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Glutathione S-transferase Catalyzed Desulfonylation of a Sulfonylfuropyridine

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Running title

Desulfonylation of a sulfonylfuropyridine

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Abbreviations: GSH, reduced glutathione; GST, glutathione S-transferase; LC/MS-MS, liquid chromatography-tandem mass spectrometry; CID, collision-induced dissociation.

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Abstract

MRL-1, a cannabinoid receptor-1 inverse agonist, was member of a lead candidate series for the treatment of obesity. In rats, MRL-1 is eliminated mainly via metabolism, followed by excretion of the metabolites into bile. The major metabolite M1, a glutathione conjugate of MRL-1, was isolated and characterized by LCMS and NMR spectroscopic methods. The data suggest that the t-butylsulfonyl group at C-2 of furopyridine was displaced by glutathionyl group. In vitro experiments using rat and monkey liver microsomes in the presence of GSH showed that the formation of M1 was independent of NADPH and molecular oxygen, suggesting that this reaction was not mediated by an oxidative reaction and a glutathione S-transferase was likely involved in catalyzing this reaction. Further, a rat hepatic glutathione-S-transferase was capable of catalyzing the conversion of MRL-1 to M1 in the presence of GSH. When a close analog of MRL-1, a p-chlorobenzenesulfonyl furopyridine derivative (MRL-2), was incubated with rat liver microsomes in the presence of GSH, p-chlorobenzene sulfinic acid (M2) was also identified as a product in addition to the expected M1. Based on these data, a mechanism is proposed involving direct nucleophilic addition of GSH to sulfonylfuropyridine, resulting in an unstable adduct which spontaneously decomposes to form M1 and M2.

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Introduction

Glutathione conjugation of xenobiotics is a detoxification pathway that inactivates reactive electrophiles, which may alternatively covalently bind to endogenous proteins and elicit potential detrimental effects (Evans and Baillie, 2005; Zhou et al., 2005). Following glutathione conjugation, the molecule may be directly eliminated via biliary excretion, or the conjugate may proceed through the so-called "mercapturic acid pathway", where the glycine and glutamic acid residues are eliminated, and the remaining cysteine residue is N-acetylated to form a mercapturic acid derivative. This mercapturic acid derivative may be eliminated in urine or via the bile into the feces (Stevens and Jones, 1989; Hinchman et al., 1998). In the process of drug discovery, the presence of glutathione conjugates serves as an indirect indication of the formation of reactive intermediates, which could not have been otherwise isolated due to their reactivity. In addition to the ability to react with xenobiotics, glutathione also plays a role in maintaining the redox state of cells by scavenging reactive oxygen species. The depletion of glutathione through binding to reactive species could lead to increased levels of ·OH, O_2^-, H_2O_2, which may damage cellular lipids, endogenous proteins and DNA (Khan et al., 1998; Marnett et al., 2003; Reid et al., 2005), although cellular levels of glutathione are quite high in the range of 2-10 mM (Anders, 2004).

Glutathione-S-transferases (GST's) are enzymes which catalyze nucleophilic attack by glutathione on compounds containing electrophilic centers. The presumed mechanism is through de-protonation of GSH to form GS^- by a tyrosinate ion in the active site which enhances the nucleophilicity and reactivity of glutathione (Atkins et al., 1993).

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GST-catalyzed displacement of several functional groups, including F, Cl, Br, NO_2, and sulfonamides, by GSH has been reported (Livesey et al., 1982; Conroy et al., 1984; Dekant et al., 1988; Zhao et al., 1999; Parkinson, 2001). In this paper, we describe a novel desulfonylation reaction of a sulfonylfuropyridine catalyzed by GST(s). Structure-activity relationship and proposed mechanism of desulfonylation are also discussed.

Materials and Methods

Materials. MRL compounds 1-6 (Figure 1) were synthesized by the Medicinal Chemistry Group, [^{3}H]MRL-1 was prepared by the Labeled Compound Synthesis Group, Merck Research Laboratories (MRL), Rahway, NJ, USA. The specific activity of [^{3}H] tracer was ~50 Ci/mmol and the radiochemical purity, as determined by HPLC, was 99.5%. Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). NADPH, reduced glutathione (GSH) and glutathione-S-transferase (GST, from rat liver) were obtained from Sigma (St. Louis, MO, USA). Rat, dog, monkey and human liver microsomes were prepared as outlined in literature (Lu and Levine, 1972). The amount of P450 enzyme present in rat, dog and human liver microsomal protein were 0.21, 0.43 and 0.60 nmol/mg, respectively. The amount of P450 enzyme present in monkey liver microsomes was not estimated.

Animal Experiments. Experiments were performed according to procedures approved by the Merck Institutional Animal Care and Use Committee. [^{3}H]MRL-1 (2 mg/mL) was formulated in Imwitor 742: Tween 80 (1:1; w/w) and dosed orally to bile duct-cannulated male Sprague-Dawley rats (n=3) at 2 mg/kg. Bile, urine, and fecal

samples were collected at room temperature for up to 72 hr post dose and stored at -80ºC until analysis.

Analysis of Rat Bile and Isolation of M1. The pH of an aliquot of rat bile (0-72 hr) was adjusted to 6 with 50% acetic acid and an equal volume of acetonitrile was added. The mixture was vortex-mixed, centrifuged at 14,000xg for 10 min and the supernatant was used for metabolite analysis by LC-MS/MS with radiometric detection. For isolation of the GSH conjugate (M1), 10 mL of rat bile (8-24 hr) was concentrated to about 1 mL under a stream of nitrogen, an equal volume of acetonitrile was added and the mixture was centrifuged in a centrifuge as described above. The M1 metabolite was isolated from the supernatant by semi-preparative HPLC.

Incubation of [^{3}H]MRL-1 and its Analogs with Liver microsomes or Cytosol. The incubation mixtures contained [^{3}H]MRL-1 or its analogs MRL-2, MRL-3, MRL-4, MRL-5 or MRL-6 (10 µM), liver microsomal or cytosolic protein from rat, dog, monkey or human (1 mg/mL), $MgCl_2$ (0.1 mM) in 500 µL of phosphate buffer (100 mM, pH 6.5), NADPH and/or glutathione (1 mM), and were incubated for 60 min at 37°C in a shaking water bath. Control incubations were run in parallel without NADPH and GSH. Additional control experiments also were conducted in the absence of microsomes and with heat inactivated microsomes. For anaerobic experiments, reaction mixtures were evacuated under reduced pressure and flushed three times with argon to ensure removal of oxygen. The NADPH and glutathione solutions also were evacuated and purged with argon, separately, before addition to the incubation mixture. The reactions were quenched with an equal volume of acetonitrile and analyzed by LC-MS/MS. In a separate experiment, to study the possibility of (non-enzymatic) base catalyzed formation of M1,

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[^{3}H]MRL-1 (10 μM) was incubated with 0.1N NaOH in the presence of GSH (1 mM) at 37°C for 60 min and processed as described above.

Incubation of [^{3}H]MRL-1 with Glutathione S-transferase. [^{3}H]MRL-1 (2 μM) was incubated with a crude preparation of rat hepatic glutathione S-transferases (100 U) in 200 μL of phosphate buffer at pH 6.5 and at pH 8.5 containing 0.1 mM $MgCl_2$ either with or without 1 mM glutathione or cysteine. The reaction mixtures were incubated for 60 min at 37°C in a shaking water bath. At the end of the incubation, they were analyzed by LC-MS/MS as described above.

Instrumentation. Conjugates of glutathione were identified by electrospray LC-MS/MS analysis using a Finnegan LTQ mass spectrometer (San Jose, CA), which was interfaced with a Shimadzu HPLC system equipped with two Series LC-10AD*VP* micro pumps and a Series SIL-10AD*VP* auto sampler. The spray voltage was maintained at 4.1 kV, and the capillary temperature was set at 250ºC. Full scan spectra, from *m/z* 200 to 800, were obtained in the positive ion mode, and product ion spectra were generated by CID of MH^+ ions of interest. The CID of MH^+ was achieved with helium as the collision gas at the collision energy of 25 eV. Separation of bile metabolites was achieved on a 5-μm Zorbax SB C3 column (4.6x250 mm) at a flow rate of 1 mL/min. The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B). The column was eluted with a linear gradient from 30 to 90% B over 40 min. One-fourth of the column eluate was directed into the mass spectrometer, and the remaining into a β-Ram radiometric detector (Inus Systems, Inc., NJ, USA) for on-line radio-profiling. These HPLC conditions also were used for the purification of the glutathione conjugate.

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For identification of p-chlorobenzenesulfinic acid, the mass spectrometer was operated in the negative ion mode from m/z 120-700, with a spray voltage of 4.0 kV, capillary temperature of 350ºC and the collision energy of 30 eV. The chromatography was performed on a 5-µm zorbax RX-C8 column, eluted at a linear rate of 1 mL/min. The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile: methanol (1:1; B). The column was eluted at a linear gradient from 10-60% B over a period of 10 min.

HRMS data was acquired using an LTQ-Orbitrap operated in positive electrospray ionization mode. Spray voltage was set at 5.0 kV, with a capillary temperature of 300°C. Sheath and auxiliary gas flow rates were set at 37, and 0, respectively. Capillary and tube lens voltages were set at 5 and 90 respectively. Resolution was set at 60,000 for full MS (*m/z* 300-800) and at 15,000 for MS^2 and MS^3 scan events. Differences between the measured mass and the calculated mass are expressed in parts per million (ppm).

[^{1}H]NMR Analysis. NMR spectra of MRL-1 and the purified metabolite were obtained in methanol-d_4 at room temperature using a Varian Inova 600 MHz NMR spectrometer (Palo Alto, CA, USA) equipped with a 3 mm probe. The chemical shifts are expressed as parts per million relative to tetramethylsilane.

Results

Identification of GSH Conjugate of MRL-1 (M1) in Rat Bile. A representative HPLC-radiochromatogram of the bile from rats dosed orally with [^{3}H]MRL-1 is depicted in Figure 2. A major polar peak eluting at ~13 min constituted about ~40% of the radioactivity excreted in the bile. LC-MS analysis of this metabolite indicated the

presence of an MH^+ ion at *m/z* 645, which is 185 Da higher than that of parent compound (MRL-1, MH^+ 460 Da). The collision induced dissociation (CID) spectrum of the MH^+ ion *m/z* 645 yielded fragment ions *m/*z 516 corresponding to the loss of 129 Da, *m/z* 570 due to the loss of 75 Da and *m/z* 627 corresponding to the loss of 18 Da (Figure 3). These neutral losses are characteristic of glutathione conjugates; 129 Da due to pyroglutamate and 75 Da due to glycine (Baillie and Davis, 1993). The mass of the metabolite corresponded well with a structure in which t-butylsulfonyl group had been replaced by a glutathionyl group. Further, HRMS analysis of the metabolite showed MH^+ ion at *m/z* 645.0980, which matched well with a molecular formula of $C_{29}H_{27}O_7N_4Cl_2S$ (ΔM 1.3 ppm). Based on the above data, a tentative structure for the metabolite (M1) was assigned as shown in Figures 1 and 3.

The structure of this conjugate (M1) was confirmed by ^{1}H NMR analysis (Table 1). The ^{1}H NMR spectrum of M1 exhibited the signals characteristic of GSH and lacked the signals due to t-butylsulfonyl group (Figure 4). The regiochemistry of glutathionyl group was deduced from the 1D ^{1}H-^{1}H NOE spectrum (Figure 5), wherein the lone pyridine proton (8.03 ppm, H-*m*) was selectively inverted and an enhancement of the lone furan proton (7.09 ppm, H-*p*) was observed. Thus, these data suggest the glutathionyl group is located on the furan carbon that was previously occupied by the t-butylsulfonyl group at C2.

In Vitro Metabolism of [^{3}H]MRL-1 in NADPH/GSH-Fortified Liver Microsomes or Cytosol. Incubation of [^{3}H]MRL-1 with liver microsomes from rats, dogs, monkeys and humans in the presence of NADPH yielded small amounts of oxidative metabolites (addition of 16 Da, data not shown). When [^{3}H]MRL-1 was

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incubated with liver microsomes from these species in the presence of GSH, formation of a new peak was observed in rat and monkey liver microsomal incubations. A representative radiochromatogram depicting metabolite of [^{3}H]MRL-1 formed upon incubation with rat liver microsomes is shown in Figure 6. The metabolite was confirmed as M1 (see above) on the basis of LC-MS analysis (MH^+ at *m/z* 645 and its CID spectra) and by comparison with that identified in rat bile. However, M1 was not formed when [^{3}H]MRL-1 was incubated with dog or human liver microsomes in the presence of GSH (data not shown). Also, M1 was not formed when [^{3}H]MRL-1 was incubated with rat, dog, monkey or human liver cytosol in the presence of GSH (data not shown).

Metabolism of [^{3}H]MRL-1 by Glutathione S-Transferase. Incubation of [^{3}H]MRL-1 with glutathione S-transferase in the presence of GSH yielded a product, the mass (MH^+ at *m/z* 645) and CID spectra of which corresponded well with those of M1 detected in rat bile, and rat or monkey liver microsomal incubations. When cysteine was used instead of GSH in the above reaction, no product was formed (data not shown).

Metabolism of MRL-1 Analogs in GSH-fortified Rat Liver Microsomes. Figures 6A and 6B depict extracted ion chromatograms of metabolites formed from MRL-2 upon incubation with rat liver microsomes and GSH. The mass and CID spectra of the peak at ~25 min corresponded well with those of M1 formed from MRL-1. The LCMS analysis of the reaction mixture under different analytical conditions (Figure 7B, see methods) indicated the presence of MH^- ion at m/z 175, the CID of which yielded a fragment ion at m/z 111 resulting from the loss of 64 Da attributed to SO_2 (Figure 7C). Based on these data and by comparison with LCMS data of an authentic standard, the metabolite M2 was identified as p-chlorobenzenesulfinic acid.

Figure 8 shows the mass and the CID spectra of metabolite M3 formed from MRL-3 upon incubation with rat liver microsomes in the presence of GSH. The metabolite displayed MH^+ ion at m/z 489, CID of which produced characteristic fragment ions of GSH conjugates (loss of 129 and 75 Da) similar to those observed for M1. Based on these data, the proposed structure for this metabolite was as shown in Figure 8, formed following displacement of chlorobenzene sulfonyl group by GSH (M3, Figure 1), similar to the formation of M1 from MRL-1. Finally, when MRL-4, MRL-5 and MRL-6 were incubated with liver cytosol or microsomes from rat, dog, monkey or human in the presence of GSH, no thiol conjugates were formed.

Discussion

This study describes a novel desulfonylation of a sulfonyl furopyridine that is catalyzed by a GST. The identification of the resulting GSH conjugate and a mechanism of its formation are also discussed. GST catalyzed displacement of several hetero groups and activated sulfonamides by GSH have been reported (Livesey et al., 1982; Conroy et al., 1984; Dekant et al., 1988; Zhao et al., 1999; Parkinson, 2001). Common characteristics of these displacement reactions are that they occur with good leaving groups and on ring systems activated by electron withdrawing groups. In the case of sulfonamides, a series of N-substituted aryl sulfonamide derivatives were evaluated for their ability to undergo GST-dependent sulfonamide cleavage (Zhao et al., 1999). The sulfonamide cleavage resulted in the formation of the corresponding aryl-SG conjugate, an amine and sulfur dioxide (Koeplinger et al., 1999; Zhao et al., 1999). Based on the metabolites identified, a mechanism was proposed for the cleavage of sulfonamide which involves direct attack of the thiolate anion of GSH on the aryl carbon attached to the

sulfonamide, with the subsequent C-S cleavage proceeding via a concerted or a non-concerted fashion (Zhao et al., 1999). Structure activity relationship of *non-enzymatic* cleavage of sulfonamides, attached to a series of heterocycles, has also been evaluated (Conroy et al., 1984). The results from these studies demonstrated that sulfonamides with pKa values above 9 were not cleaved by GSH or L-cysteine.

The structural features of the M1, detected in rat bile following MRL-1 dosing or formed in vitro following incubation with microsomes and GSH, were evident from its NMR and LCMS analyses which indicated the absence of the t-butyl group and the presence of glutathionyl group. The data obtained by NMR spectroscopy also suggested that the glutathionyl group in M1 was present on the same carbon (C2) of the furan ring on which the t-butylsulfonyl group was present in MRL-1. The latter finding was rather unexpected, because it was reasonable to hypothesize that nucleophilic attack of the thiolate anion of GSH would have been blocked at C2 (Figure 9) due to steric hindrance by the t-butylsufonyl group. This hypothesis was further fueled by observations with MRL-6 which differed from MRL-1 by only an additional methyl group at the C3 position and did not form the analogous glutathionyl conjugate; initially this was presumed to be due to the blockade of the C3 position to nucleophilic attack by the thiolate anion.

To study the mechanism of this reaction further, the formation of M1 was investigated in rat, dog, monkey and human liver microsomes in vitro. The data showed that the M1 metabolite was formed only in rat and monkey liver microsomes, but not in dog and human liver microsomes. Also, formation of M1 was independent of NADPH and molecular oxygen, and GSH could not be replaced by L-cysteine as a co-substrate (a

cysteine conjugate similar to M1 was not formed, data not shown). These data and the experiments conducted with partially purified GST (from rat liver) confirmed that the formation of M1 is catalyzed by one or more GST enzymes. There are three distinct families of glutathione transferases that are localized in mitochondria, cytosol and/or microsomes with varying substrate specificity (Dejong, 1988; Picket and Lu, 1989; Jakobsson, 1999; Hayes, 2005). In the present study, the glutathione conjugation of MRL-1 appeared to be catalyzed by a microsomal GST, as formation of M1 was not observed in liver cytosolic fractions obtained from rat, dog, monkey and human (data not shown).

In many instances, the nucleophilic attack on electrophilic centers by GS^- does proceed at a slow rate even in the absence of GST. In the present investigation, M1 was not detectable in incubations of MRL-1 with GSH and heat-inactivated rat liver microsomes. However, M1 was detectable in trace amounts when MRL-1 was incubated with GSH and sodium hydroxide, suggesting that C-S bond in MRL-1 is strong and harsh reaction conditions are required to break the bond.

On the basis of the above data, a mechanism was proposed for the formation of M1 that involves direct attack of the enzymatically stabilized (by GST) thiolate anion on the electron deficient C2, as shown in Figure 9. The resulting adduct spontaneously loses t-butylsulfinic acid to produce M1. Because of its small mass, it was difficult to directly identify t-butylsulfinic acid by LCMS analysis in support of this proposed mechanism. Therefore, MRL-2, a p-chlorobenzenesulfonic acid analog of MRL-1 (Figure 1), was used to study the nature of co-product formed in this reaction. In GSH-supplemented rat and monkey liver microsomal incubations, MRL-2 underwent metabolism similar to

MRL-1 to produce M1. More importantly, p-chlorobenzenesulfinic acid (M2) was identified as a co-product in these incubations. Thus, formation of M2 from MRL-2 supports the proposed mechanism as shown in Figure 9. This mechanism of nucleophilic displacement of sulfonyl group by a thiolate anion, which occurs like an S_N2 addition-elimination process, is similar to that proposed for the non-enzymatic displacement of sulfonamide by GSH or L-cysteine in 1,3,4-thiadiazole-2-sulfonamides (Conroy et al., 1984). However, as mentioned above, the formation of M1 from MRL-1 (or M2 from MRL-2) does not proceed in the absence of GST enzyme in the present study.

It is interesting to note that MRL-4 and MRL-5 were not substrates for GST and did not produce M1; both are analogues of MRL-1 differing only in the moiety at the C2 position on the furan ring. This can be explained by the fact that the carbonyl moieties at C2 would make poor leaving groups. Of interest, however, was the fact that MRL-6 (3-methylated analog of MRL-1) did not produce the corresponding GS-conjugate. This stands in stark contrast to the *non-enzymatic* GSH additions to related benzofuran systems substituted with 2-sulfonamides (Graham et al., 1990). In these benzofuran systems, 3-methylated analogues were appreciably more reactive to GSH than the desmethyl compounds. It is unclear why in our investigations with this furopyridine chemical series MRL-6 is not reactive towards GSH conjugation. One could speculate that the C3 methyl group blocks a key enzymatic transition state or destabilizes the C3 carbanion due to its positive inductive effect, thus making the nucleophilic attack of thiolate anion at C2 less favorable (Fig 9). It might be intriguing to investigate if electron withdrawing groups at C3 will allow desulfonylation in related structures. The electrophilicity of sulfonamides, attached to various heterocycles, has been assessed by

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measuring their rate of reaction with GSH (Graham et al., 1989; Woltersdorf OW et al., 1989; Graham et al., 1990; Hartman et al., 1992). Sulfonamides attached to benzothiazole were more reactive with GSH compared to those attached to benzofuran, benzoxazole, or indole. Contrary to these sulfonamides, benzothiophenesulfonamides were practically inert toward glutathione. The GSH reactivity of these sulfonamides was found to be well correlated with dermal sensitization reaction in guinea pigs. On the other hand, benzothiophenesulfonamides, which were inert toward glutathione, were devoid of dermal sensitization potential assessed in guinea pigs. In the present study, no dermal sensitization was observed in rodents following administration of MRL-1 or related compounds. Clinical significance of glutathione conjugation with MRL-1 is not known. As discussed above, only rat and monkey liver microsomes were active in GST mediated conjugation reaction described in this study. It is possible that there is a species difference in GST activities and MRL-1 may be cleared in human via non-GST mediated metabolism routes.

In conclusion, we have identified a novel GST-catalyzed desulfonylation of a 2-sulfonylfuropyridine series that appears to proceed via addition-elimination reaction. Initial nucleophilic attack of glutathionyl sulfur on the C-2 position of the sulfonylfuropyridine produces an unstable adduct which leads to the formation of rearomatized 2-glutathionylfuropyridine conjugate with the elimination of corresponding sulfinic acid derivative.

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Figure legends

Figure 1. Chemical structures of MRL-1, related analogs and metabolites.

Figure 2. Representative HPLC radiochromatogram of bile from rats dosed with [^{3}H]MRL-1, which was formulated in Imwitor 742: Tween 80 (1:1) and orally dosed to bile duct-cannulated rats at 2 mg/kg. Bile was collected and analyzed as described in the text (peaks A and B are the oxidative metabolites of MRL1).

Figure 3. High-resolution product ion mass spectrum obtained by collision induced dissociation of the MH^{+} ion of metabolite M1 at *m/z* 645.

Figure 4. ^{1}H NMR spectrum of (600 MHz) of M1 (A) and MRL-1 (B).

Figure 5. 1D ^{1}H-^{1}H NOE NMR spectrum of M1 (lower trace) with the corresponding region of the ^{1}H NMR spectrum (upper trace).

Figure 6. Radiochromatographic profile of products formed upon incubation of [^{3}H]MRL-1 (10 μM) with rat liver microsomes and GSH.(1 mM). Incubation mixtures were extracted with acetonitrile and analyzed by HPLC with radio detection as described in the text.

Figure 7. Extracted ion chromatograms of products formed upon incubation of MRL-2 (10 μM) with rat liver microsomes and GSH (1 mM). Incubation mixtures were extracted with acetonitrile and analyzed by HPLC with radio detection as described in the text; (A) positive ion mode, (B) negative ion mode. (C) Product ion spectrum of the metabolite M2 obtained by collision induced dissociation of the MH^{-} ion at *m/z* 175.

Figure 8. Product ion mass spectrum obtained by collision induced dissociation of the MH^{+} ion of metabolite M3 at *m/z* 489.

Figure 9. Proposed mechanism of formation of M1 and M2 from MRL-2.

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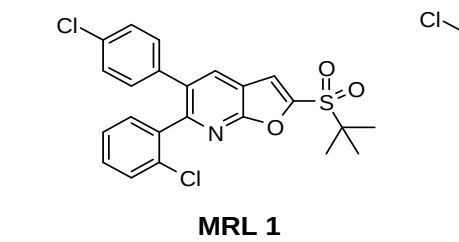
Table-1

Proton and ^{13}C NMR data[1] for MRL-1 and the glutathionyl conjugate M1

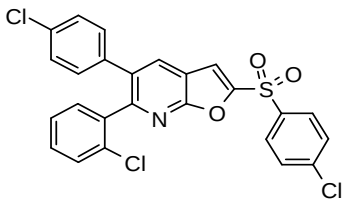
	MRL-1		M1	
atom	^{1}H	^{13}C	^{1}H	^{13}C
j	7.19 (m, 2H, *J*=8.6)	131.2	7.15 (m, 2H, *J*=8.6)	131.1
k	7.23 (m, 2H, *J*=8.6)	128.1	7.18 (m, 2H, *J*=8.6)	127.9
m	8.36 (s, 1H)	135.0	8.03 (s, 1H)	131.1
p	7.81 (s, 1H)	116.3	7.09 (s, 1H)	109.5
s	1.47 (s, 9H)	22.3	--	--
b'	--	--	3.65, 3.72 (AB, 2H, *J*=17.2)	43.2
d'	--	--	4.70 (dd, 1H, *J*=4.8,8.5)	29.2
e'	--	--	3.30, 3.58 (m, 2H)	34.9
g'	--	--	2.48 (m, 2H)	53.5
h'	--	--	2.10 (m, 2H)	26.6
i'	--	--	3.58 (t, 2H, *J*=5.9)	54.6

[1] in CD_3OD at 600 MHz. Data as follows: assignment, chemical shift (ppm) , multiplicity, integrals, coupling constants (Hz).

Figure 1



MRL 1



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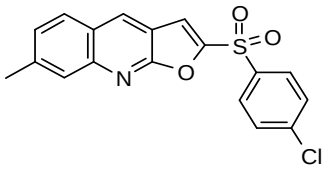

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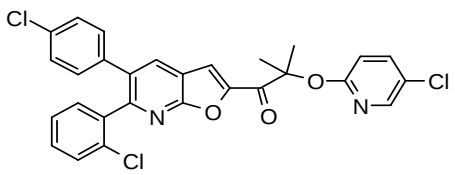

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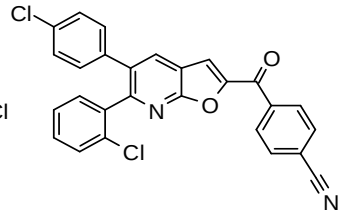

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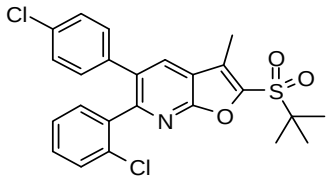

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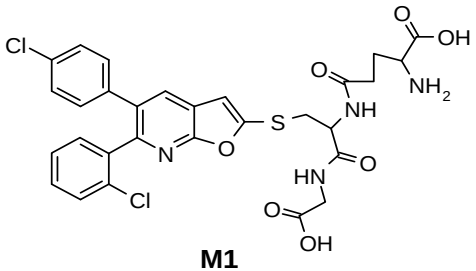

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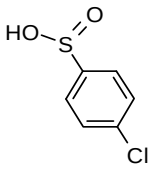

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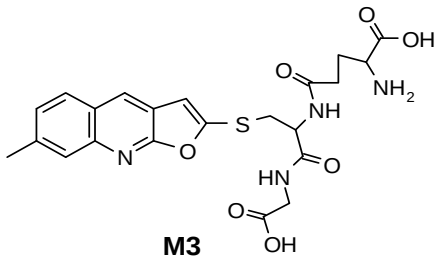
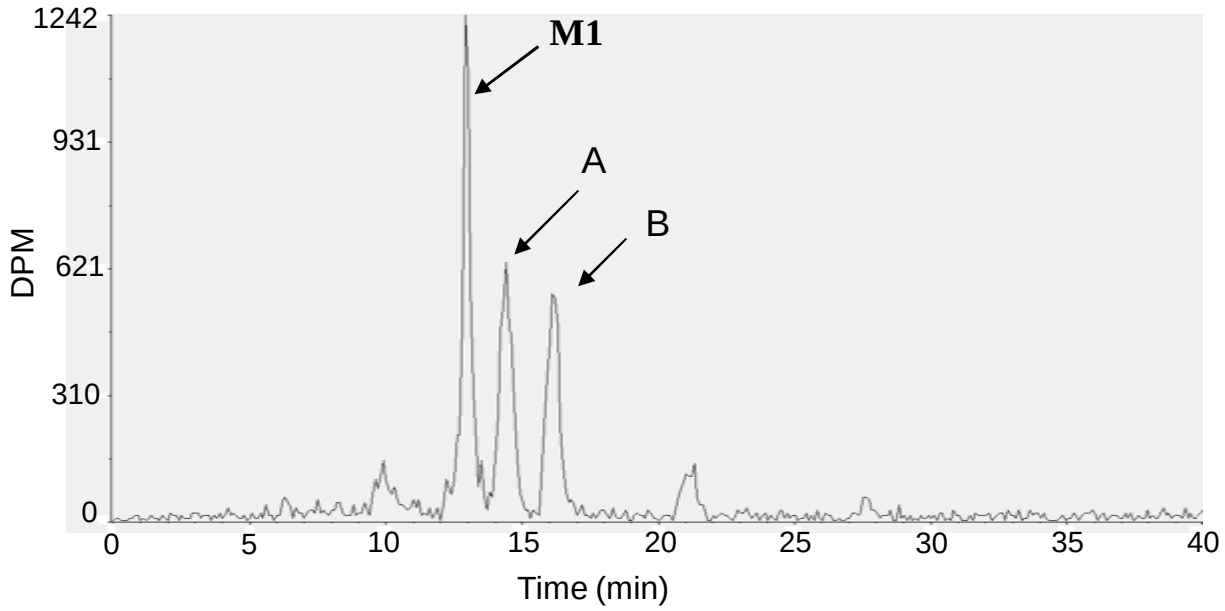


M3

Figure 2



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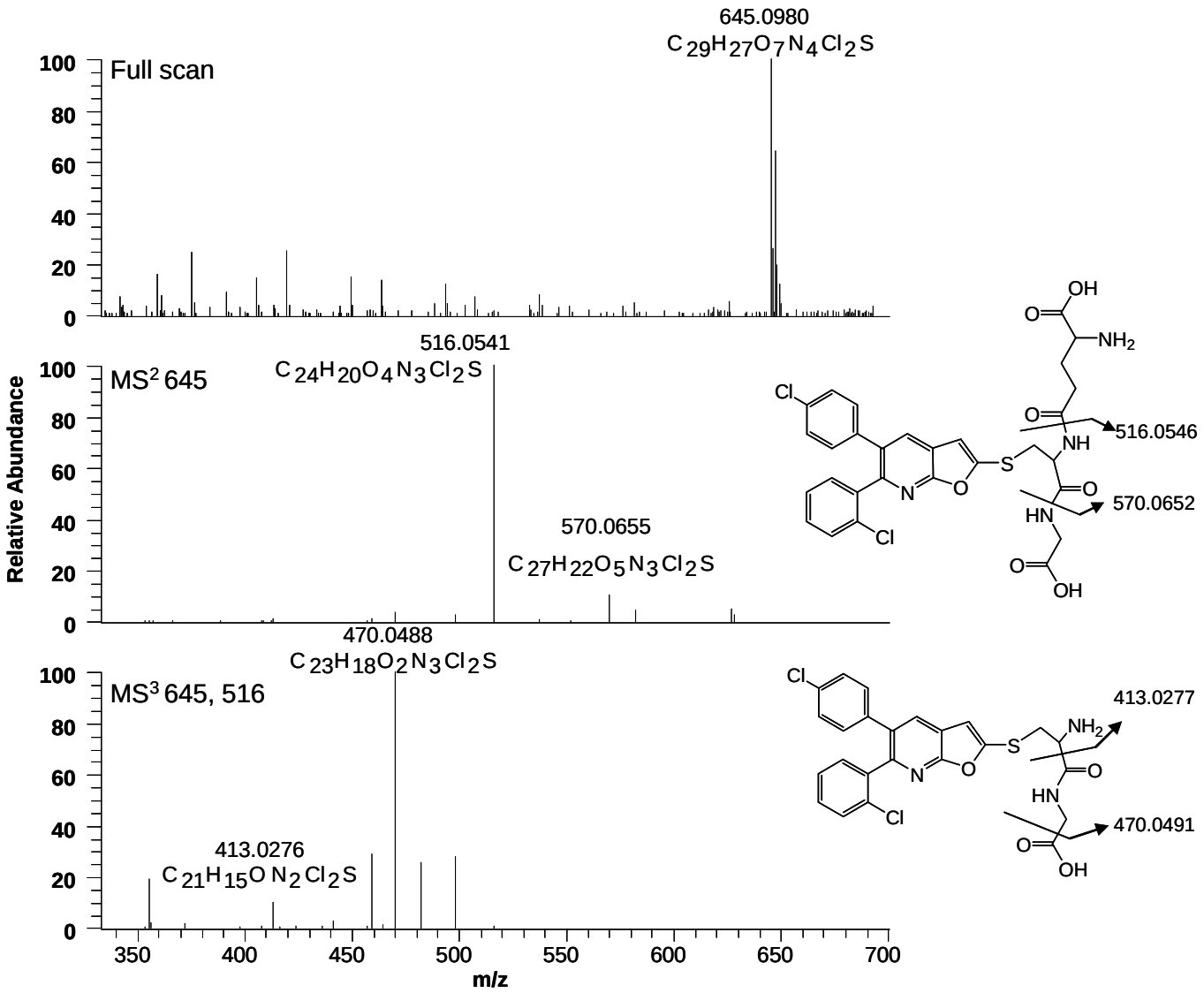

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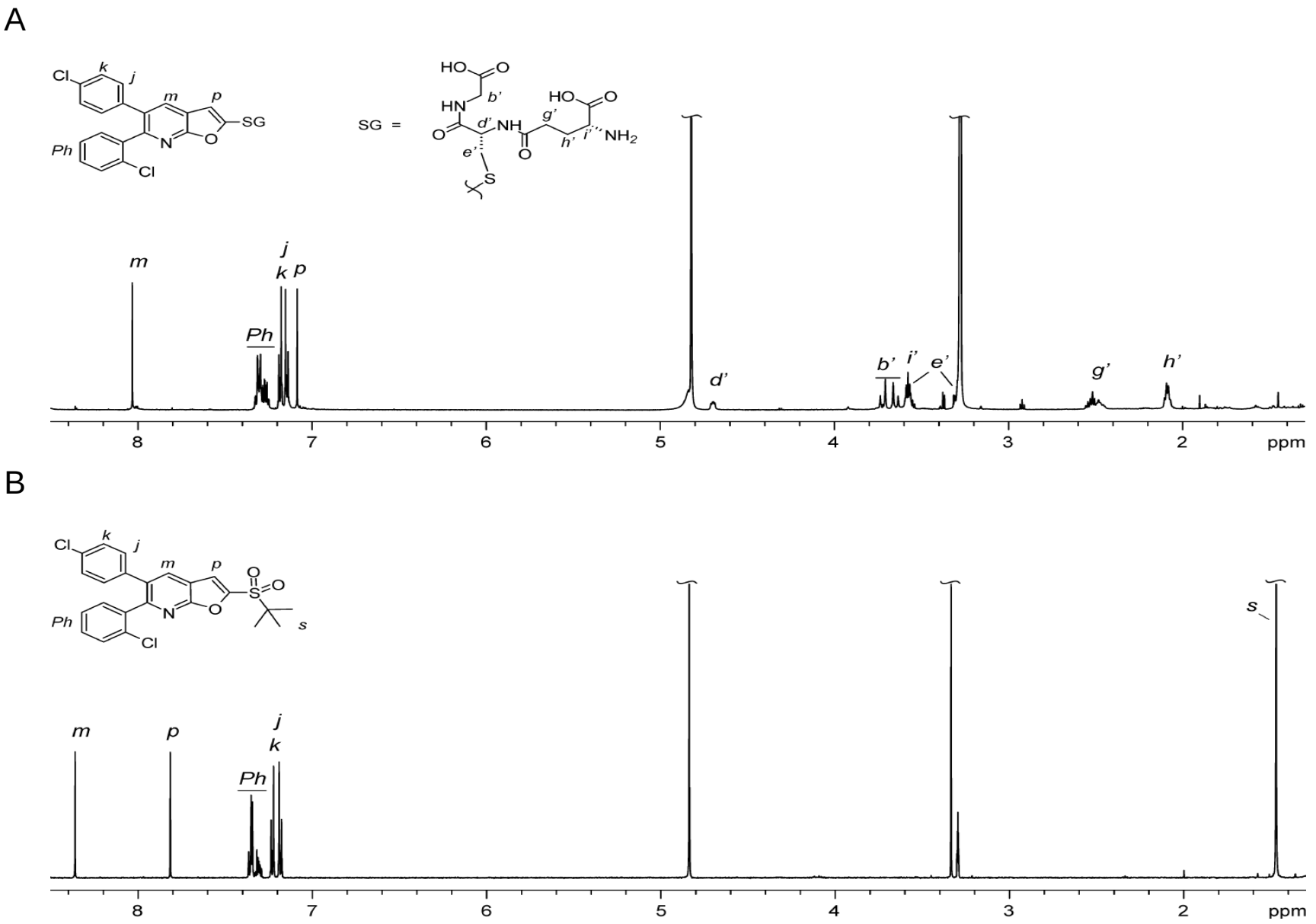

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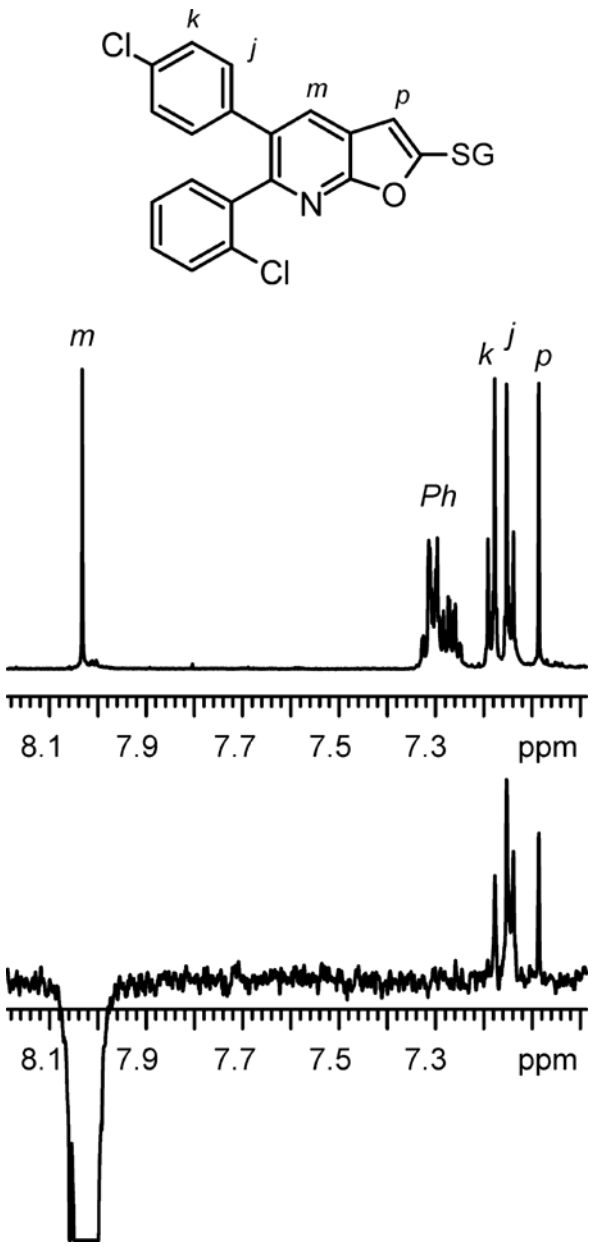

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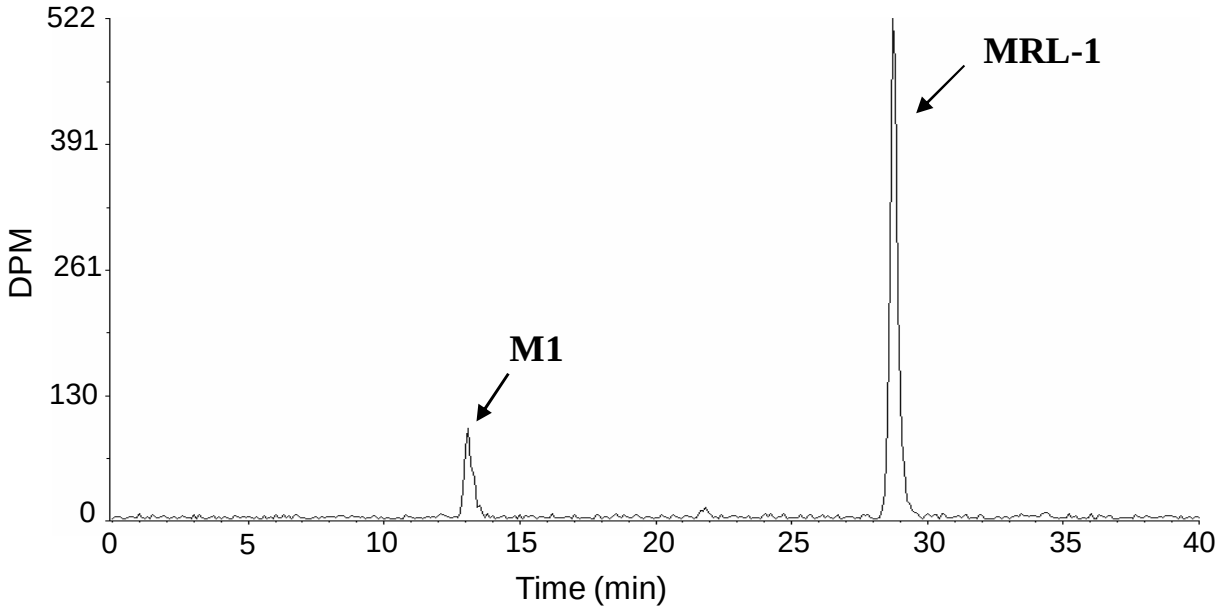

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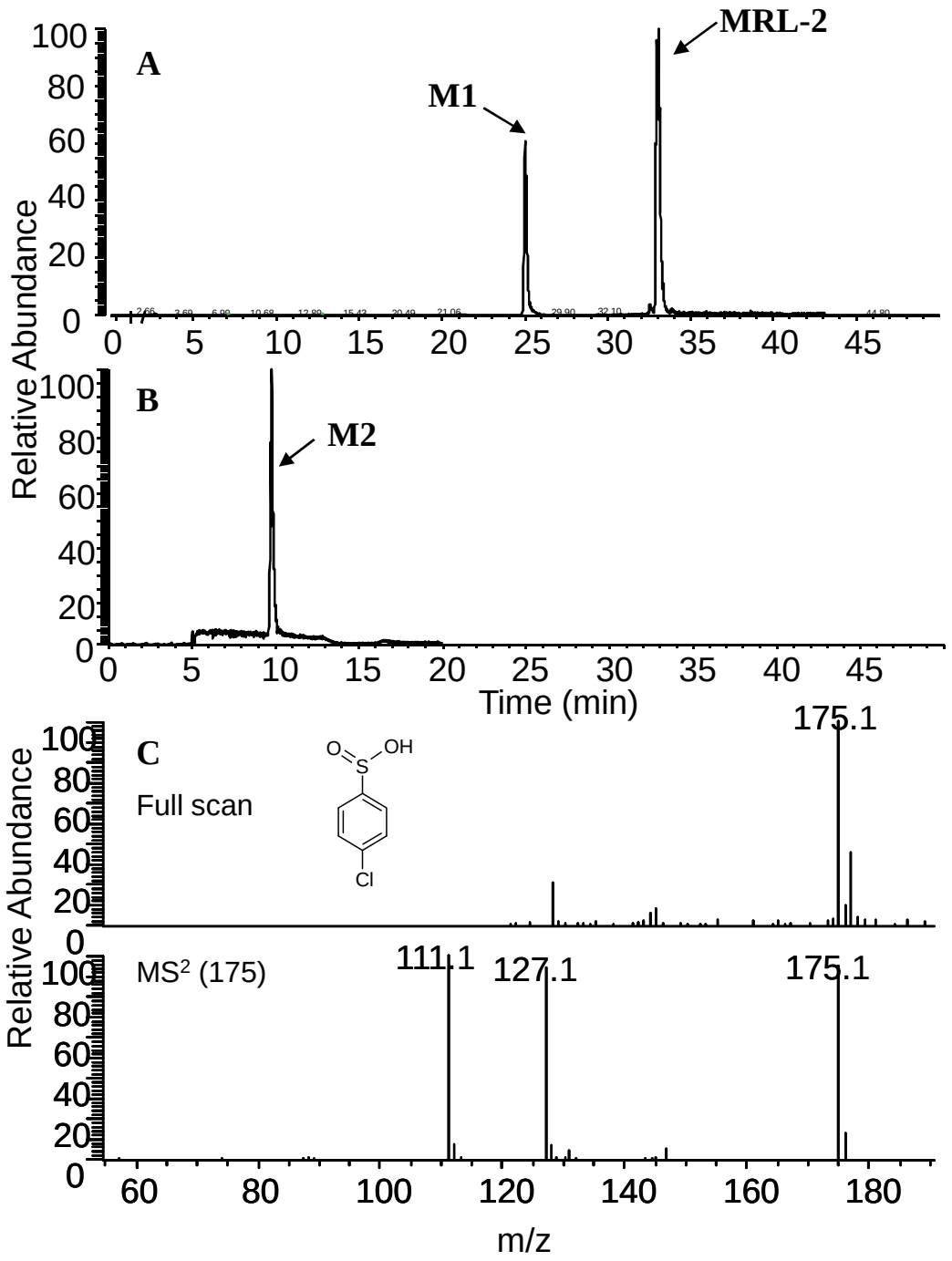
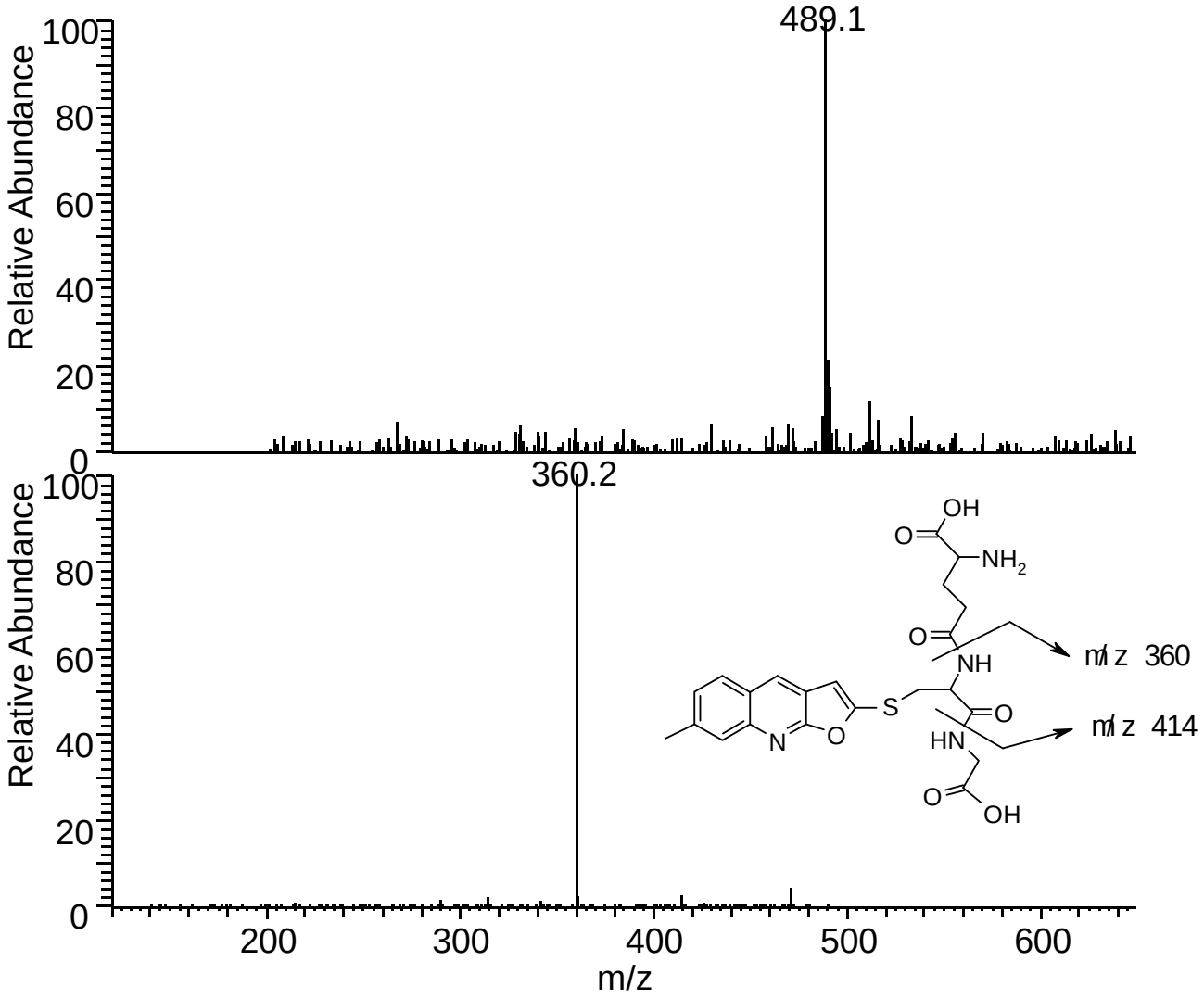


Figure 8



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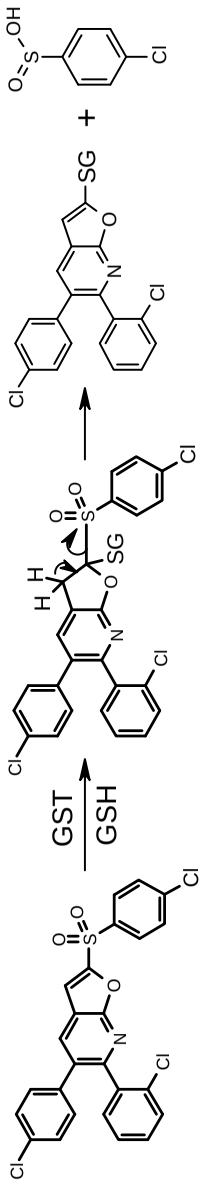

Figure 9