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Short Communication

Transamination in the Metabolism of the Nephrotoxicant *N*-(3,5-Dichlorophenyl)succinimide in Rats

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Number of words in Introduction: **265**

Number of words in Results and Discussion: **1220**

[1]Abbreviations: AOAA, (aminooxy)acetic acid hemihydrochloride; CID, collision induced dissociation; ESI, electrospray ionization; 2-NDASA, *N*-(3,5-dichlorophenyl)-2-aminosuccinamic acid; *N*-acetyl-2-NDASA, *N*-(3,5-dichlorophenyl)-2-*N*-acetylaminosuccinamic acid; 2 and 3-NDHSA, *N*-(3,5-dichlorophenyl)-2- and -3-hydroxysuccinamic acid; NDPSA, *N*-(3,5-dichlorophenyl)succinamic acid; NDPS, *N*-(3,5-dichlorophenyl)succinimide; PLP, pyridoxal 5'-phosphate; SRM, single reaction monitoring; XIC, extracted ion chromatogram.

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Abstract

The agricultural fungicide *N*-(3,5-dichlorophenyl)succinimide (NDPS) is nephrotoxic in rats. Due to the involvement of NDPS metabolism in its mechanism of toxicity, the detailed biotransformation of ^{14}C-NDPS in rats was previously evaluated using HPLC-electrospray ionization (ESI)-mass spectrometry. In the present report, we describe the identification of two novel amino metabolites of NDPS, which were present in significant amounts in rat kidney tissues. Using LC-MS/MS and synthetic standards, the two metabolites were identified as *N*-(3,5-dichlorophenyl)-2-aminosuccinamic acid (2-NDASA) and its *N*-acetylated derivative (*N*-acetyl-2-NDASA). The mechanism of formation of 2-NDASA was studied in vitro. Incubations were carried out in rat liver and kidney cytosols using the major oxidative metabolite of NDPS, *N*-(3,5-dichlorophenyl)-2-hydroxysuccinamic acid (2-NDHSA), as the substrate. Formation of 2-NDASA in vitro was confirmed using mass spectrometry. Inhibitors of alcohol dehydrogenase (4-methylpyrazole) and aldehyde dehydrogenase (disulfiram) reduced 2-NDASA formation by 40 to 50%. Menadione (an inhibitor of aldehyde oxidase) and quercetin (an inhibitor of carbonyl reductase) did not show any effects. (Aminooxy)acetic acid (AOAA), an inhibitor of pyridoxal 5'-phosphate (PLP)-containing enzymes such as aminotransferases, almost completely abolished the formation of 2-NDASA. Using LC-MS, the transamination mechanism was further supported by the incorporation of an ^{15}N-amino group in 2-NDASA when ^{15}N-glutamic acid was included in the incubation mixture. Results from these studies show that transamination is a metabolic pathway in the clearance of NDPS in rats, and that cytosolic dehydrogenases and aminotransferases may be involved in this process.

Introduction

N-(3,5-Dichlorophenyl)succinimide (NDPS, Figure 1) was originally synthesized as an agricultural antifungal agent (Fujinami et al., 1972). In subsequent testing, NDPS was shown to be nephrotoxic in male rats at doses ≥ 0.4 mmol/kg following acute administration (Rankin, 1982; Rankin et al., 1985). Although not widely used, NDPS remains on the market as an agricultural fungicide, and it has been studied extensively as a model compound for chemically-induced kidney damage (Rankin, 2004).

Studies in rats have shown that NDPS is well absorbed, widely distributed (highest in kidney), and rapidly eliminated, mainly as metabolites in urine (Ohkawa et al., 1974; Griffin and Harvison, 1998). The major in vivo metabolites of NDPS are *N*-(3,5-dichlorophenyl)succinamic acid (NDPSA), *N*-(3,5-dichlorophenyl)-2- and 3-hydroxysuccinamic acids (2- and 3-NDHSA) and *N*-(3,5-dichlorophenyl)malonamic acid (Ohkawa et al., 1974; Griffin and Harvison, 1998). Glucuronide/sulfate and glutathione-derived conjugates of 2-/3-NDHSA and NDPS/NDPSA, respectively, have also been reported (Cui et al., 2005). Both Phase I and II metabolic pathways may be involved in the formation of a nephrotoxic species from NDPS (Rankin et al., 1987; Nyarko et al., 1997; Hong et al., 1999a,b; Cui et al., 2005). Using LC-MS/MS (Cui et al. 2005), we tentatively identified two novel amino-derived metabolites of NDPS, *N*-(3,5-dichlorophenyl)-2-aminosuccinamic acid (2-NDASA) and its *N*-acetylated derivative (*N*-acetyl-2-NDASA). These two metabolites accounted for 34.5±5.3% and 11.5±2.0% of the total radioactivity in rat kidney and liver, respectively. A potential scheme for the formation of these two metabolites from 2-NDPSA is shown in Figure 1. The

experiments described in this report were conducted to further characterize the amino metabolites and investigate the mechanism of formation of 2-NDASA in vitro.

Materials and Methods

Chemicals. 2-NDHSA and NDPSA were prepared by the methods of Kellner-Weibel et al. (1997) and Fujinami et al. (1972), respectively. All reagents were of the highest purity commercially available and were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted. Protected aspartic acid analogs, Z-*O*-methyl-*N*-carbobenzoxyaspartic acid (Z-Asp-OMe) and Z-*O*-methyl-*N*-acetylaspartic acid (Ac-Asp-OMe), were obtained from Bachem Bioscience, Inc. (King of Prussia, PA).

Animals. Male Fischer 344 rats (200-250 g) were obtained from Charles River Laboratories (Wilmington, MA) and were given a one week acclimation period before use. All experiments were approved by the Institutional Animal Care and Use Committee of the University of the Sciences in Philadelphia and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health).

In Vitro Formation of 2-NDASA. Rat liver and kidney cytosols were prepared using a standard method (Rodrigues et al., 1994). Final incubation mixtures (1 mL) contained 0.1 M (pH 7.4) potassium phosphate buffer, 2.5 mM $MgCl_2$, 100 μM 2-NDHSA and 2 mg/mL cytosolic proteins. Control incubations were performed with boiled cytosols or in the absence of cytosolic fractions. Mixtures were pre-warmed at 37^{o}C for 3 min and reactions were started with the addition of 2-NDHSA in DMSO (in 5 μL DMSO to give the final concentration of 100 μM). The mixtures were incubated at 37^{o}C for 60 min in a shaking water bath and were stopped with the addition of 2 mL of ice-cold acetonitrile containing NDPSA (2 μM) as the internal standard. The mixtures were then centrifuged

and the supernatants were evaporated to dryness under vacuum. Residues were reconstituted into 30% acetonitrile in water for HPLC-MS/MS analysis.

The involvement of different enzymes in the formation of 2-NDASA was studied using a variety of enzyme inhibitors. 4-Methylpyrazole, disulfiram, menadione, quercetin, and aminooxyacetic acid (AOAA) were dissolved in acetonitrile and 5 μL was used (to give a final concentration of 100 μM for each inhibitor). Involvement of a transamination reaction was further evaluated using L-glutamic acid (a common amino group donor) and ^{15}N-L-glutamic acid. The amino group donors were dissolved in water and 100 μL was used for a final concentration of 100 μM. Incubations in the absence of enzyme inhibitors (5 μL of acetonitrile only) were used as the no-inhibitor controls. After a 10 min preincubation, reactions were started with the addition of 2-NDHSA (in 5 μL DMSO for a final concentration of 100 μM). Incubation conditions and sample treatments were the same as described above.

HPLC-MS/MS. Formation of 2-NDASA in rat liver and kidney cytosols was determined using an HPLC-MS/MS assay. The system consisted of Agilent HP1100 HPLC coupled to a Finnigan TSQ7000 triple quadrupole mass spectrometer. Analytes were eluted on a Phenomenex Luna^{TM} C18-2 column (2.0 x 50 mm, 5 μm, Phenomenex, Torrance, CA) using 25 mM ammonium formate, pH 3 (A) and 0.1% formic acid in acetonitrile (B) as mobile phase components. The HPLC was operated at a flow rate of 0.25 mL/min and the elution gradient was as follows: 0-10 min, 10%-90% B; 10-11 min, 90% B; and 11-12 min, 90% -10% B. The system was re-equilibrated at 10% B for 8 min prior to the next injection.

2-NDASA formation was monitored using positive ESI-MS. Single reaction monitoring (SRM) scans with the precursor ion at *m/z* 277 and product ion at *m/z* 162 were performed to detect 2-NDASA. The internal standard, NDPSA, was monitored under negative ESI and SRM scans from *m/z* 260 to *m/z* 160 were used. Under the HPLC conditions described, NDPSA and 2-NDASA had retention times of 7.2 min and 9.4 min, respectively. Collision energy at ± 25 eV and collision gas (argon) at 1.7 mtorr were used in all scans. The assay exhibited a linear dynamic range of 0.05 to 64 μM for 2-NDASA.

Chemical Syntheses.

N-(3,5-Dichlorophenyl)-2-aminosuccinamic Acid (2-NDASA).

The protected aspartic acid, Z-Asp-OMe (281.3 mg, 1 mmol), was dissolved in 20 mL of dichloromethane containing 0.5% triethylamine. 3,5-Dichloroaniline (162.0 mg, 1 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (155.2 mg, 1 mmol) were then added into the solution and the resulting mixture was stirred at room temperature for 2 h. The mixture was cooled to -10^{o}C and BBr_3 (10 mL of a 1 M solution in hexane) was added dropwise while stirring. After stirring for another hour at room temperature, the reaction was stopped with the addition of water (50 mL, dropwise). The dichloromethane layer was washed 3 times with water (25 mL) and the combined water layers were dried under vacuum. Residues were then purified using a semi-preparative HPLC system equipped with Agilent HP1100 pumps and a Zorbax Rx-C8 column (9.6 mm x 25 cm, 5 μm). Water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) were used as the mobile phase components, and the flow rate was 2 mL/min. The gradient was as follows: 0-15 min, 10-40%B; 16-19 min, 80%B; 19-20 min, 80-

10%B; and the column was re-equilibrated at 10%B for 6 min before the next injection. Structure of the isolated product was confirmed by ^{1}H NMR (300 MHz) and LC-MS/MS. ^{1}H-NMR (d_6-DMSO): δ 2.99 (m, 2H, -C*H*$_2$CONH-), 4.13 (m, 1H, -C*H*NH_2), 7.33 and 7.65 (s, 3H, $C_6H_3Cl_2$-). MS/MS (CID of $(M-H)^-$ at m/z 275): 258 ($(M-H)^-$-NH_3), 214 ($(M-H)^-$-NH_3-CO_2), 160 (dichloroaniline-H), 114 ($(M-H)^-$-dichloroaniline).

N-(3,5-Dichlorophenyl)-2-*N*-Acetylaminosuccinamic Acid (*N*-acetyl-2-NDASA).

N-Acetyl-2-NDASA was synthesized using the same method as described above for the synthesis of 2-NDASA, except that Ac-Asp-OMe (189.2 mg, 1 mmol) was used as the starting protected aspartic acid. The reaction product was isolated using the same preparative HPLC system described above. ^{1}H-NMR and MS/MS characteristics of the *N*-acetyl-2-NDASA product were as follows: ^{1}H-NMR (d_6-DMSO): δ 1.83 (s, 3H, C*H*$_3$CO-), 2.73 (m, 2H, -C*H*$_2$CONH-), 4.58 (m, 1H, -C*H*$NHCOCH_3$), 7.27 and 7.64 (s, 3H, $C_6H_3Cl_2$). MS/MS (CID of $(M-H)^-$ at m/z 317): 299 ($(M-H)^-$-H_2O), 257 ($(M-H)^-$-H_2O-C_2H_2O), 160 (dichloroaniline-H), 156 ($(M-H)^-$-dichloroaniline).

Statistical Analyses. Statistical tests were performed using the SigmaStat, version 2.03 (Copyright Jandel Corporation, 1986-1992) software package. Analyses included descriptive statistics and comparisons of group means by a one-way ANOVA followed by the Student-Newman-Keuls test. A 5% level of significance was used for all analyses.

Results and Discussion

In a previous study (Cui et al., 2005), two amino-derived metabolites of NDPS were detected in male Fischer 344 rats and we numbered them **M6** and **M8**. The two metabolites were detected in higher concentrations in rat kidney tissues than in liver (**M6**, 0.3 vs. 0.1 μmol/g of tissue; **M8**, 0.2 vs. 0.1 μmol/g of tissue) (Cui et al., 2005). The purpose of the current study was to further characterize these metabolites and investigate the mechanism of formation of **M6** in vitro.

HPLC-extracted ion chromatograms of 2-NDHSA and its incubation product with rat liver cytosols are shown in Figures 2A and 2B, respectively. Under the HPLC conditions previously used for in vivo samples, the incubation product had an identical retention time and MS spectrum as that of **M6** observed in rat urine and tissue homogenates (Cui et al., 2005). In negative Q1 scans the incubation product (**M6**) had an $(M-H)^-$ ion at m/z 275. The negative CID mass spectra of m/z 275 (Figure 2C) for **M6** and the synthetic standard of 2-NDASA (see Materials and Methods) exhibited major MS/MS fragments at m/z 258, 214, 160 and 114. Loss of ammonia in **M6** (from m/z 275 to m/z 258, loss of 17 Da) and the subsequent loss of a carboxylic group (44 Da to m/z 214) suggested the structure of **M6** as an amino derivative of NDPSA. To further characterize the position of the amino group, positive CID scans of m/z 277 for **M6** were performed (Figure 2D). Fragment ions at m/z 231 and m/z 74 provided evidence that the amino group was attached to the carbon at the 2 position of NDPSA. The fragment ion at m/z 116 also supported that an amino group was added to the succinamic acid moiety. Since the HPLC retention times and MS/MS spectra of **M6** formed in vitro

(Figure 2C) and in vivo (Cui et al, 2005) were identical to those obtained from synthetic 2-NDASA, **M6** was identified as 2-NDASA.

Metabolite **M8** was previously observed in rat urine and liver and kidney homogenates, and was tentatively identified as the *N*-acetyl derivative of **M6** (Cui et al., 2005). In the current study, we used LC-MS and a synthetic standard to confirm the proposed structure of **M8** as *N*-acetyl-2-NDASA. Major MS/MS fragments at m/z 299, 257 and 160 were found in the negative CID mass spectrum of m/z 317 for synthetic *N*-acetyl-2-NDASA (see Materials and Methods) and **M8** (Cui et al., 2005). Although **M6** and **M8** were detected in hepatic and renal homogenates prepared from rats that received NDPS (Cui et al., 2005), only **M6** was produced by rat liver and kidney cytosols. This may be due to insufficient cofactor, i.e. acetyl co-enzyme A, being present in the cytosolic preparations to support the acetylation of **M6** to **M8** in vitro. Fortifying acetyl co-enzyme A in rat cytosolic incubations has been shown to produce acetylated metabolites that were absent without the cofactor (Martire et al., 1991). Alternatively, it is also possible that the acetylation reaction is not cytosolic. For example, evidence exists that aliphatic amines may be acetylated by liver and kidney microsomal enzymes in the presence of acetyl co-enzyme A (Green and Elce, 1975).

A scheme depicting the metabolic pathways involved in the formation of 2-NDASA (**M6**) and its acetylated derivative (**M8**) is proposed in Figure 1. To study the enzymes involved in the formation of **M6**, in vitro incubations were performed in the presence of a variety of chemical inhibitors. The α-keto acid intermediate (Figure 1) was not monitored because of its poor ionization potentials in mass spectrometry. Suppression of 2-NDASA formation in rat liver cytosol by various enzyme inhibitors is

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shown in Figure 3. Only a minimal amount (just above the limit of quantitation of 50 nM) of 2-NDASA was produced when 2-NDHSA was incubated with boiled rat liver cytosol, which suggests the reaction was enzyme catalyzed. Inhibitors of alcohol dehydrogenase (4-methylpyrazole) and aldehyde dehydrogenase (disulfiram) reduced 2-NDASA formation by 40 to 50%. Menadione (an inhibitor of aldehyde oxidase) and quercetin (an inhibitor of carbonyl reductase) did not show any effects. AOAA, an inhibitor of PLP-containing enzymes such as aminotransferases, almost completely abolished the formation of 2-NDASA in vitro. These data suggest that oxidative enzymes, such as cytosolic alcohol and aldehyde dehydrogenases and aminotransferases, may be involved in the biotransformation of 2-NDHSA to 2-NDASA in rat liver cytosol. To further confirm that transamination is involved in the formation of 2-NDASA, 2-NDHSA was incubated with rat liver cytosol in the presence of an ^{15}N-labeled amino group donor (^{15}N-glutamic acid). A positive full scan mass spectrum obtained from the incubation mixture with the addition of a non-labeled glutamic acid is shown in Figure 4A, while the corresponding MS spectrum in the presence of the ^{15}N-glutamic acid is shown in Figure 4B. A molecular ion at m/z 277 in Figure 4A is consistent with the formation of ^{14}N-2-NDASA. With the addition of ^{15}N-glutamic acid, approximately one-half of the $(M+H)^+$ ion shifted from m/z 277 to m/z 278, and the chlorine isotope peak shifted from m/z 279 to m/z 280. CID product ion spectra of the ^{14}N- and ^{15}N- products were consistent with the incorporation of the amino group at the C-2 position of the succinamic acid ring (Figures 4C and 4D). These data suggest that an approximately 1:1 mixture of ^{14}N-2-NDASA and ^{15}N-2-NDASA were formed in the

presence of ^{15}N-glutamic acid. The partial incorporation of ^{15}N can be explained by the presence of endogenous amino group donors in the liver cytosol preparations.

Formation of 2-NDASA in rat kidney was also evaluated using 2-NDHSA as the substrate. Under the same incubation conditions, formation of 2-NDASA was significantly higher in rat kidney than in liver (2.9±0.2 vs. 1.0±0.2 nmol/mg protein/hr, respectively). As in liver cytosol, *N*-acetyl-2-NDASA was not produced by the renal preparations. In rat kidney cytosol fractions, the involvement of alcohol and aldehyde dehydrogenases and aminotransferases in the formation of 2-NDASA was indicated by the inhibitory effects of 4-methylpyrazole (39.0±8.8% inhibition), disulfiram (36.9±6.7% inhibition), and AOAA (88.3±2.3% inhibition).

The identification of 2-NDASA and its acetylated product suggested that a transamination reaction is involved in the metabolism of NDPS in rats. Similar metabolic pathways involving oxidation and transamination have been proposed in the metabolism of several xenobiotics. Examples include the formation of an amino metabolite of 3-(phenylamino)-1,2-propanediol in human liver preparations in studies of its involvement in Spanish toxic oil syndrome (Mayeno et al., 1995); and identification of an *N*-acetylated metabolite of phenyl glycidyl ether (PGE) in rat urine in studies of PGE-induced dermatitis (de Rooij et al., 1998). The observation that formation of 2-NDASA was significantly higher in rat kidney compared to liver may be partially explained by the presence of two different isozymes of glutamine transaminases (L and K) in the two tissues. Kidney glutamine transaminase (glutamine transaminase K, also known as cysteine conjugate β-lyase) was shown to have higher activity than glutamine transaminase L, and has high affinity towards aromatic α-keto acids (Cooper and

Meister, 1981). Although 2-NDASA was detected in higher amounts in kidney homogenates than in liver (Cui et al., 2005), its role in NDPS-induced nephrotoxicity, if any, remains to be determined. Recently, we found that 2-NDASA (up to 1 mM) was not toxic to isolated renal cortical cells (unpublished results). However, the in vivo effects of 2-NDASA in rats have not been studied.

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Figure Legends

Figure 1. Proposed metabolic scheme for the formation of the amino metabolites of NDPS. The α-keto acid intermediate (in parenthesis) was not monitored in the experiments.

Figure 2. Mass spectral analysis of an incubate of 2-NDHSA (substrate) with rat liver cytosols. (A) HPLC-Extracted ion chromatograms (XIC) of m/z 276 for 2-NDHSA, (B) XIC of m/z 275 showing the formation of 2-NDASA, (C) negative CID product ion spectrum of m/z 275 and (D) positive CID product ion spectrum of m/z 277.

Figure 3. Effect of various enzyme inhibitors on the formation of 2-NDASA in rat liver cytosols. Results are expressed as means ± SD (n=6). *Values that are significantly different ($p < 0.05$) from the corresponding results in the complete incubations.

Figure 4. Mass spectra of the 2-NDHSA incubation products in rat liver cytosol in the presence of either ^{14}N-glutamic acid or ^{15}N-glutamic acid. (A) Positive full scan MS spectra of ^{14}N-2-NDASA and (B) a mixture of ^{14}N- and ^{15}N-2-NDASA. (C) Positive CID product ion spectrum of m/z 277 for ^{14}N-2-NDASA and (D) CID product ion spectrum of m/z 278 for ^{15}N-2-NDASA.

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Figure 1

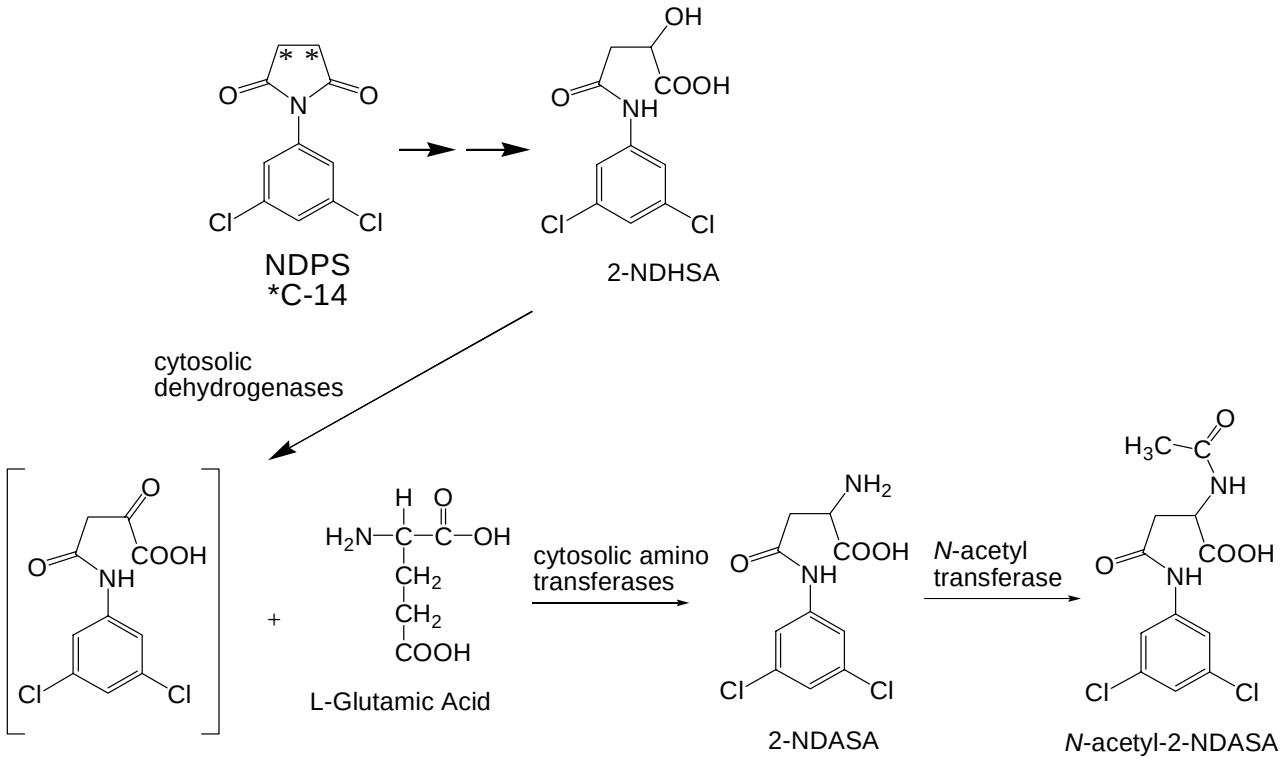

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Figure 2

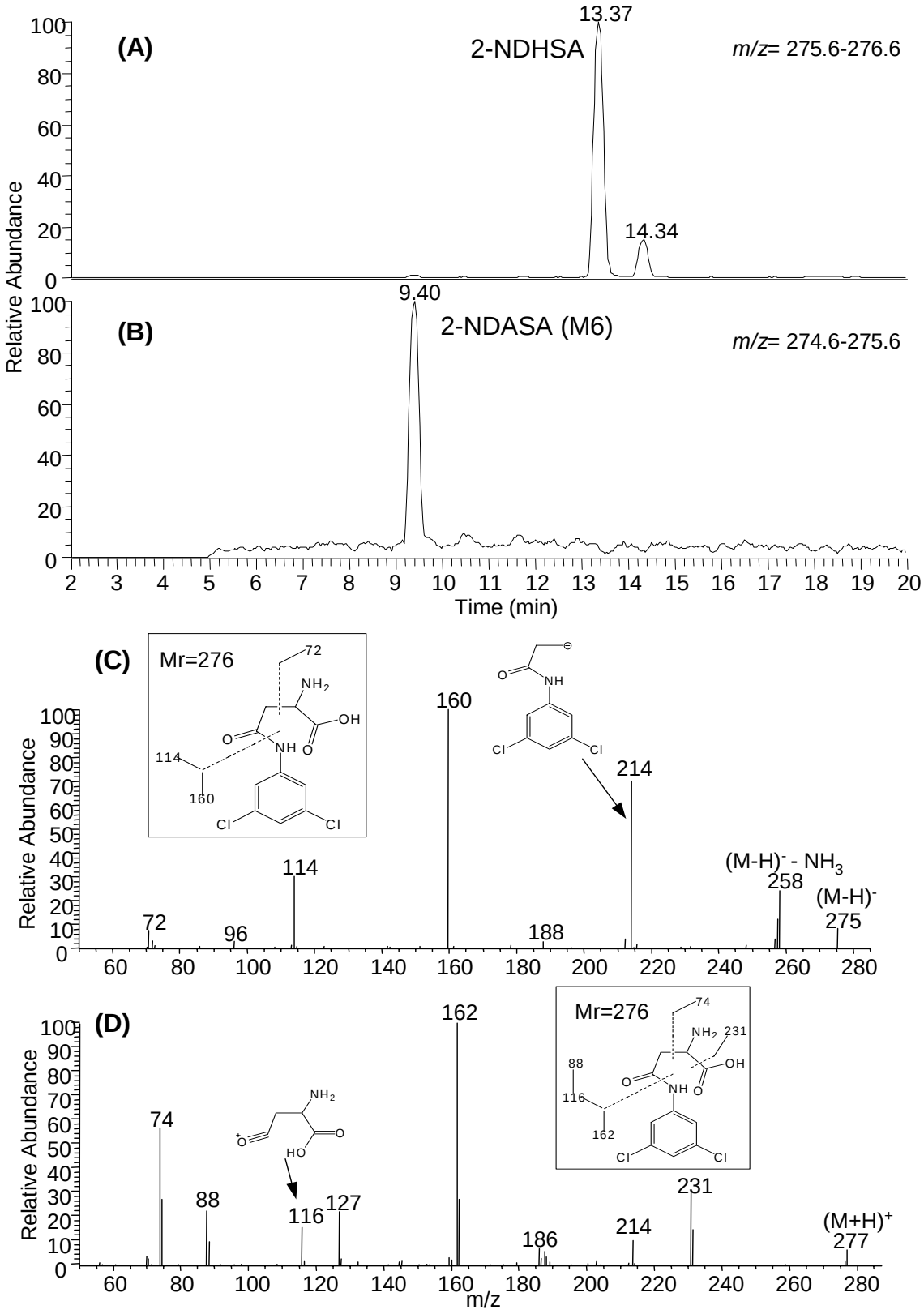

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Figure 3

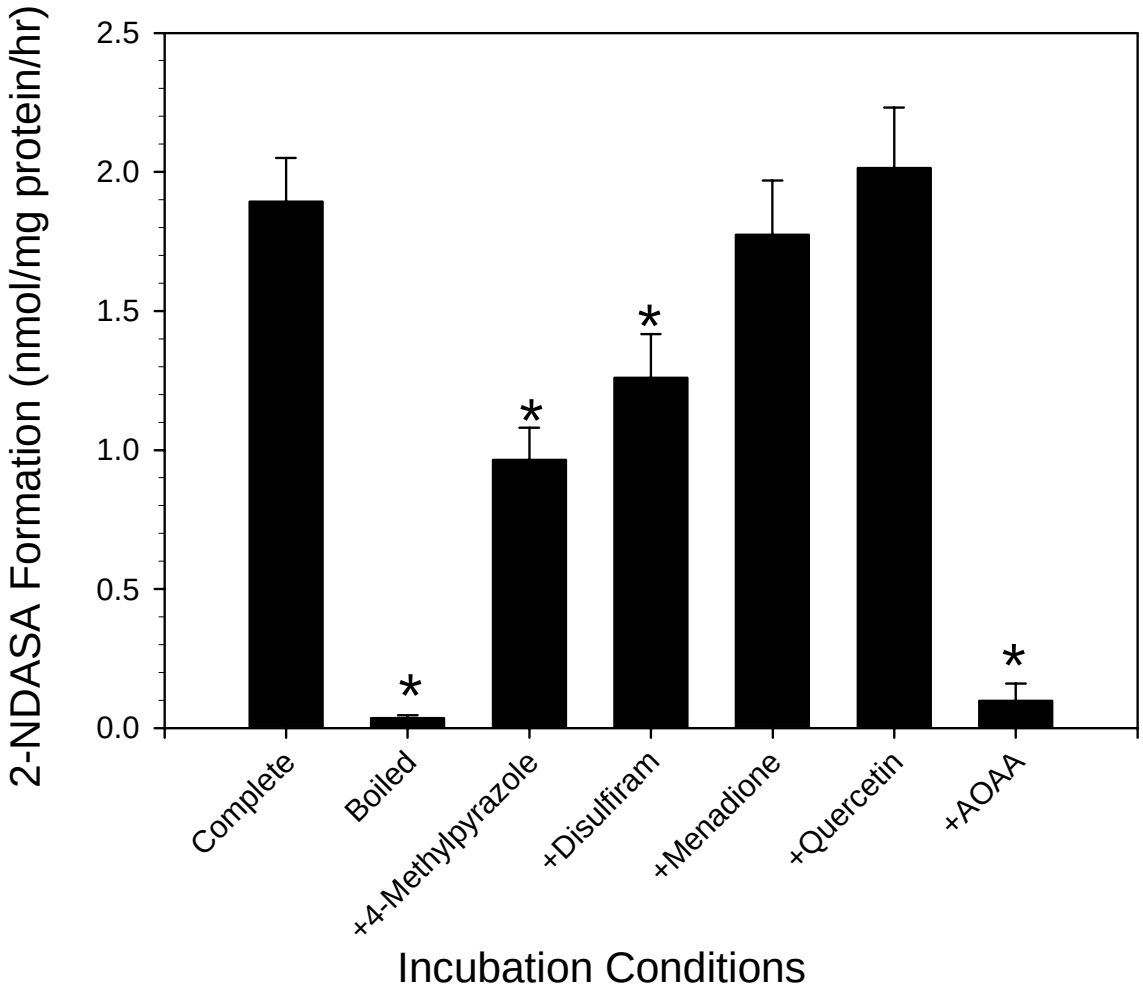

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Figure 4