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Identification of a Novel Glutathione Conjugate of Flutamide in Incubations with Human Liver Microsomes

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Running Title:

A novel glutathione conjugate of flutamide.

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Flutamide, 2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide; GSH, reduced glutathione; GSSG, oxidized glutathione; ESI, electrospray ionization; LC/MS, liquid chromatography/ mass spectrometry; FLU-1, 4-nitro-3-(trifluoromethyl)phenylamine.

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Abstract

Flutamide, a nonsteroidal antiandrogen drug widely used in the treatment of prostate cancer, has been associated with rare incidences of hepatotoxicity in patients. It is believed that bioactivation of flutamide and subsequent covalent binding to cellular proteins is responsible for its toxicity. Current in vitro studies were undertaken to probe the P450-mediated bioactivation of flutamide and identify the possible reactive species using reduced glutathione (GSH) as a trapping agent. NADPH- and GSH-supplemented human liver microsomal incubations of flutamide gave rise to a novel GSH conjugate where GSH moiety was conjugated to the flutamide molecule via the amide nitrogen, resulting in a sulfenamide. The structure of the conjugate was characterized by LC-MS/MS and NMR experiments. The conjugate formation was primarily catalyzed by heterologously expressed CYP2C19, CYP1A2, and to a lesser extent by CYP3A4 and CYP3A5. The mechanism for the formation of this conjugate is unknown; however, a tentative bioactivation mechanism involving a P450-catalyzed abstraction of hydrogen atom from the amide nitrogen of flutamide and the subsequent trapping of the nitrogen-centered radical by GSH or GSSG was proposed. Interestingly, the same adduct was formed when flutamide was incubated with human liver microsomes in the presence of GSSG and NADPH. This suggests that P450-mediated oxidation of flutamide via a nitrogen-centered free radical could be one of the several bioactivation pathways of flutamide. Even though the relationship of the GSH conjugate to flutamide-induced toxicity is unknown, the results have revealed the formation of a novel, hitherto unknown, GSH adduct of flutamide.

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Introduction

Flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide, Scheme 1) is a nonsteroidal antiandrogen drug that is widely used for the treatment of prostate cancer. When used in combination with luteinizing hormone-releasing agonists or orchiectomy, flutamide has been shown to increase survival time of prostate cancer patients (Brogden and Clissold, 1989; McLeod 1993, Schmitt et al. 2001). However, the therapeutic effects of flutamide have been overshadowed by reports of temporary increases in transaminase markers and rare incidences of severe liver dysfunction (Wysowski and Fourscroy, 1996, Osculati and Castiglioni, 2006, Gomez et al., 1992, Cetin et al., 1999, Nakagawa et al., 1999). Some cases have been associated with blood eosinophilia (Hart and Stricker, 1989), which indicates an immune-mediated mechanism in some patients.

Investigations into the disposition of flutamide indicate that it is rapidly absorbed from the gastrointestinal tract in humans and rats (Schulz et al. 1988; Zuo et al. 2002; Katchen and Buxbaum 1975). Maximum plasma concentrations up to 20 ng/mL have been attained in humans within 1 hr of oral administration of a single dose of 250 mg dose. However, administration of repeated 250 mg doses of flutamide resulted in the mean maximum plasma concentrations of 130 ng/mL. Flutamide undergoes extensive hepatic first-pass metabolism in humans and rats. The primary route of metabolism is the CYP1A2-mediated oxidation to 2-hydroxyflutamide although the formation of other oxidative metabolites and the involvement of CYP1A1 and CYP1B1 in the metabolism of flutamide have also been reported (Schulz et al. 1988, Shet et al. 1997, Watanabe et

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al. 2001; Katchen and Buxbaum 1975). 2-Hydroxyflutamide (M1, Scheme 1) appears to be largely responsible for the anti-androgenic activity of flutamide. In addition to oxidative metabolism, the esterase-catalyzed 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1) metabolite has also been detected in human plasma (Schulz et al. 1988). More recently, a new N-oxidized metabolite of flutamide, N-[4-nitro-3-(trifluoromethyl)phenyl]hydroxylamine (possibly derived from FLU-1) and its glucuronide, have been identified in the urine of prostate cancer patients (Tevell et al. 2006; Goda et al. 2006). Interestingly, reduction to a 4-amino derivative appears to be a minor pathway in the metabolism of flutamide in humans and preclinical species.

The mechanisms of flutamide-induced hepatotoxicity have not been precisely elucidated. Although nitro groups are generally implicated in various toxicities via reduction to an hydroxylamine or nitroso functionalities, studies with flutamide indicate that it is not detectably reduced by microsomal NADPH-cytochrome P450 reductase (Berson et al. 1993). In contrast, oxidative bioactivation to a reactive metabolite is believed to be the cause of flutamide-induced toxicities. Studies by Berson et al. and Fau et al. suggest that flutamide undergoes a CYP3A4- and CYP1A2-catalyzed metabolism to reactive metabolite(s) that are covalently bound to hepatic proteins (Berson 1993, Fau 1994). Ichimura and co-workers have also illustrated the necessity of enhanced flutamide metabolism for development of severe hepatotoxicity (Ichimura et al. 1999). More recently, Matsuzaki and co-workers have demonstrated the flutamide-induced toxicity after dosing flutamide to CYP1A2 knockout SV129 mice (Matsuzaki et al. 2006).

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Although several mechanistic studies have been performed to prove the involvement of metabolic activation in flutamide-induced hepatotoxicity, the nature of the reactive metabolite has yet to be identified. In vitro conjugation with GSH is a widely used method in the characterization of reactive metabolites and in probing the mechanism of bioactivation (Samuel et al 2003). Recently, Soglia and co-workers have detected a GSH conjugate of hydroxylated flutamide in human liver microsomal incubations (Soglia et al. 2006). Similarly, Tevell and co-workers (Tevell et al. 2006) have also detected a mercapturic acid conjugate of hydroxylated flutamide in the urine of prostate cancer patients. However, no definitive structural information of the conjugates or a proposed mechanism of bioactivation was presented in these reports. The present study was conducted to further probe the bioactivation of flutamide using GSH as a trapping agent and it has revealed the formation of a novel GSH conjugate in the incubations with human liver microsomes as well as heterologously expressed P450 isoforms. A tentative bioactivation mechanism was also proposed for the formation of this GSH conjugate.

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Materials and Methods

Materials. Flutamide and reduced GSH were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes were prepared from human livers (International Institute for the Advancement of Medicine, Jessup, PA) using standard protocols and were characterized using P450-specific marker substrate activities. Aliquots from the individual preparations from 56 individual human livers were pooled on the basis of equivalent protein concentrations to yield a representative microsomal pool with a protein concentration of 20.4 mg/ml. Recombinant P450 isozymes CYP1A2, 3A4, 2C8, 2C9, 2C19, and 2D6 supersomes were obtained from BD Gentest (Woburn, MA). Respectively the protein concentration and P450 content of the recombinant enzymes were 4.5 mg/mL and 1 nmol/mL for CYP1A2; 7.6 mg/mL and 1 nmol/mL for CYP3A4; 4.9 mg/mL and 2 nmol/mL for CYP3A5; 3.2 mg/mL and 1 nmol/mL for CYP2C8; 2.6 mg/mL and 2 nmol/mL for CYP2C9; 6.9 mg/mL and 1 nmol/mL for CYP2C19; 8.3 mg/mL and 1 nmol/mL for CYP2D6. All other commercially available reagents and solvents were of either analytical or HPLC grade.

Incubation of flutamide with human liver microsomes. Flutamide (5 μM to 100 μM) was incubated for 1 h at 37 °C in an incubation system consisting of 100 mM potassium phosphate buffer (pH 7.4), 2 mg human liver microsomes and 5 mM GSH in a final volume of 1 mL. After 3 min preincubation, incubations were initiated by the addition of 1 mM NADPH. Reactions were terminated by the addition of 5 mL acetonitrile. Samples were mixed on a vortex mixer and centrifuged for 5 min. The supernatants were transferred into conical glass tubes for evaporation to dryness under N_2 at 30 °C. The

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residues were reconstituted in 200 μL of 30:70 (v/v) methanol:20 mM ammonium acetate (pH 4) and aliquots (100 μL) were injected into an HPLC-MS system. Metabolite profiling was performed on an Agilent 1100 HPLC system (Wilmington, DE) coupled with a Finnigan LCQ-Deca ion-trap mass spectrometer (San Jose, CA). Separation was achieved using a kromasil C4 100A column (3.5 μm, 150 x 2.0 mm, Phenomenex, Torrance, CA) at a flow rate of 0.2 mL/min. A gradient of (A) water with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid was as follows: initiated with 1 % B for 5 min and then increased in a linear manner to 30% at 20 min and to 50% at 25 min, held at 50% until 28 min, changed linearly to 90% at 40 min, maintained at 90% for up to 43 min and then decreased to 1% at 45 min. The column was allowed to equilibrate at 1% solvent B for 5 min prior to the next injection. The HPLC effluent going to the mass spectrometer was directed to waste through a divert valve for the initial 5 min after sample injection. Major operating parameters for the ion-trap ESI-MS method are shown as follows: capillary temperature 270 °C; spray voltage 5.0 kV; capillary voltage -14 V; sheath gas flow rate 90 (arbitrary units) and auxillary gas flow rate 30 (arbitrary units). The mass spectrometer was operated in a negative ion mode with data-dependent scanning. The ions were monitored over a full mass range of *m/z* 125-1000. For a full scan, the automatic gain control was set at 5.0×10^8, maximum ion time was 100 ms and the number of microscans was set at 3. For MS^n scanning, the automatic gain control was set at 1.0×10^8, maximum ion time was 400 ms and the number of microscans was set at 2. For data dependent scanning, the default charge-state was 1, default isolation width was 3.0 mass units, and normalized collision energy was 45.0%.

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Incubations with cDNA-Expressed Human P450 Enzymes. Flutamide (50 μM) was incubated for 1 h at 37 °C in an incubation system consisting of 100 mM potassium phosphate buffer (pH 7.4), recombinant P450 CYP1A2, CYP3A4, CYP2C8, CYP2C9, CYP2C19, or CYP2D6 supersomes (50 pmol), 5 mM GSH, and 1 mM NADPH in a final volume of 0.5 mL. After a 3-min preincubation, incubations were initiated by the addition of 1 mM NADPH. Reactions were terminated by the addition of 1 mL acetonitrile. Nilutamide was added as an internal standard. Formation of the glutathionyl adduct was quantified by Shimadzu LC-10AD VP binary pumps (Columbia, MD, UDA) coupled with a Q-Trap 4000 (Applies Biosystems/MSD Sciex, Concord, ON, Canada). The adduct was separated by a kromasil C4 100A column (3.5 mm, 150 x 2.0 mm, Phenomenex, Torrance, CA) at a flow rate of 0.2 mL/min. A gradient of (A) water with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid was as follows: initiated with 0% B for 3 min and then increased in a linear manner to 90% at 15 min and then decreased to 0% at 17 min. The column was allowed to equilibrate at 0% solvent B for 3 min prior to the next injection. The HPLC effluent going to the mass spectrometer was directed to waste through a divert valve for the initial 3 min after sample injection. The Q-trap 4000 ESI-MS was operated in the negative ionization mode, by applying to the capillary a voltage (IS) of -4.5 kV. Nitrogen was used as curtain gas (CUR), as well as nebulizing (GS1) and turbo spray gas (GS2, heated at 450 °C), with the optimum values set, respectively, at 36, 50, and 40 (arbitrary values). Collisionally activated dissociation (CAD) was performed at 6 (arbitrary value) with nitrogen as the collision gas. Declustering potential (DP) was set at -90 V, whereas entrance potential (EP) was set at -10 V; collision energy (CE) was optimized at -34 eV. The MRM transitions used

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were 580→307 for the glutathionyl adduct M2 and 316→205 for internal standard nilutamide, respectively. The amounts of M7 produced by CYPs were represented by the ratio of peak areas of M7 to that of internal standard, nilutamide. The CYP-mediated formation of M7 was normalized to CYP2C19.

Isolation of GSH Adduct and NMR Characterization. Rat liver microsomes were used to scale up the formation and isolation of the GSH conjugate. The incubation with flutamide was carried out in a final volume of 10 mL. The incubation conditions were similar to those described above. The corresponding GSH conjugate was isolated with the LC conditions as follows. Separation was achieved using a COSMOSIL 5PYE column (150 x 4.6 mm, Waters, Milford, MA) at a flow rate of 1.0 mL/min with an Agilent 1100 HPLC system (Wilmington, DE). A gradient of (A) water with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid was as follows: initiated with 100% A for 5 min, changed to 80% A from 5 to 10 min, changed to 50% A from 10 to 60 min, change to 10% A from 60 to 70 min, held at 10% A from 70 to 75 min, changed to 100% A from 75 to 76 min, and held at 100% A from 76 to 80 min for the column to be equilibrated. Approximately 6 µg of the material was isolated from the incubation mixture. All NMR spectra were acquired on a Bruker-Biospin AV700 spectrometer running TopSpin 1.3 software and equipped with a Bruker 5-mm TCI z-gradient Cryoprobe (Bruker, Rheinstetten, Germany). ^{1}H NMR spectra were acquired with water suppression using a Watergate W5 pulse sequence with gradients and a double echo. 2D COSY and HSQC spectra were acquired without solvent suppression using gradient pulses for coherence selection. Chemical shifts are reported in ppm relative to tetramethylsilane.

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Results

Incubation of flutamide with human liver microsomes in the presence of NADPH and reduced GSH yielded 8 peaks in the UV chromatogram as shown in (Figure 1). LC-MS analysis of the incubation mixture indicated the formation of mono- and di- hydroxylated metabolites of flutamide (Scheme 1). The peaks at retention time of 30 and 32 min (M5 and M1) gave a molecular ion $[M-H]^-$ at *m/z* 291 which corresponded to the monohydroxyflutamide. Further mass spectral analysis of these two peaks using MS/MS and MS^n mode in the ion trap indicated that the isopropyl group was the primary site of hydroxylation in both metabolites. The site of hydroxylation in M1 was further confirmed by ^{1}H-NMR spectroscopy (data not shown) and indicated that it was the 2-hydroxyflutamide metabolite (Scheme 1). This result was in concordance with previous reports which suggested that 2-hydroxyflutamide is a primary circulating metabolite in humans and preclinical species (Schulz et al. 1988; Shet et al. 1997). The position of the hydroxyl group in M5 could not be confirmed by mass spectral analysis; however the metabolite was presumably the hydroxymethylflutamide from hydroxylation of the terminal methyl of the isopropyl group (Scheme 1). Metabolites M3 and M6 (Figure 1) gave molecular ions at $[M-H]^-$ at *m/z* 305 and 307, respectively. This suggested an addition of 30 and 32 amu, respectively, to *m/z* 275 (the molecular ion $[M-H]^-$ of flutamide). The mass spectral analysis was consistent with the formation of the carboxylic acid (M3) and a dihydroxyflutamide metabolite (M6) as shown in Scheme 1. The molecular ions of metabolite M2 and M4 at *m/z* 205 and 221, respectively, were consistent with hydrolyzed product, 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), and its hydroxylated metabolite observed previously (Schulz et al. 1988, Tevell et al. 2006).

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Formation and Characterization of GSH Conjugate of Flutamide (M7): LC-MS/MS analysis of the peak M7 at retention time of 27.3 min displayed a molecular ion $[M-H]^-$ at *m/z* 580 and indicated an addition of 305 amu (addition of one molecule of GSH) to the molecular ion of flutamide (Figure 2A). The peak was absent in incubations that did not contain NADPH suggesting that its formation was P450-dependent (Figure 2B). The mass spectrum (MS^2) of M7 at *m/*z 580 (Figure 3A) showed a fragment ion at *m/*z 451 resulting from a loss of 129 amu corresponding to pyroglutamic acid. This suggested that M7 was a GSH conjugate of flutamide. Other major fragment ions in the mass spectrum (Figure 3A) were observed at *m/z* 307, 306 and 272. The fragment ions at *m/z* 306 and 272 resulted from the cleavage of the GSH moiety while the ion at *m/z* 307 was derived from the cleavage of the carbon-sulfur bond, as shown in Figure 3A. Further fragmentation of *m/z* 307, obtained by data dependent scanning (Figure 3B), resulted in ions at *m/z* 273 and 205. While *m/z* 273 was produced by the loss of H_2S moiety, the fragment ion at *m/z* 205 was generated by subsequent loss of the isobutyryl moiety, as shown in Figure 3B. This mass spectral data suggested that the GSH moiety was conjugated to the flutamide molecule via the amide nitrogen, resulting in a sulfenamide, and not on the aromatic ring or the isopropyl group of flutamide.

The proposed structure of M7 was further confirmed by NMR experiments to obtain more definitive structural information on the GSH conjugate. Since M7 was formed in larger amounts in rat liver microsomal incubations with flutamide (data not shown), rat liver microsomes were used to produce sufficient amounts of M7 for NMR experiments.

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The presence of glutathionyl moiety in M7 was confirmed by the appearance of proton signals between 2.0 and 4.5 ppm in ^{1}H NMR (Figure 4). The presence of resonances at 8.30 (d, 1H, J = 1.82 Hz), 8.07 (doublet of doublet, 1H, J = 9.01 and 1.82 Hz) and 8.04 (d, 1H, J = 9.01 Hz) ppm, corresponding to the aromatic protons H3, H5 and H6, respectively, and those at 2.84 (m, 1H, J = 6.84 Hz) and 1.84 (d, 6 H, J = 6.84Hz) corresponding to the protons H29, H37 and H30, clearly suggested that the aromatic ring and the isopropyl group were not modified. Furthermore, the differences in carbon-13 chemical shifts, proton chemical shifts and coupling constants were minimal between flutamide and M7 (Table 1), suggesting no major modification of the chemical environment of flutamide when the GSH conjugate was formed. The ^{1}H-^{1}H COSY (Figure 5) experiment with M7 showed coupling of the methine proton H29 at 2.66 ppm with the protons of the two methyl groups H30 and H37 at 1.22 ppm and allowed further confirmation of the structure.

Enzymes Involved in the Formation of GSH Conjugate M7. Incubation of flutamide with human liver microsomes in the presence of GSH, NADPH and specific chemical inhibitors of P450s indicated that furafylline strongly inhibited the formation of M7, suggesting that CYP 1A2 plays a major role in the oxidation of flutamide to M7 (data not shown). However, incubation of flutamide with recombinant enzymes indicated that in addition to CYP1A2, CYP2C19 is another major P450 isozyme catalyzing the formation of M7. CYP3A4 and 3A5 played a minor role in the bioactivation pathway leading to the observed adduct (Figure 6).

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Formation of G1. Incubation of flutamide with human liver microsomes in the presence of NADPH and reduced GSH also yielded a peak (G1) at 26.9 min with a molecular ion ($[M-H]^-$) of *m/z* 451 (Figure 1). The peak was absent in incubations that lacked NADPH (Figure 7A and B). MS analysis of *m/z* 451 indicated that it was 129 amu less than the molecular ion of M7 (*m/z* 580), suggesting that G1 was most probably derived from M7 (following the loss of the glutamate moiety). The MS/MS spectrum of G1 at *m/z* 451 showed a major fragment ion at *m/z* 307 that was the same as the fragment ion observed in the mass spectrum of M7 and was possibly formed by cleavage of the sulfur-carbon bond of the adduct (Figure 8A). Furthermore the MS/MS spectrum of *m/z* 307 in the data dependent scanning mode (Figure 8B), gave fragment ions that were the same as those observed in the MS^3 spectrum of M7 (Figure 3B). This data suggested that G1 was probably formed following hydrolysis of the glutamate moiety from M7.

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Discussion

In the present study, a novel GSH conjugate of flutamide was identified following human liver microsomal incubations of flutamide in the presence of NADPH and GSH. The structure of the conjugate was unambiguously determined by LC-MS/MS and NMR analyses. These experiments suggested attachment of GSH moiety to the amide nitrogen of flutamide via the N-S bond formation, thus forming a novel sulfenamide derivative. Sulfenamides are compounds containing trivalent nitrogen bonded to divalent sulfur (Craine and Raban, 1989). Several synthetic routes to sulfenamides have been developed over the years; however, information on the enzyme-catalyzed formation of these derivatives is relatively sparse. Mulder and Gallemann have previously reported the formation of a sulfenamide derivative of GSH. This conjugate was formed by reaction of 4-nitrosophenetole, derived from the oxidative metabolism of phenacetin via N-hydroxy-p-phenetidine, and GSH (Mulder et al. 1984; Gallemann et al 1998). However, the formation of N-acylsulfenamide, similar to the one described in this report, is not known. In the present study, the conjugate M7 was stable enough to be isolated and characterized by NMR. This was consistent with literature reports which have indicated modest stability of N-GSH conjugates in water (Mulder et al. 1984).

Since previous studies have reported the oxidative bioactivation of flutamide to chemically reactive intermediates, all incubations in this study were performed under aerobic conditions. The NADPH-dependent GSH conjugate formation indicated that one or more P450s were involved in the production of the reactive intermediate by human liver microsomes. Experiments with bacculovirus-expressed recombinant P450

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enzymes revealed that both P450 2C19 and 1A2 were involved in the formation of M7 when GSH was present in the incubation mixtures. CYP 3A4 and 3A5 were also able to catalyze the oxidation of flutamide to yield M7, but to a lesser degree. The CYP1A2 and 3A4-mediated formation of M7 was consistent with the previous reports that suggested involvement of these two enzymes in the bioactivation of flutamide (Berson et al. 1993; Matsuzaki et al 2006). Recent reports on metabolism of flutamide to hydroxyflutamide (M1) have demonstrated the involvement of CYP2C19 in its formation (Goda et al., 2006); however, the involvement of 2C19 in the bioactivation of flutamide has not been previously reported.

The relevance of this conjugate to hepatotoxicity following oral administration of flutamide to humans is not clear. Even though detailed kinetic studies were not performed on the formation of M7 in liver microsomes, preliminary concentration-dependent incubations of flutamide with NADPH and GSH supplemented human liver microsomes suggested that M7 was observed only at > 5 μM concentrations of flutamide (data not shown). The above assessments were qualitative and were made by comparing the peak areas of M7 in the extracted ion chromatogram of the MS at *m/z* 580. Although the maximum total plasma concentration of flutamide in patients after repeated doses at 250 mg (t.i.d.) is approximately 0.5 μM (free concentrations of 0.03 μM), the liver concentrations could be significantly higher at this dose. Also, given that flutamide undergoes extensive hepatic first pass metabolism, the importance of this bioactivation pathway cannot be completely ignored.

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The mechanism for formation of M7 is unknown at this time. However, several mechanisms that result in conjugation of GSH and flutamide can be speculated. Reports on the chemistry of sulfenamides have indicated that aromatic thiols can react with amines in the presence of oxygen and a metal catalyst to result in the corresponding sulfenamides possibly via a radical mechanism (Craine and Raban, 1989). Additionally, syntheses of sulfenamides from disulfides and amines have also been suggested. Since M7 formation is P450-catalyzed, the first step could involve generation of a nitrogen-centered radical **1**, via hydrogen atom abstraction (HAT) from the amide moiety of flutamide (Scheme 2). It is possible that the nitro group at the 4-position can possibly stabilize radical **1** by delocalization. P450-mediated oxidation of amides via hydrogen atom abstraction from NH to form nitrogen centered radicals is well known (Testa 1995). Generally, this intermediate further undergoes combination with hydroxyl radicals to form N-hydroxylamide. However, the N-hydroxyflutamide (**2**) was not detected in incubations of flutamide with human liver microsomes in our study. Although speculative, one pathway for the formation of M7 could involve the combination of radical **1** with the thiolate anion of GSH (that is in equilibrium with GSH) to give the corresponding thiolate anion radical **3** which is then oxidized to M7 (pathway A, Scheme 2). Thiolate anion radicals have been previously proposed as intermediates during formation of GSH disulfide (GSSG) (Buettner, 1993). Alternatively, **1** could recombine with GSH radical to yield M7 (pathway B, Scheme 2). Interestingly, incubation of flutamide with human liver microsomes in the presence of NADPH and oxidized glutathione (GSSG) also resulted in the formation of M7 (data not shown). It is known that glutathione reductase in the presence of NADPH reduces GSSG to GSH

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which could trap the proposed intermediate **1** to form M7. Although this experiment does not prove the mechanism for the formation of M7 it suggested that pathway B illustrated in Scheme 2 could be one of the ways to form M7. Previous reports have shown a decrease in the GSH and thiol content and an increase in the GSSG/GSH ratio following incubation of flutamide with isolated rat hepatocytes (Fau D 1994). It is possible that the reaction is mediated by the nitrogen-centered radical proposed in this study which can oxidize GSH or thiol containing proteins.

Microsomal incubation of flutamide in the presence of GSH and NADPH also resulted in the formation of G1, a deglutamyl derivative of M7. It is known that GSH conjugates formed in vivo undergo γ-glutamyl-transpeptidase catalyzed hydrolysis to form adducts similar to G1 (Horvath, 1992; Meister and Tate, 1980, Horiuchi et al. 1978). However, such hydrolytic cleavages are rarely observed within in vitro microsomal incubations. Since this was a minor metabolite in the incubation mixture, no attempt was made to further characterize G1 in this study.

In summary, a novel GSH conjugate of flutamide was identified and characterized in this study. Although the relevance of this pathway in flutamide-induced toxicity is not known, the P450-mediated formation of this conjugate suggests that the proposed radical formation could possibly be one of the several bioactivation pathways by which flutamide induces its hepatotoxicity.

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Legends for Figures

Figure 1. UV chromatogram (306 nm) of flutamide and its metabolites following incubation of flutamide with human liver microsomes supplemented with NADPH and reduced GSH.

Figure 2. Extracted ion chromatograms (XIC) of M7 at *m/z* 580 ($[M-H]^-$) in human liver microsomal incubations, A) with NADPH and GSH, and B) with GSH but without NADPH.

Figure 3. (A) MS/MS spectrum of the glutathione conjugate of flutamide M7, at *m/z* 580 ($[M-H]^-$). B) MS^3 mass spectrum of M7 (the fragment ion at *m/z* 307) in data-dependent scanning mode on an ion trap mass spectrometer.

The origins of the characteristic ions are indicated.

Figure 4. 1H NMR spectrum of the glutathione conjugate of flutamide (M7).

The insert is the expanded region of the aromatic group. The spectrum was obtained by dissolving the isolated M7 in deuterated methanol.

Figure 5. Expanded region of the chemical shifts exhibiting the glutathionyl and isopropyl groups in the $^1H^1H$-COSY spectrum of M7.

Figure 6. The relative formation of the glutathione conjugate M7 mediated by heterologously expressed P450 isoforms CYP1A2, CYP3A4, CYP3A5, CYP2C8, CYP2C9, CYP2C19, and CYP2D6. The amounts of M7 produced by CYPs were

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represented by the ratio of peak areas of M7 to that of internal standard, nilutamide. The CYP-mediated formation of M7 was normalized to CYP2C19.

Figure 7. Extracted ion chromatograms (XIC) of G1 at *m/z* 451 ($[M-H]^-$) in human liver microsomal incubations. A) with NADPH and GSH, and B) with GSH but without NADPH.

Figure 8. A) MS/MS spectrum of the adduct G1, at *m/z* 451 ($[M-H]^-$). B) MS^3 mass spectrum of G1 (the fragment ion at *m/z* 307) in data-dependent scanning mode on an ion trap mass spectrometer.

The origins of the characteristic ions are indicated.

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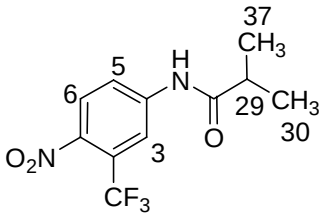
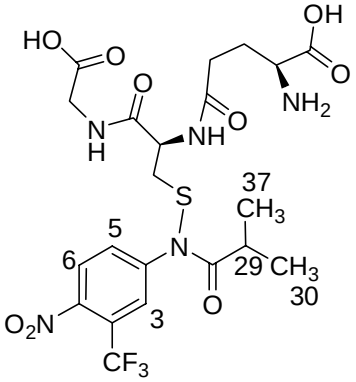
Table 1. ^{1}H and ^{13}C NMR data for flutamide and flutamide-GSH adduct M7





	Proton chemical shift (ppm)	J coupling constant (Hz)	Carbon-13 chemical shift (ppm)	Proton chemical shift (ppm)	J coupling constant (Hz)	Carbon-13 chemical shift (ppm)
3	**8.25**	**-**	**118.7**	**8.30**	**1.82 (d)**	**117.5**
5	**8.03**	**-**	**122.4**	**8.07**	**9.01, 1.82 (dd)**	**122.2**
6	**8.03**	**-**	**127.7**	**8.04**	**9.01 (d)**	**126.7**
29	**2.66**	**6.85 (m)**	**37.1**	**2.84**	**6.84 (m)**	**41.6**
37	**1.22**	**6.85 (d)**	**19.6**	**1.28**	**6.84 (d)**	**16.0**

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30	**1.21**	**6.85 (d)**	**19.6**	**1.28**	**6.84 (d)**	**16.0**

Multiplicity of proton signals: d, doublet; dd, doublet of doublet; m, multiplet;

^{13}C chemical shifts were extracted from HSQC spectra of flutamide and M7.

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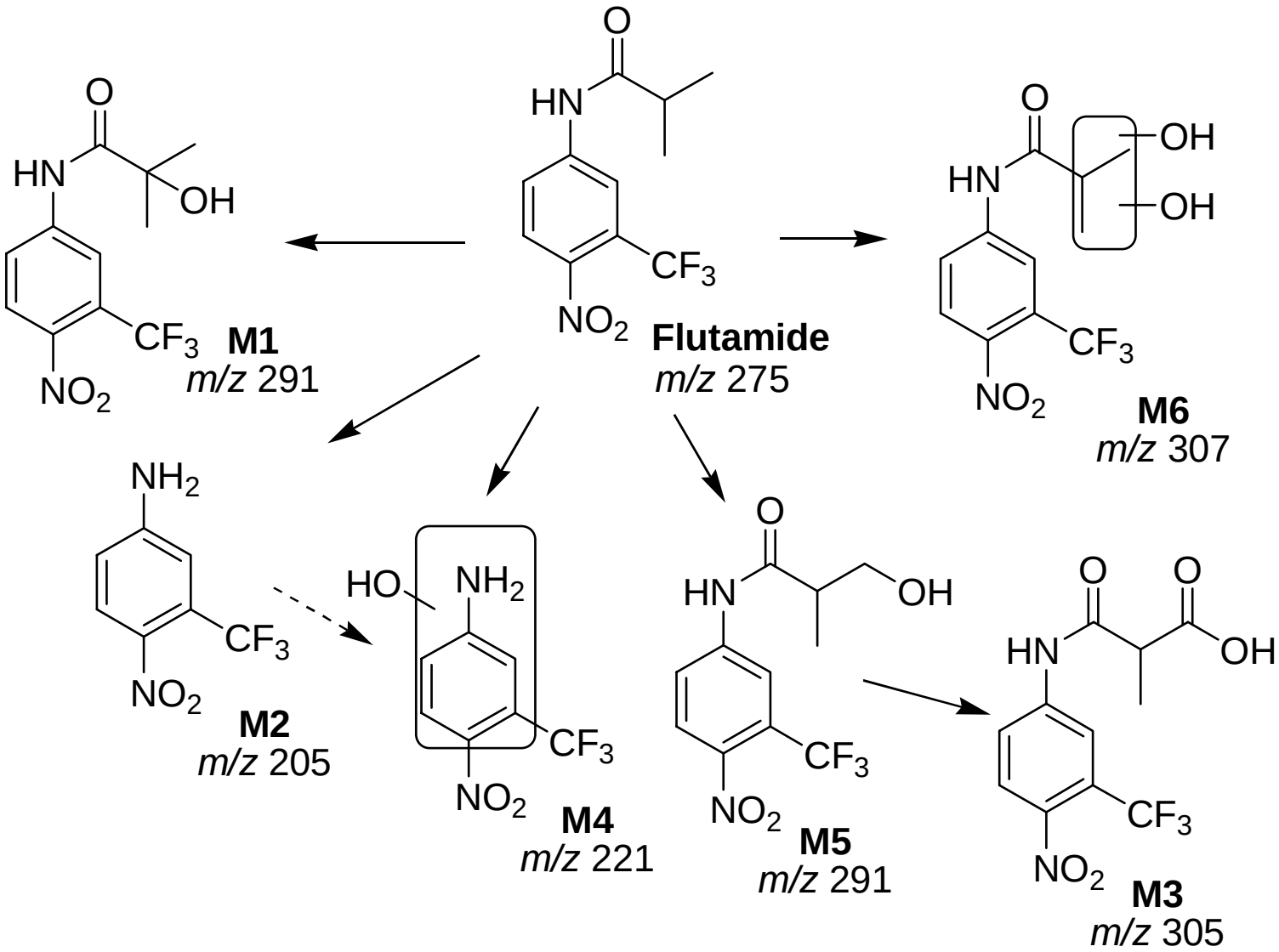
Scheme 1. Proposed metabolic scheme of flutamide following incubation with human liver microsomes in the presence of NADPH and reduced GSH.



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Scheme 2. Proposed mechanism for the formation of N-(glutathio-S-yl) flutamide M7 from flutamide in human liver microsomal incubations supplemented with NADPH and GSH or GSSG.

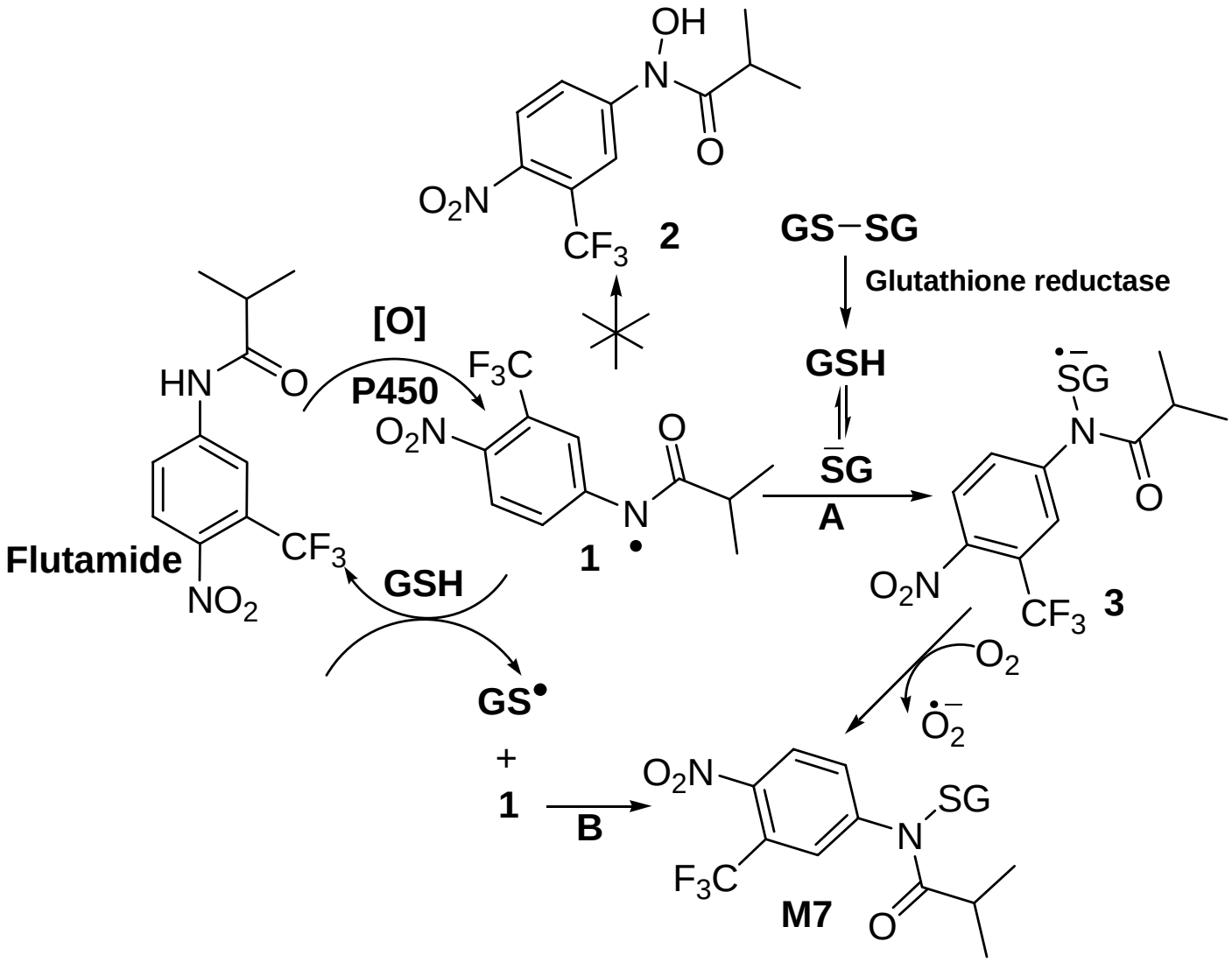

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Figure 1

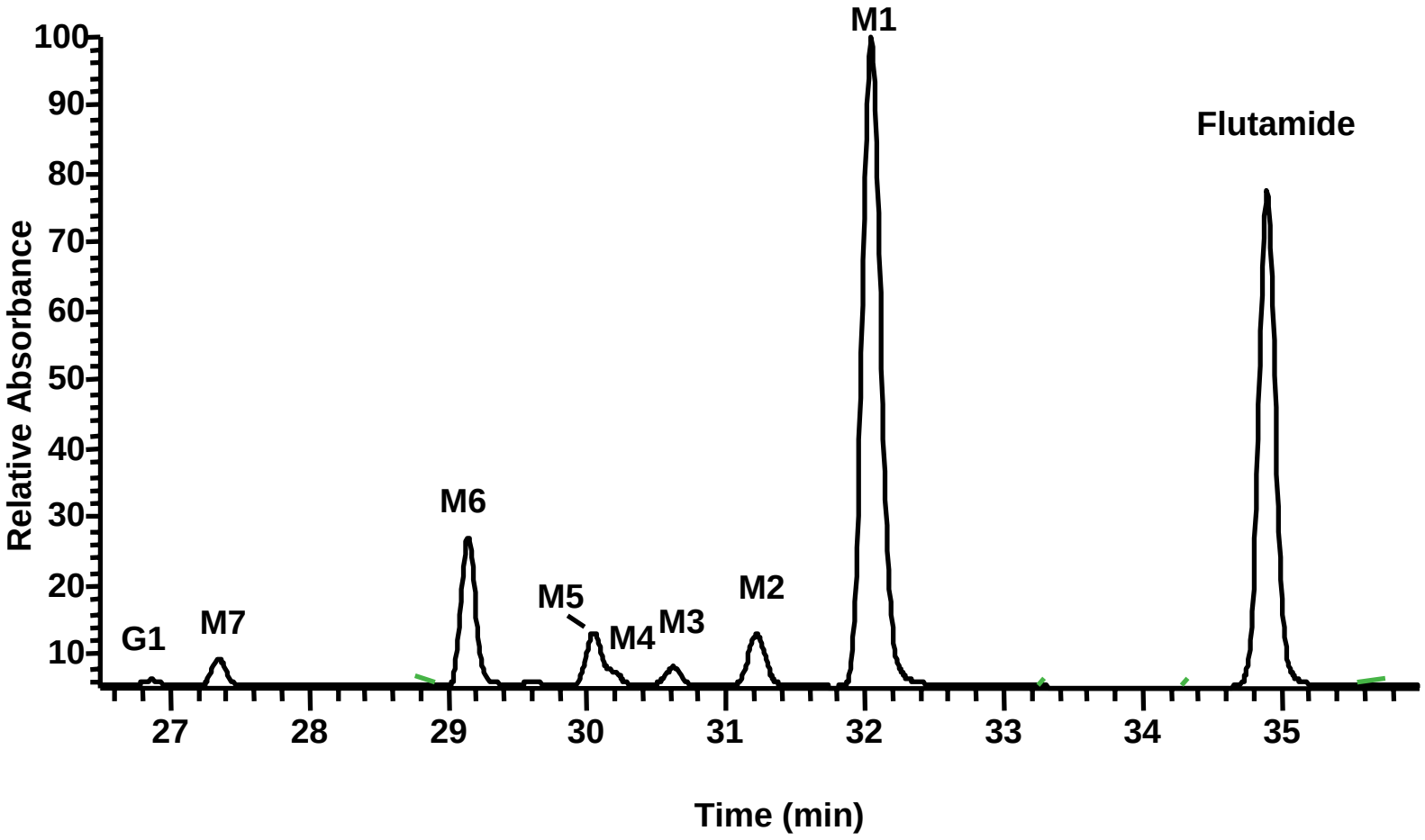

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Figure 2

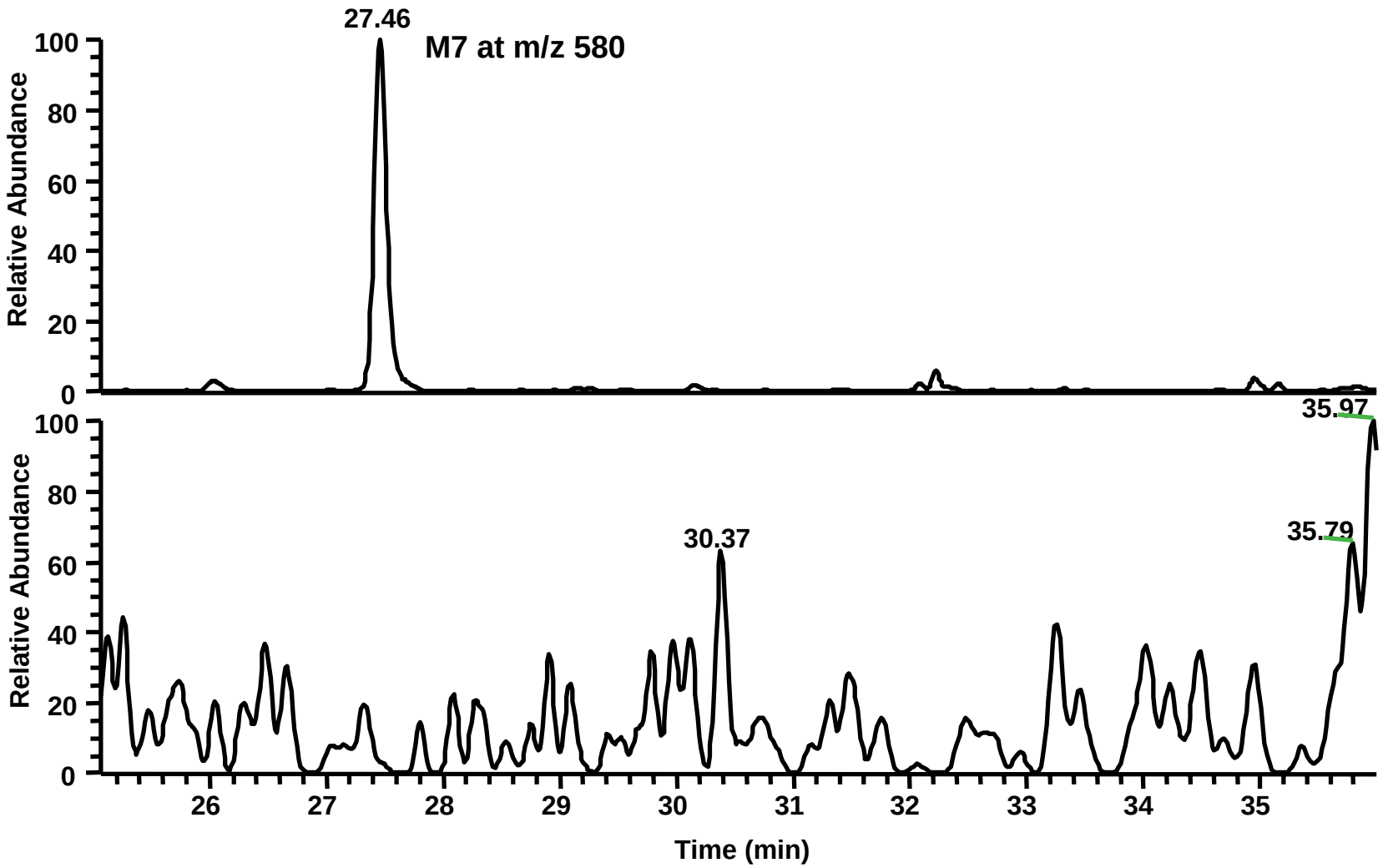

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Figure 3

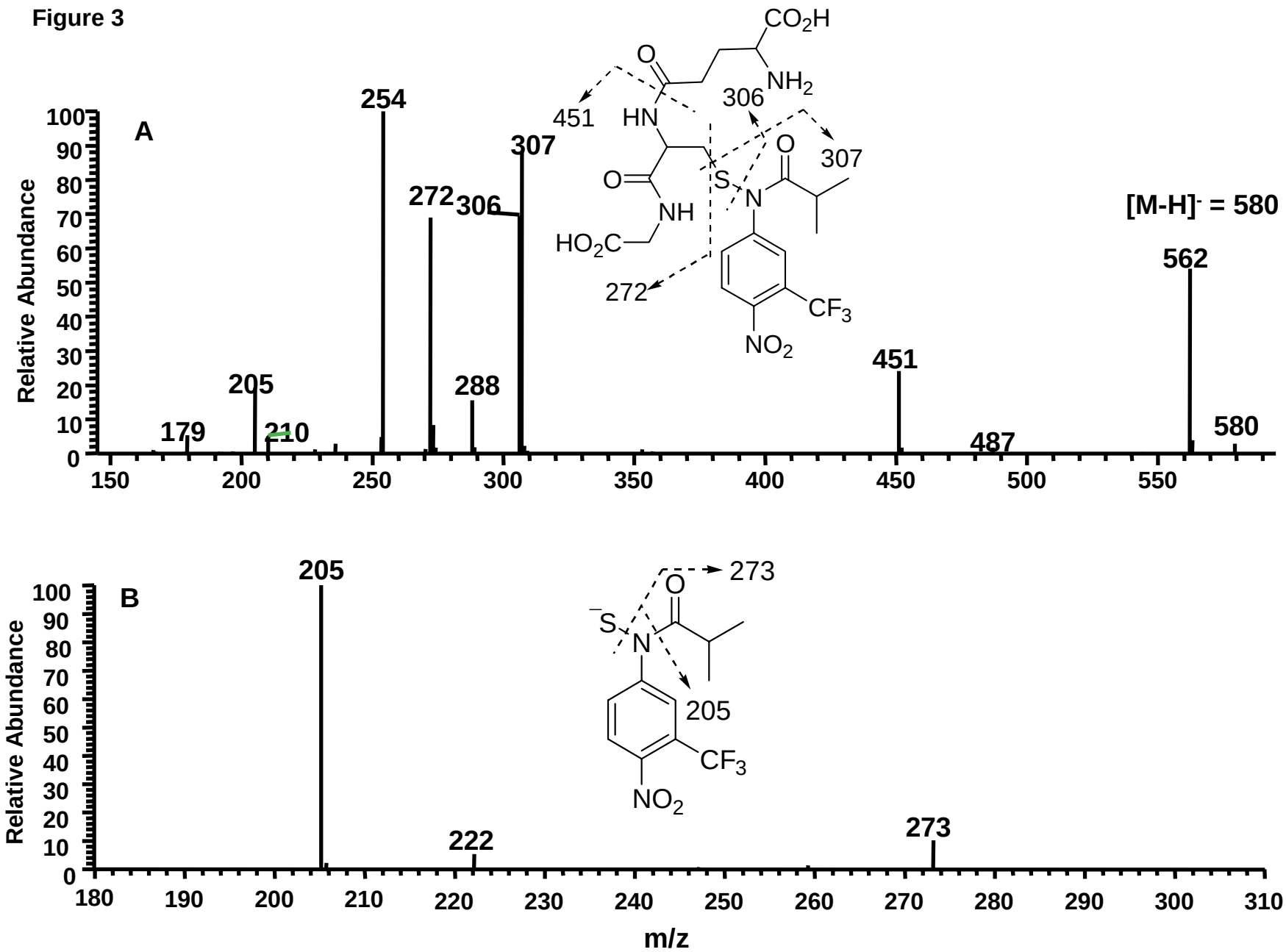

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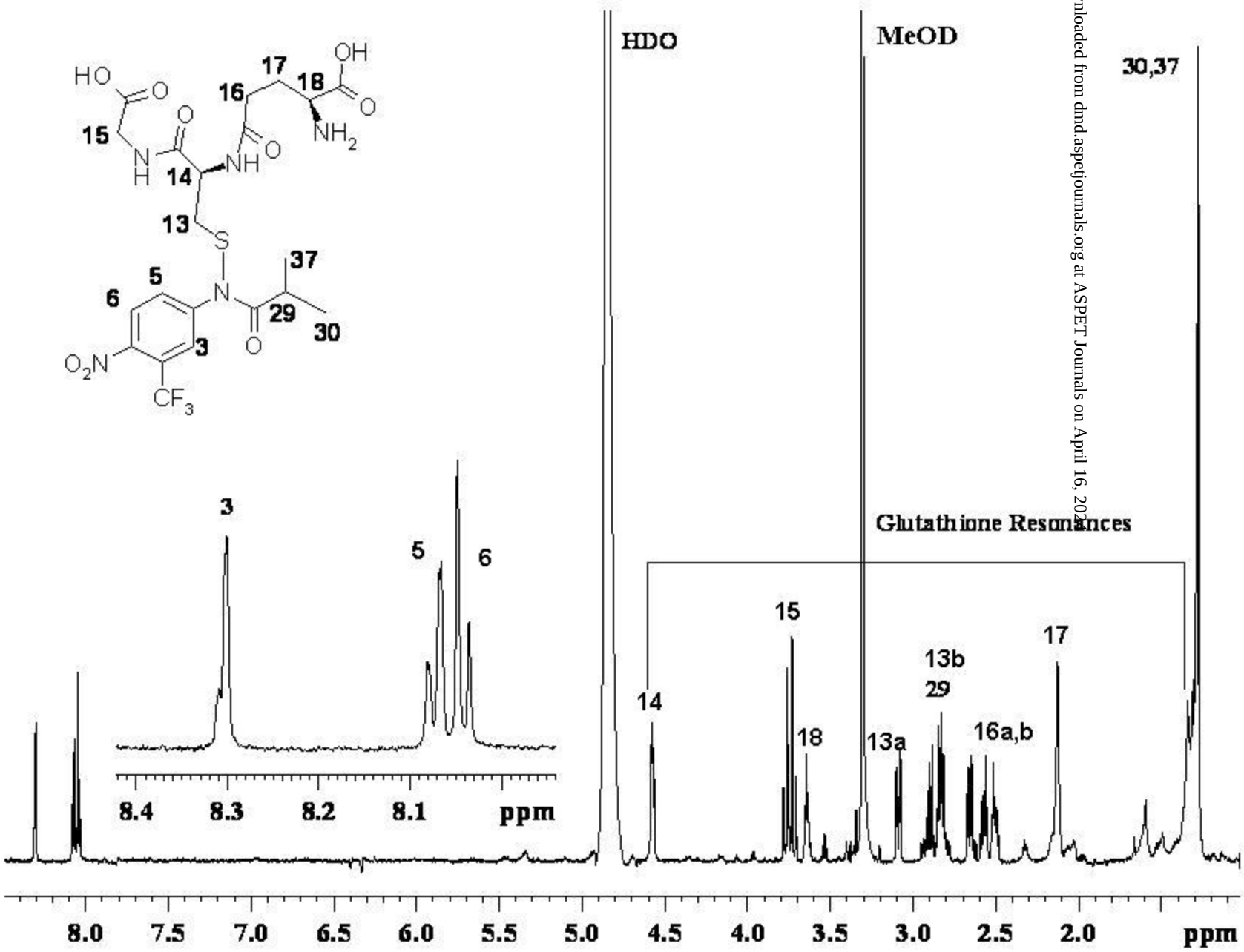
Figure 4



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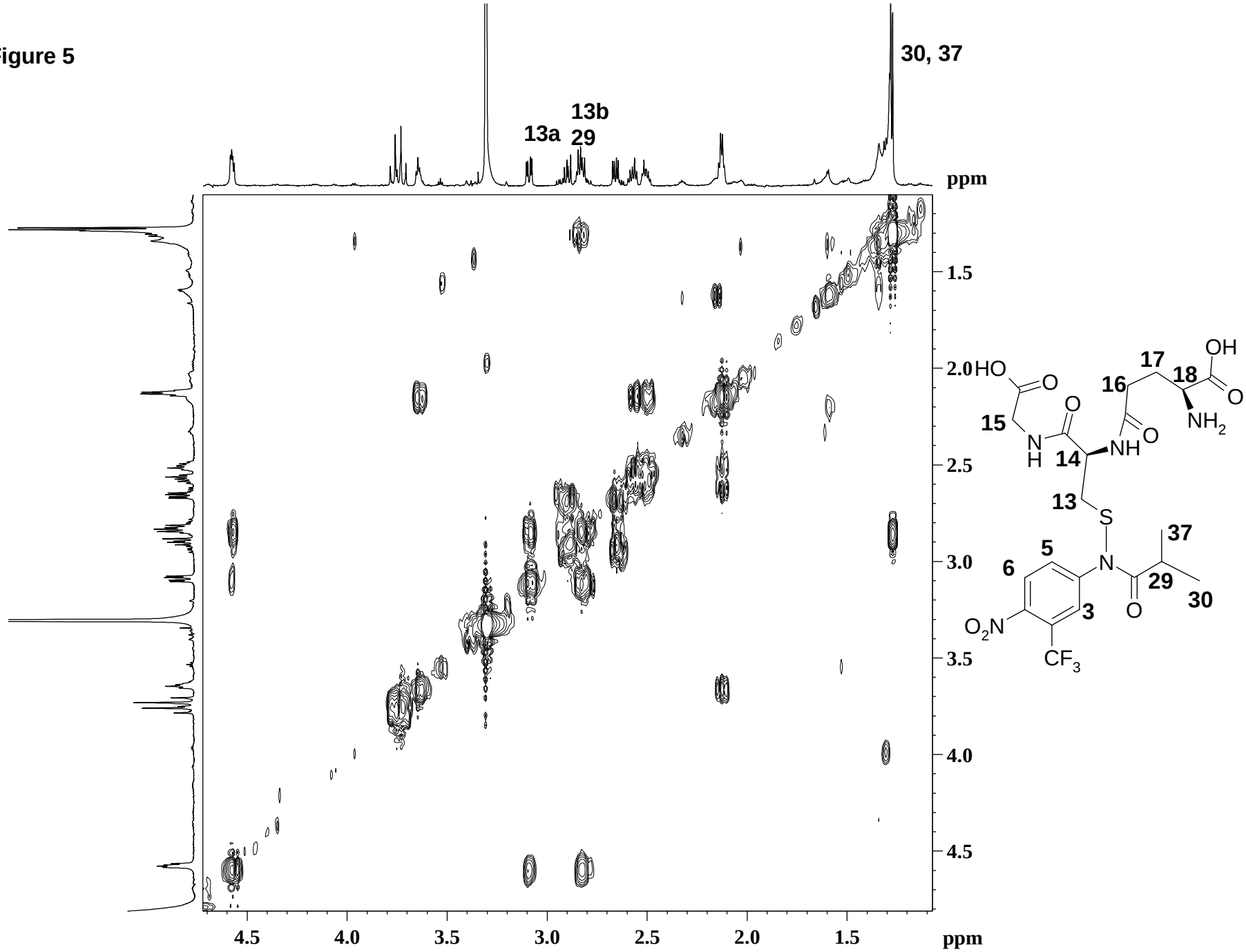

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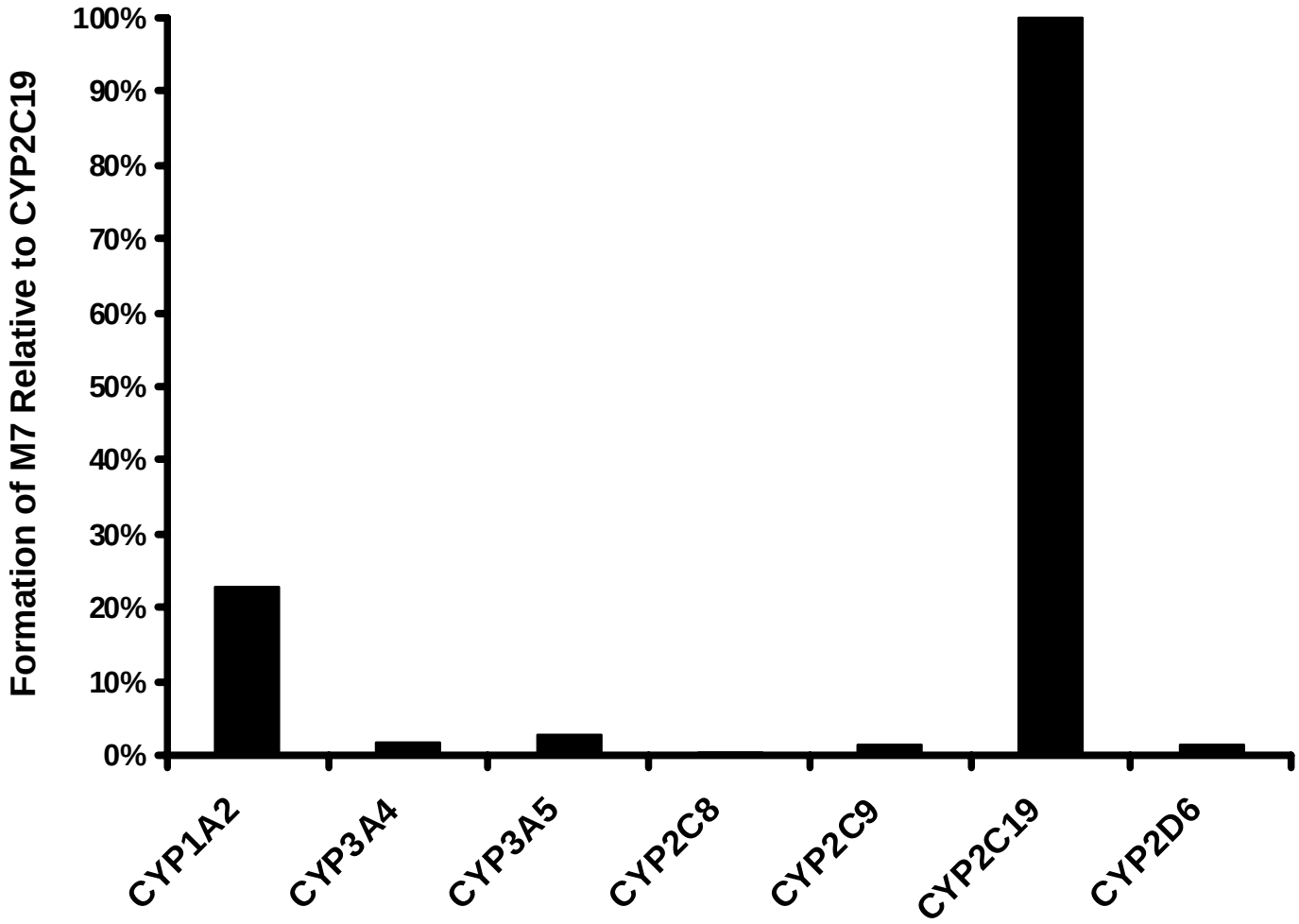

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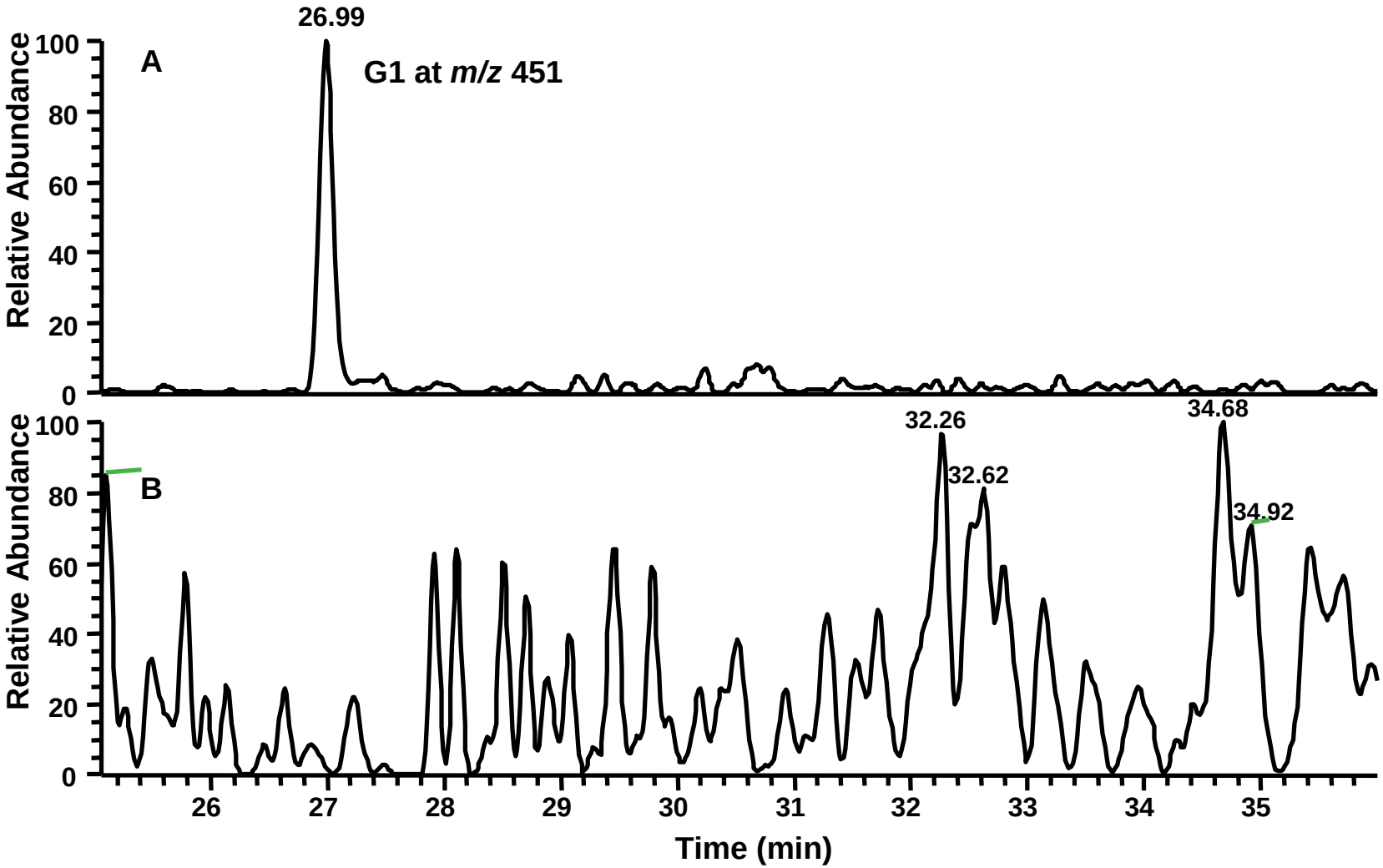

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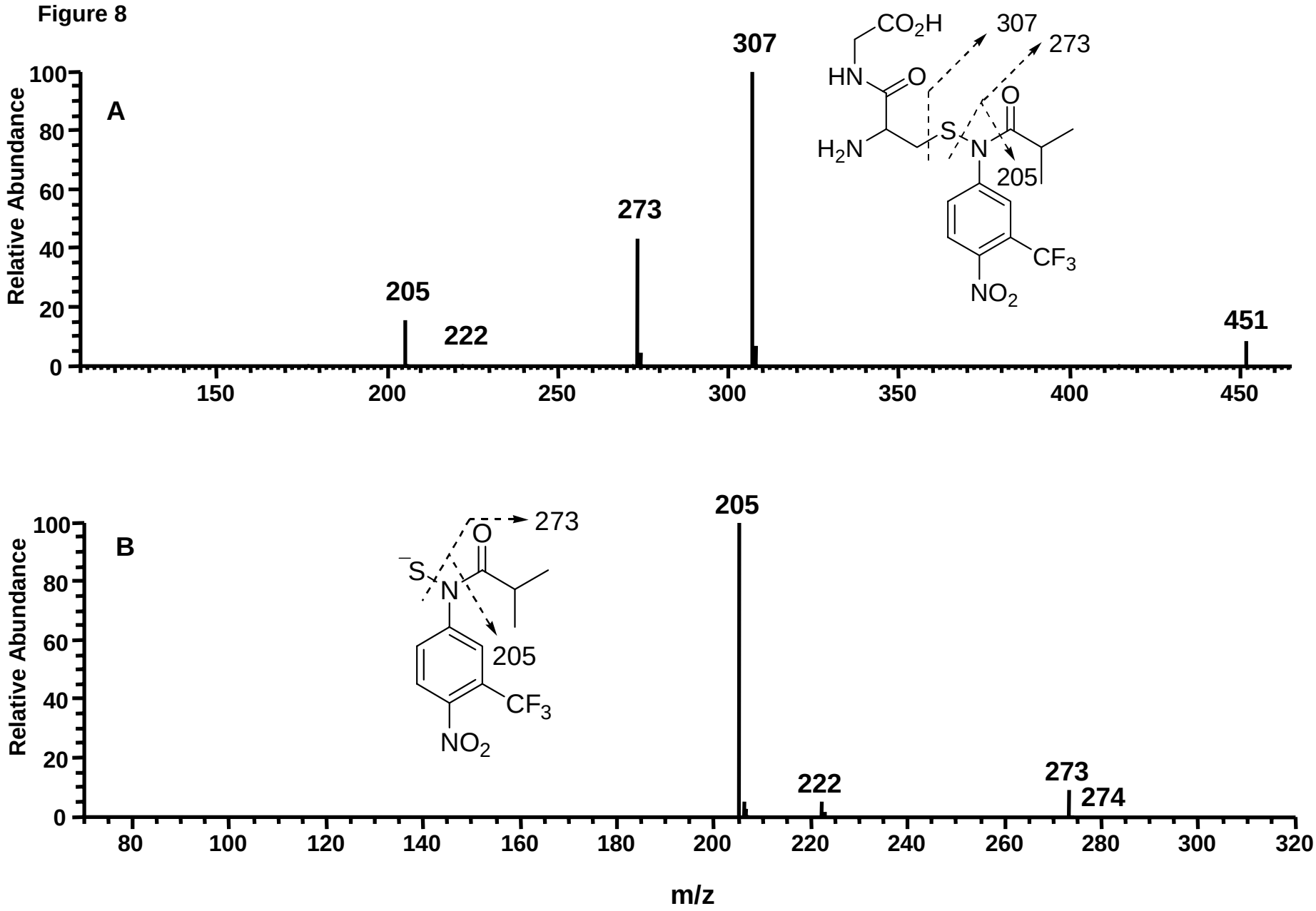

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