

Identification of A Novel *In Vitro* Metabonate from Liver Microsomal Incubations

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(d) List of abbreviations:

NADPH, Nicotinamide Adenine Dinucleotide Phosphate

HPLC, High Pressure Liquid Chromatography

LC/MS, Liquid Chromatography / Mass Spectrometry

Q-TOF, Quadrupole Time-Of-Flight

Abstract

In vitro drug metabolism studies during early drug discovery stage are becoming increasingly important. With the increasing demand on high throughput and quick turn around time for *in vitro* metabolism studies, however, careful examination of the results and proper design of the experiments are still crucial. In this communication, we report the identification and mechanism of formation of a novel metabonate from incubations of a di-amine containing compound with liver microsomes. The metabonate appeared to be the major product, and its formation was NADPH and microsomal protein dependent. LC/MS and NMR analysis of the metabonate indicated an extra carbon and unusual formation of an imidazolidine ring. Further studies revealed that this metabonate was not a true biotransformation product from the di-amine compound itself in the microsomal incubation, but rather a product resulting from a condensation reaction between the compound and a metabolite of the solvent (alcohol) used in the incubation. When the microsomal incubations contained a small amount of methanol or ethanol as solvent, the alcohols were metabolized to formaldehyde or acetaldehyde, which then condensed with the di-amine compound through an imine intermediate to form the metabonate. The compound itself was metabolically stable *in vitro* when acetonitrile or DMSO was used as solvent. During the study of *in vitro* microsomal stability and metabolite identification of amine containing compounds, the use of alcohol as solvent should be avoided.

Introduction

In vitro metabolite identification studies during the drug discovery stage are an important part of lead optimization. Often, high metabolic “soft spots” in a molecule contribute to poor pharmacokinetic properties. Early identification of the metabolically labile sites in a particular structural series can help the medicinal chemists to better design compounds with improved metabolic stability. In addition, characterization of potentially active or toxic metabolites can potentially enable lead optimization programs to design more potent and safer drug candidates.

The advances in liquid chromatography and mass spectrometry have allowed the *in vitro* drug metabolism studies to be carried out in a fairly high throughput fashion. High throughput microsomal stability assays have become standard screening assays in the pharmaceutical industry for reducing metabolic liability in discovery stage. Further *in vitro* metabolite identification studies are carried out for lead compounds to address liabilities associated with pharmacokinetic properties or reactive metabolite formation, and, therefore, have become crucial in providing synthetic directions. Typically, the *in vitro* incubations contain a small amount of organic solvent in which the compound of interest is dissolved. The effect of common organic solvents on *in vitro* cytochrome P450 enzyme activities has been reported (Chauret et al., 1998). DMSO was found to inhibit several P450-mediated reactions, while methanol and acetonitrile exhibited less inhibitory effect when present at less than 1%. As a result, methanol and acetonitrile have been used as the preferred solvents for performing *in vitro* metabolism studies.

In this study, we report the formation of novel metabonates of 1,2 ethylene diamine containing compounds. Beckett has used the term “metabonate” to describe a

substance, which appears to be a metabolite but which in reality, is an artifact, formed during experimental conditions, isolation and/or storage (Beckett et al., 1971).

Methods

Chemicals and Reagents:

Compounds A and B (R1-NH-CH₂-CH(NH₂)-CH₂-R2) were synthesized by the Amgen Chemical Research Department, Thousand Oaks, CA. NADPH was purchased from Sigma-Aldrich (St. Louis, MO). Human and rat liver microsomes were obtained from Xenotech (Lenexa, KS).

***In Vitro* Incubations:**

Compound of interest (10 μM) was pre-incubated for 5 minutes with rat or human liver microsomes (1 mg/mL) in 0.1 M phosphate buffer (pH 7.4) at 37°C in a total volume of 0.5 mL. NADPH (1 mM) or buffer was added to the incubation, and incubated at 37°C for 30 minutes. Reactions were terminated using equal volume of acetonitrile. Samples were centrifuged at 14,000 rpm for 10 minutes and supernatants were analyzed by LC/MS.

Mass Spectrometric Analysis:

LC/MS analysis was carried out using a YMC ODS-AQ column (3 mm, 2.0x100 mm) at a flow rate of 0.25 mL/min. Mobile phase contained A: H₂O, 0.1% formic and B: CH₃CN, 0.1% formic. A linear gradient elution (0-5 min, 5% B, 5-25 min, linear to 65% B) was used for separation. The entire flow after UV detector was directed to the electrospray source of a Q-TOF (Waters) mass spectrometer, operated in the positive ion mode and V configuration of TOF. The electrospray voltage was set to 3.5 kV, while the

cone voltage was tuned to 35 V. The Z-spray source block and desolvation temperatures were at 100 °C and 350 °C, respectively. Nebulizing gas flow was set to maximum, and desolvation gas flow was set to 750 L/h. A solution containing 2 μM leucine enkephalin in 50:50 acetonitrile and water was infused to the lock spray source. MH⁺ of leucine enkephalin at m/z 556.2771 was used as lock mass for exact mass measurement correction. Mass spectra were acquired from 100 to 1000 amu with 1 s scan cycle time. For MS/MS experiments, collision energy was set to 25 eV with argon as collision gas.

NMR Analysis:

Isolated metabolite was dissolved in MeOH-d₄ and manually loaded into a MRM Protasis microcoil NMR probe with a total volume of 5 μL. Spectra were acquired on a Bruker AV600 NMR spectrometer.

Results and Discussion

Compound A (R1-NH-CH₂-CH(NH₂)-CH₂-R2) and several structural analogs showed moderate *in vitro* clearance in rat and human microsomal stability assays, and moderate to high *in vivo* clearance in rats. *In vitro* metabolism studies were then carried out using rat and human liver microsomes to identify the metabolic liabilities.

Compound A appeared to be metabolized to one major metabolite (Ma) in both human and rat liver microsomes, as shown in Figure 1. The formation of Ma was microsomal protein, time and NADPH dependent. Metabolite Ma appeared to be less hydrophilic than the parent compound A, as it eluted after compound A on the reversed phase HPLC. Full scan and tandem mass spectra of compound A and metabolite Ma are shown in Figure 2. Metabolite Ma displayed a molecular ion 12 amu higher than that of compound

A. Accurate mass measurement indicated an additional carbon and one extra double bond equivalence (DBE) over the parent compound A. Tandem mass spectrum of Ma was identical to that of parent drug A, with major fragment ions at m/z 232, 244 and 348 which corresponded to $R1-NH_3^+$, $R1-NH-CH_2^+$ and $R1-NH-CH_2-CH^+-CH_2-R2$. The addition of one extra carbon of Ma was likely on the primary amine of compound A based on the tandem mass spectra. Metabolite Ma was isolated from the human liver microsomal incubation of compound A in the presence of NADPH. NMR analysis of isolated Ma showed all proton signals as in compound A, plus two additional doublets 10' and 10'', as shown in Figure 3. The two doublets had chemical shifts at 4.34 and 4.58 ppm, and a coupling constant of 7.9 Hz, indicative of the methylene protons adjacent to two heteroatoms (imidazolidine protons at C-10 position). The NMR data is consistent with information obtained from mass spectral data. The formation of Ma was quite unusual, and can not be explained from any common biotransformation pathways.

The *in vitro* microsomal incubations contained 1% methanol in which compound A was dissolved. We hypothesized that the source of one additional carbon might be coming from the solvent methanol. The incubations were then repeated using different solvents, as illustrated in Figure 4 for a structural analog compound B. The formation of the metabolite was clearly solvent dependent, as shown in Figure 5. When the incubation contained 1% methanol, metabolite Mb1 whose molecular ion was 12 amu higher than that of parent compound B was detected. When the incubation contained 1% d₄-methanol, a similar metabolite (Mb2), which had the same retention time as Mb1, but had 2 amu mass increments over Mb1, was observed. When the incubation contained 1%

ethanol, a metabolite (Mb2) with longer retention time than Mb1 and a mass increment of 26 amu over the parent compound was detected, but was formed to a lesser extent.

The “metabolites” identified were not true biotransformation products from the parent compound itself, but rather from chemical reaction between the parent compound and metabolites of the solvents used. We propose a mechanism of formation of the novel metabonates as condensation reaction between formaldehyde (or acetaldehyde) and the amine containing xenobiotic through an imine intermediate, as shown in Figure 6.

Formaldehyde (or acetaldehyde) was formed as consequence of alcohol dehydrogenase catalyzed metabolism of methanol or ethanol (Dawidek-Pietryka et al, 1998), which was present as solvent for compound in the microsomal incubations. A similar finding has been also reported by Yin et al. (2001) for compounds containing 1,2-diamino and 1,2-amino hydroxyl groups in microsomal incubations containing methanol.

In order to test the hypothesis that the reactive species is aldehyde, a small amount of formaldehyde was added to the solution containing the di-amine compound A (or B), which resulted in the instantaneous formation of product Ma (or Mb1). This reaction is a typical Mannich reaction (Mannich reaction, Thompson, 1968). When acetonitrile or DMSO was used as solvent in the microsomal incubation, compound A and B had little turn over, and were metabolically stable. A very minor Mb1 was detected for compound B (Figure 4), but it was also present in similar amount in all control incubations without NADPH, likely due to trace amount of formaldehyde in the incubation solutions or in HPLC mobile phases.

There have been a few examples of metabonate formation in the literature. A new imidazolidone metabolite was discovered during the course of studying the

biotransformation of lidocaine in man (Breck and Trager, 1971; Nelson et. al., 1973).

This metabolite was a product of the condensation reaction between acetaldehyde and a major N-dealkylated metabolite of lidocaine. A diarylimine was detected as a metabolite of N-benzyl-4-chloroaniline, which was a chemical artifact from two debenzylated metabolites (Low et. al., 1994).

Our results indicated that if careful examination of the metabolite structure and its mechanism of formation were not done, wrong conclusions might be drawn. The moderate to high *in vitro* micromal clearance (in methanol) of the di-amine compounds was initially thought to be the cause of moderate to high *in vivo* clearance of these compounds in rats. The clearance mechanism was explored further using bile-duct-cannulated rats, and significant amount of unchanged parent drug was detected in the bile, metabolism played a minor role in the elimination of the di-amine compounds. In the Caco-2 assay, these compounds also showed significant efflux ratios. Whether or not the imidazolidine metabolite is formed *in vivo* or has any implication in the *in vivo* clearance of the di-amine compounds is not clear. The presence of alcohol can strongly affect the liability and interpretation of *in vitro* metabolism of these di-amine containing compounds. During the study of *in vitro* microsomal stability and metabolite identification of amine containing compounds, the use of alcohol as solvent should be avoided.

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Footnotes

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Legends for Figures

- Figure 1 HPLC/UV Trace of *In Vitro* Human Liver Microsomal Incubation of Compound A With (a) or Without (b) NADPH
- Figure 2 Accurate Mass LC/MS Spectra of (a) Compound A, and (b) Metabolite Ma, and tandem mass spectra of (c) Compound A and (d) Metabolite Ma
- Figure 3 Selected Regions of NMR Spectra of (a) Metabolite Ma and (b) Compound A
- Figure 4 Solvent Effects on the Formation of Metabonate of Compound B
- Figure 5 Mass Spectra of (a) Compound B, (b) Mb1, (c) Mb2 and (d) Mb3
- Figure 6 Mechanism of Metabonate Formation

Figure 1

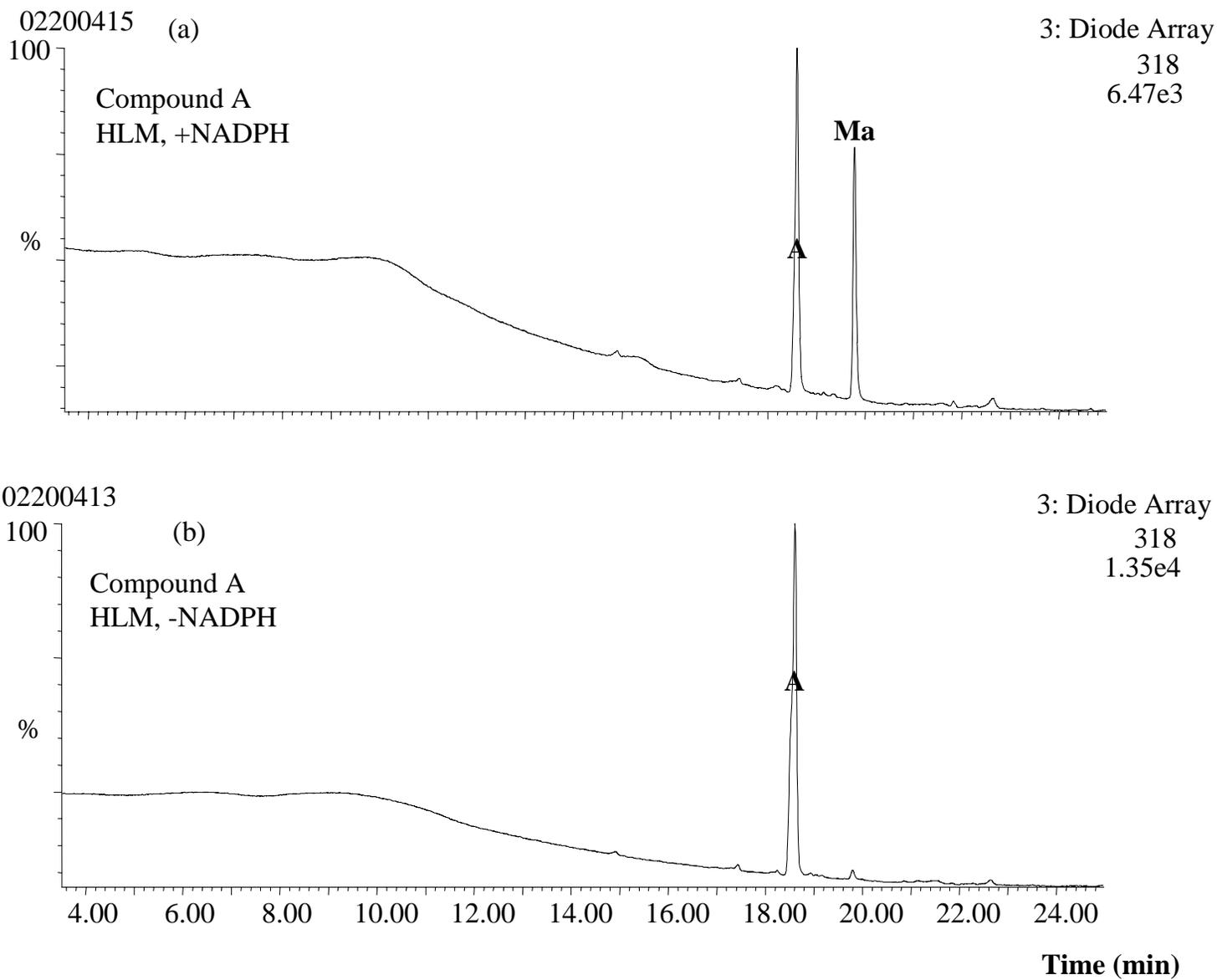
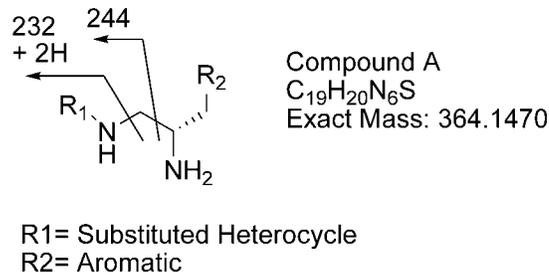
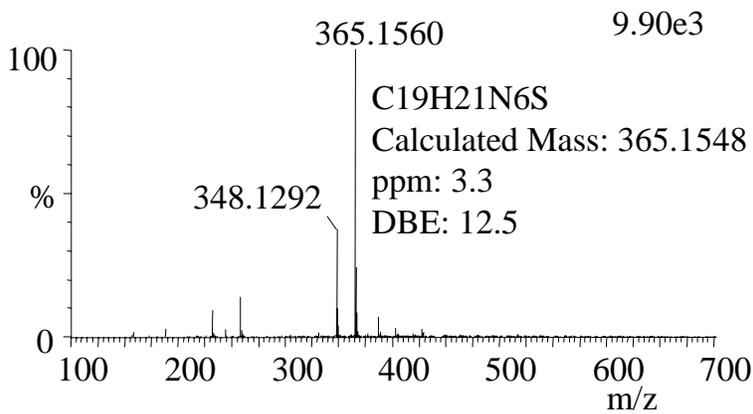


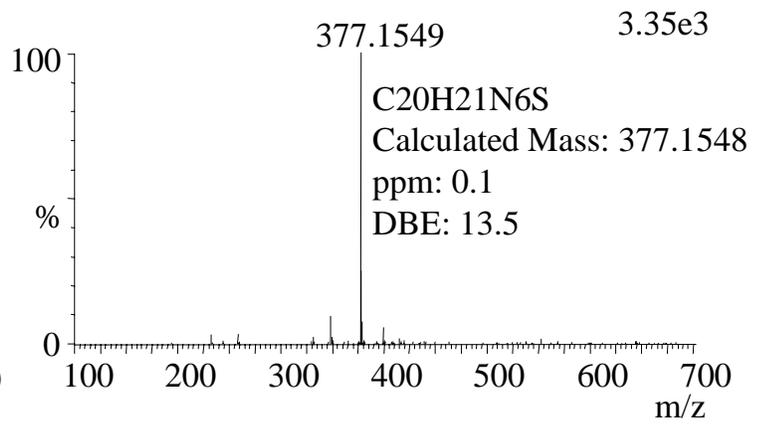
Figure 2



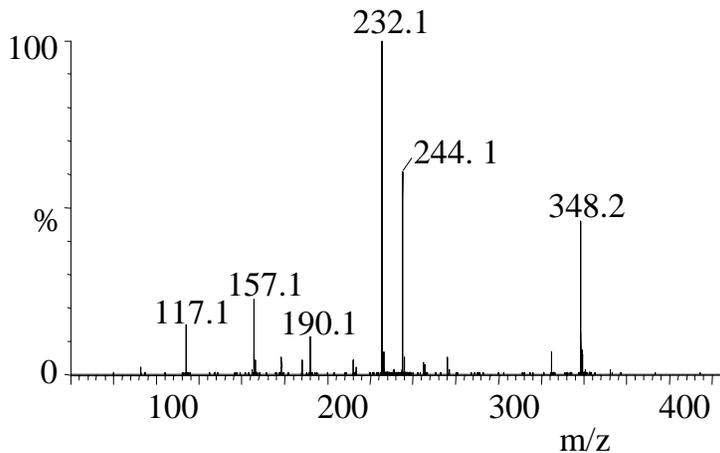
(a)
Compound A



(b)
Metabolite Ma



(c)
Compound A



(d)
Metabolite Ma

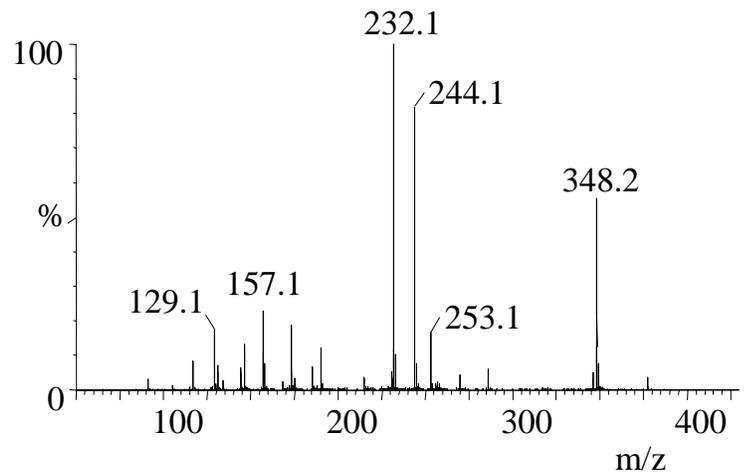


Figure 3

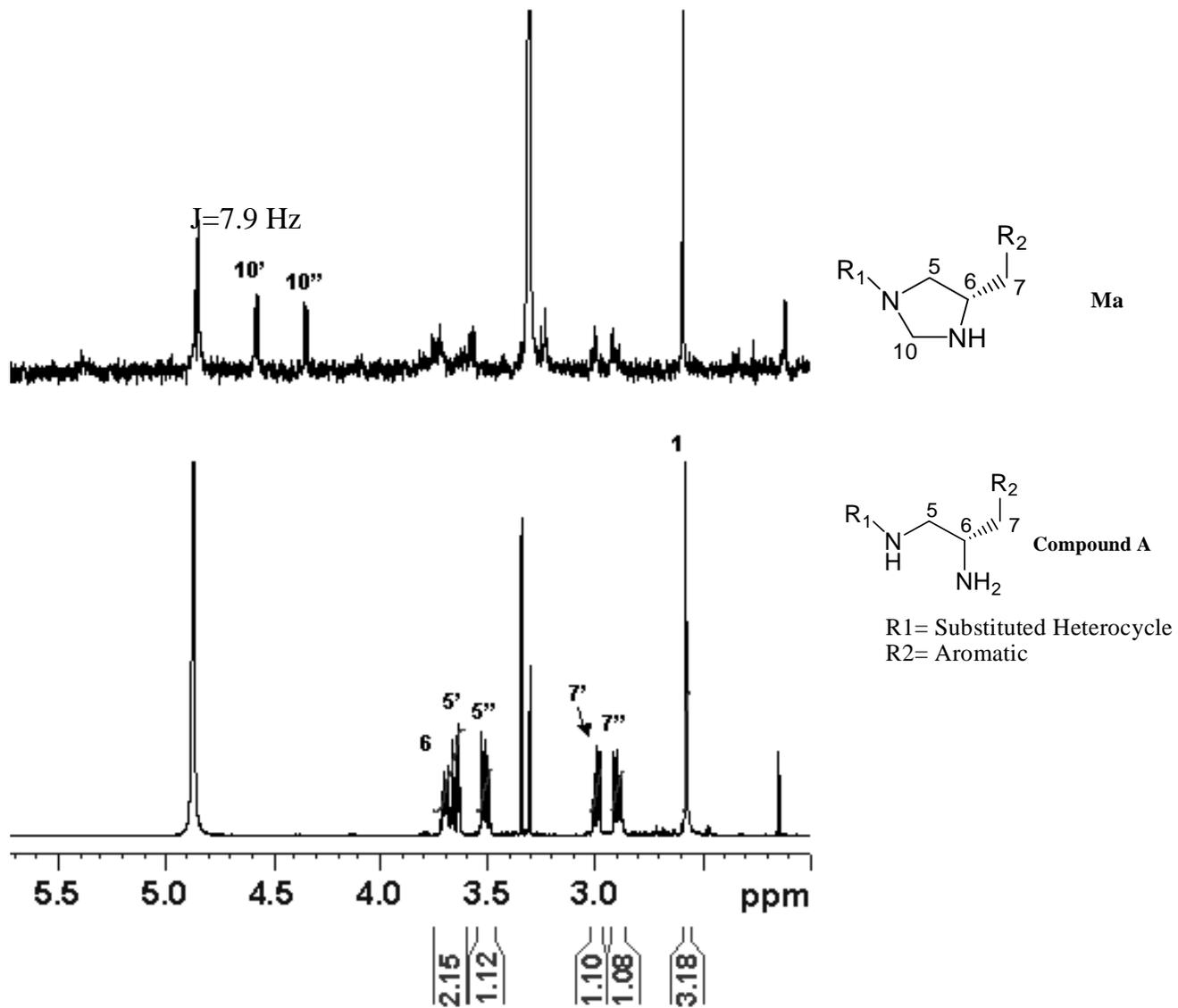


Figure 4

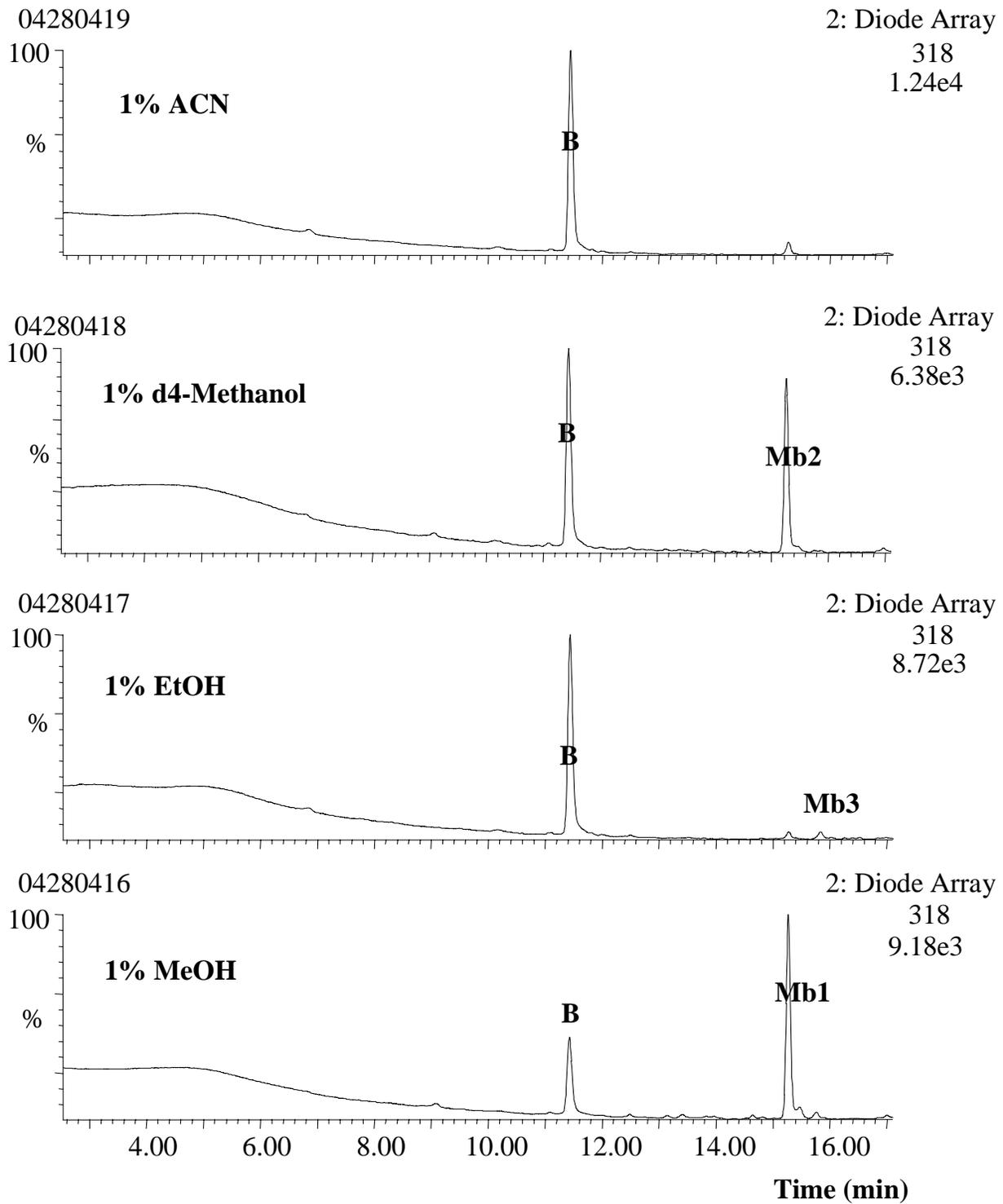


Figure 5

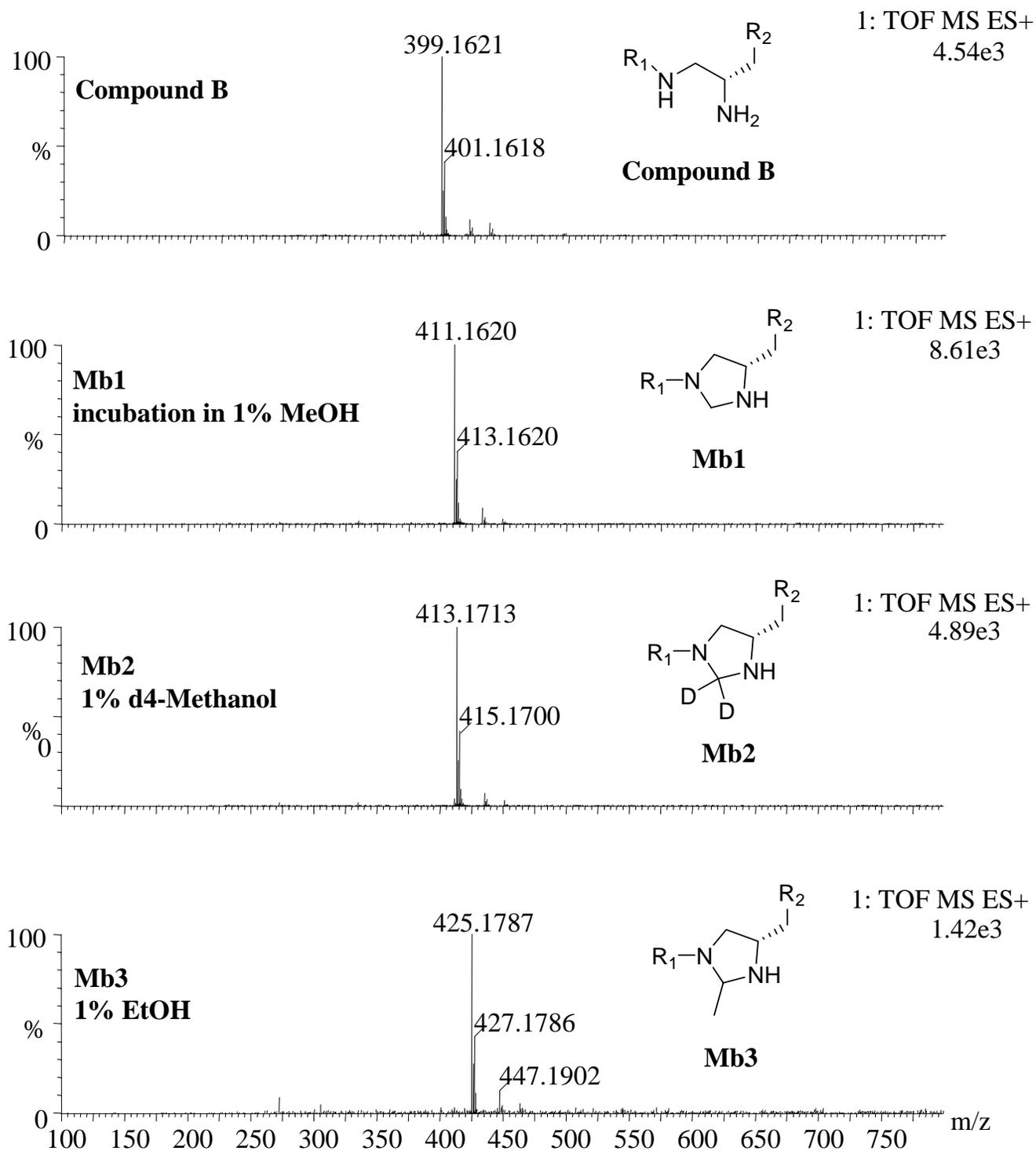


Figure 6

