (t, J = 3.8), 162.0, 141.43, 132.0, 131.2, 130.8, 128.9, 127.5, 113.8 (t, J = 257), 89.5, 52.8; ^{19}F NMR (DMSO-d_6, $CFCl_3$) δ −87.3 (d, J = 71.5); MS (FAB positive and negative spectra in magic bullet matrix (5:1 = dithiothreitol/dithioerythritol) positive, 889 [5, $(M + Na)^+$], 867 [68, $(M + H)^+$], 669 (15), 435 (83), 411 (69), 385 (37), 220 (100), negative 865 [3, $(M - H)^-$], 650 (15), 433 (89), 365 (100), 218 (51). Anal Calcd for $C_{10}H_8N_6O_2F_4S_2$: C, 38.80; H, 2.56; N, 12.81. Found: C, 38.90; H, 2.64; N, 12.54.

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