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A Novel Biotransformation of Alkyl Amino Pyrrolidine to Amino Piperidine Ring by Human CYP3A

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Running Title: Mechanistic studies on enzymatic conversion of alkyl amino pyrrolidine to amino piperidine

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Abbreviations: CYP, cytochrome P450; HLM, human liver microsomes; human liver S9, human liver post-mitochondrial supernatant; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ESI, electrospray ionization; amu, atomic mass units; CID, collision-induced dissociation.

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Abstract

The novel biotransformation of an aminopyrrolidine to an aminopiperidine during the metabolism of AMG657417, 5-(4-chlorophenyl)-3-methyl-2-((2R)-2-(((1-methylethyl)amino)methyl)-1-pyrrolidinyl)-6-(4-pyridinyl)-4(3H)-pyrimidinone, was investigated using the NADPH-fortified S9 fraction from human liver. The major metabolite (M18) had a protonated molecule (MH^+ *m/z* 438) identical to that of AMG657417 except that it eluted earlier on a reverse phase HPLC. The structure of M18 had been identified as 5-(4-chlorophenyl)-3-methyl-2-((1-(1-methylethyl)-3-piperidinyl)amino)-6-(4-pyridinyl)-4(3H)-pyrimidinone (**I**) by LC/MS and proton NMR. M18 was not observed when AMG657417 was incubated with either microsomal or cytosolic fraction from human liver, suggesting the involvement of both microsomal and cytosolic enzymes in the biotransformation. The reaction mechanisms have been elucidated by trapping the intermediates formed during the biotransformation. An aldehyde intermediate was initially produced by hydroxylation and opening of the pyrrolidine ring of the parent molecule, followed by intramolecular Schiff-base formation between the exocyclic isopropylamine nitrogen and the aldehyde carbonyl to form a piperidinyl iminium ion. The iminium ion was then reduced to the piperidine product. The presence of the aldehyde intermediate was verified by the formation of semicarbazide conjugates in human liver microsomal, S9, and recombinant CYP3A4 incubations of AMG657417. The presence of the piperidinyl iminium ion intermediate was confirmed by the formation of cyanide conjugates in the incubations in human liver S9. Two cyanide conjugates with identical protonated molecule and product ion mass spectra were observed, indicating the likelihood of diastereomer formation. A chemical

inhibition study in NADPH-fortified S9 fraction indicated that the oxidation of AMG657417 was catalyzed almost exclusively by CYP3A.

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Introduction

The inhibition of p38α and reduction of its downstream pro-inflammatory cytokines is a promising therapeutic strategy to suppress inflammation and prevent joint damage caused by rheumatoid arthritis (RA). AMG657417, 5-(4-chlorophenyl)-3-methyl-2-((2R)-2-(((1-methylethyl)amino)methyl)-1-pyrrolidinyl)-6-(4-pyridinyl)-4(3H)-pyrimidinone, is a potent inhibitor of p38α, a mitogen-activated protein kinase (MAPK) which plays a key role in signaling transduction in response to cellular stress and mediates the biosynthesis of a number of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Lee and Dominguez, 2005). One of the major metabolites of AMG657417 was designated as M18, 5-(4-chlorophenyl)-3-methyl-2-((1-(1-methylethyl)-3-piperidinyl)amino)-6-(4-pyridinyl)-4(3H)-pyrimidinone. The structure of M18 was characterized by mass spectrometry and proton NMR and confirmed with synthesized standard in earlier studies. The biotransformation from AMG657417 to M18 (Figure 1) involved the conversion of the five-membered pyrrolidine (isopropylpiperidinylamino moiety) in AMG657417 to a six-membered piperidine (methylethyl-aminomethyl-pyrrolidinyl moiety), which requires the metabolism to proceed through a ring-opening followed by ring-closing process during the conversion. Pyrrolidine and piperidine rings are saturated azaheterocycles, which often form lactams and their corresponding ring-opened carboxylic acids. Examples of this include (*S*)-nicotine. The major metabolic route of (*S*)-nicotine is α-carbon hydroxylation leading to ring-opening by C-N cleavage (Peterson, 1988). Another example is phencyclidine. The piperidine ring of phencyclidine undergoes α-carbon hydroxylation to form an iminium ion and a carbinolamine (Ward D. P., 1982), the later can tautomerize to a ring-opened

amino aldehyde, which can then either be reduced to an open chain alcohol or be oxidized to an open chain carboxylic acid. Upon identification of M18 our interest was to understand the reaction mechanisms for the conversion and to identify the enzymes responsible for the biotransformation. We report structural characterization of the product (M18) and associated reactive intermediates trapped with diagnostic nucleophiles. The mechanisms for ring conversion are proposed as shown in Figure 2, and definitive identification of the enzyme(s) involved in the metabolism is described.

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Materials and Methods

Chemicals. AMG657417 was synthesized by chemists at Amgen Inc. M18 metabolite standard (**I**) was synthesized by chemists at Amgen Inc. following the procedures described in PCT Int. Appl. WO 2003099808, **2003,** and characterized by mass spectrometry (MH^+ *m/z* 438) and proton NMR (^{1}H-NMR (400 MHz, *Methanol-D_4*) δ ppm 8.43 (2 H, m), 7.35 (2 H, m), 7.27 (2 H, d, *J*=8.6 Hz), 7.12 (2 H, d, *J*=8.6 Hz), 4.45 (1 H, m), 3.53 (3 H, s), 3.45 (1 H, br.), 3.16 (1 H, br.), 2.67 (1 H, br.), 2.55 (1 H, br.), 2.10 (1 H, m), 2.00 (1 H, m), 1.58 - 1.88 (2 H, m), 1.24 (6 H, d, *J*=6.3 Hz), 0.92 (1 H, m)). Trapping reagents (semicarbazide and potassium cyanide), ketoconazole, and NADPH were purchased from Sigma-Aldrich Co. (St Louis, MO). Zorbax SB-C18 (2.1 x 100 mm, 1.8 μm) and Luna C18-HST (2.0 x 100 mm, 2.5 μM) HPLC columns were purchased from Agilent (Palo Alto, CA) and Phenomenex (Woodstock, IL), respectively. HLM, S9 fraction, cytosol, and recombinant CYP3A4 were purchased from either XenoTech, LLC (Lenexa, Kansas) or BD Gentest (Bedford, MA).

Hepatic S9, HLM, and recombinant CYP3A4 incubations. AMG657417 (10 μM) was incubated with HLM (1.0 mg/mL), human liver S9 fraction (2 mg/mL), or recombinant CYP3A4 (100 pmol/mL) in phosphate buffer (66.7 mM, pH 7.4). The reactions were initiated by the addition of NADPH (1 mM) and proceeded at 37°C for 45 minutes. The incubations were then quenched with equal volume of cold acetonitrile, and the samples were vortexed and centrifuged at 3000 rpm for 10 min. The supernatants were analyzed by LC-MS. For trapping studies, semicarbazide ($NH_2NHCONH_2$, 6 mM) or potassium cyanide (KCN, 5 mM) was added to the incubation mixture and incubated under similar conditions as described above.

Liquid Chromatography - Mass Spectrometry. All samples were analyzed by a LC-MS system consisting of a LC-10A HPLC (Shimadzu Scientific Instruments, Columbia, MD) and a mass spectrometer (API 4000 Q-trap mass spectrometer, Applied Biosystems, Foster City, CA) interfaced with an electrospray ion source (ESI, TurboSpray®). Chromatography was performed on a Luna C18 column (2.0 x 100 mm, 2.5 μm, Phenomenex, Woodstock, IL) with a binary mixture of solvent A (water with 10 mM ammonium acetate at pH 5) and B (acetonitrile/water with 10 mM ammonium acetate at pH 5, 95:5, *v/v*). The flow rate was 0.2 mL/min. Two HPLC gradients were used. Gradient program 1: the gradient started with 5% B, followed by linear increase to 15% B in 1 min, 25% B in 19 min, 95% B in 1 min and maintained at 95% for 5 min, then returned to the initial condition. The system was kept at the initial condition for 8 min to equilibrate the system prior to the next injection. Gradient program 2: the gradient started with 5% B, followed by linear increase to 50% B in 0.5 min, 80% B in 40 min, and 95% B in 1 min and maintained at 95% for additional 5 min before returned to the initial condition. The initial conditions were held for 6 min to equilibrate the system prior to the next injection. Gradient 1 was employed to detect the metabolites and determine their structures. Gradient 2 was employed to analyze the samples from cyanide trapping studies since the cyanide conjugates co-eluted with hydrophobic interferences if gradient 1 was used. Conditions for mass spectral analysis were as follows: mass range of 50 to 600 amu, TurboSpray temperature at 450ºC and voltage at 4000 V, declustering potential at 56 V, curtain gas at 20 psi, nebulizer (GS1) and TSI (GS2) gases at 45 psi.

AMG 657417 contains a chlorine atom. The isotopic pattern of the natural

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abundance of ^{35}Cl *vs* ^{37}Cl (3:1) was used to detect and identify the protonated molecule (MH^+) of the metabolites. The ^{35}Cl monoisotopic MH^+ ions were selected for fragmentation in product ion scan mode with CID offsets at 50 V; the product ion scan ranged from 50 amu to a few amu above that of the MH^+.

Samples from the semicarbazide trapping experiment were also analyzed on an LC-MS system consisting of a HP1100 binary pump (Agilent, Palo Alto, CA) interfaced by ESI with an LTQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Chromatography was performed on a Agilent SB-C18 column (2.1 x 100 mm, 1.8 μm, Waters, Milford, MA) with a binary mixture of solvent A (water with 10 mM ammonium acetate at pH 5), and solvent B (acetonitrile/water with 10 mM ammonium acetate at pH 5, 95:5, *v/v*) at a flow rate of 0.2 mL/min. The HPLC gradient (gradient 3) started with 5% B, followed by linear increase to 50% B in 25 min, 95% B in 1 min, and maintained at 95% B for additional 4 min before returning to the initial condition. The system was kept at the initial condition for 6 min prior to the next injection. The ESI conditions were as follows: spray voltage, +5 kV; sheath and auxiliary gas (N_2), 80 (arbitrary units); capillary voltage, 9 V; capillary temperature, 275°C. Tandem mass spectrometric (MS^2 or MS^3) data were collected using an isolation mass range of 2 amu and a collision energy of 30%. The fragmentation pattern of AMG657417 from the ion trap (data not shown) was similar to that obtained from the triple quadrapole (API 4000 Q-trap).

Ketoconazole assay. AMG657417 (10 μM) was incubated with human liver S9 (2 mg/mL) in the absence or presence of ketoconazole (1 μM). The reaction was initiated by the addition of NADPH (1 mM) and proceeded at 37ºC for 45 min. The reaction was terminated with the addition of acetonitrile containing 0.5% formic acid and internal

standard (quench solution). All samples were analyzed by LC/MS (API 4000 Qtrap) operated in positive mode with selective reaction monitoring (SRM). LC gradient program 1 was used to separate M18 from AMG657417. The SRM transition for M18 was 438.1/379.1.

Results

Identification of Piperidinyl Metabolite. AMG657417 was incubated with human hepatocytes or liver S9 fraction and resulted in the formation of the major metabolite M18, which had identical protonated molecule (*m/z* 438) to AMG657417, and eluted earlier than its parent molecule on a reverse phase HPLC. The metabolite was further identified by comparing its MS and NMR spectra with the synthetic standard (**I).** The fragment ions of M18 (Figure 3b) were identical to those of AMG657417 (Figure 3a) except for the two fragment ions at *m/z* 84 and *m/z* 126 that were not observed in the mass spectrum of AMG657417. These two characteristic fragment ions were derived from the isopropyl-amino-piperidinyl (*m/z* 126) and amino-piperidinyl (*m/z* 84) moieties, and were used to distinguish the 6-membered piperidinyl derivates from the 5-membered pyrrolidinyl derivates. M18 was not formed in the incubations of AMG657417 with NADPH-fortified HLM, recombinant CYP3A4, or cytosolic fraction suggesting that the formation of M18 required enzymes present both in HLM and cytosol.

Identification of Semicarbazide Conjugates. Semicarbazide was used to detect the formation of aldehyde intermediate during the course of pyrrolidine (parent) conversion to piperidine (M18). The reaction resulted in the formation of new molecular entities with MH^+ ions at *m/z* 511 that were consistent with nucleophilic addition of semicarbazide (75 Da) to an aldehyde formed from AMG657417 (454 Da), followed by intramolecular cyclization and dehydration as shown in Figure 2. An extracted ion chromatogram of *m/z* 511 revealed the presence of two semicarbazide conjugates (Figure 4) in the S9 incubation. Two of the four peaks in the chromatogram (marked as Ma1 and Ma2) had the expected chlorine isotopic pattern (data not shown) and each was

fragmented for further characterization. The product ion spectra of Ma1 and Ma2 are shown in Figure 5a and Figure 5b, respectively. Ma1 and Ma2 both formed fragment ions at *m/z* 494, 436, and a MS^3 ion at *m/z* 377 (fragment of 436) corresponding to the neutral loss of ammonia (NH_3), semicarbazide, and isopropylamine, respectively. The diagnostic MS^3 ions for Ma2 were *m/z* 313 and *m/z* 394 (fragment ions of 436) formed by loss of the neutral fragments shown in Figure 5b. The fragmentation patterns of Ma1 and Ma2 were consistent with a pyrrolidine semicarbazide conjugate and a piperidine semicarbazide conjugate, respectively. These conjugates of AMG657417 were formed in NADPH-fortified HLM, human liver S9 fraction, or CYP3A4 incubation (data not shown), suggesting that formation of the aldehyde intermediates of AMG657417 was mediated by CYP-catalyzed oxidation.

Identification of Cyanide Adducts. Cyanide anion (CN^-) is a hard nucleophile, which can be used to trap iminium ions (hard electrophiles) formed by dehydrogenation of an aliphatic secondary amine. One plausible mechanism of M18 formation is via a piperidinyl iminium ion, an intramolecular Schiff base resulting from cyclization of the exocyclic isopropylamine nitrogen to the carbonyl group of the aldehyde metabolic intermediate. Formation of such an iminium intermediate (Figure 2) can be verified by trapping it with cyanide. Two cyanide conjugates (Mb1 and Mb2) were detected in NADPH-fortified HLM, S9, and CYP3A4 incubations of AMG657417 and identified by LC-MS. The protonated molecules of the two conjugates were *m/z* 463 (Figure 6a), and they had identical MS^2 spectra. The representative product ion spectrum (Figure 6b) had a fragment ion at *m/z* 436, consistent with the neutral loss of hydrogen cyanide (-27 Da). Two fragment ions at *m/z* 124 and 82 were consistent with the loss of HCN from the

isopropyl piperidine and piperidine, respectively. M18 was not detected in the trapping reaction in S9 indicating that cyanide quenched its formation.

Ketoconazole inhibition. The CYP enzymes responsible for the formation of M18 were investigated using chemical inhibitors. Ketoconazole (1 μM) is a selective inhibitor of CYP3A and was used to inhibit the formation of M18 in human liver S9 fraction. The ratio of M18 peak response vs. internal standard (IS) was used to calculate the relative amount of M18 formation in the presence and absence of ketoconazole. The formation of M18 was 94% inhibited by ketoconazole, indicating that CYP3A was responsible for the initial oxidation of AMG657417 to form M18.

Discussion

Alicyclic amines are commonly encountered in natural products and synthetic drugs. The pyrrolidine moiety is found in natural alkaloids such as nicotine and hygrine, and in many drugs such as procyclidine and bepridil. The basicity of the nitrogen is sometimes required for pharmacological activity. For example, pyrrolidino, piperidino and morpholino moieties are core structures critical for drugs that target the central nervous system (Mirzadegan et al., 2000; Tran et al., 2008). Alicyclic amines are commonly metabolized via *N*-dealkylation or oxidation of the α-carbon to form a lactam (Hucker et al., 1972). The presence of primary or secondary basic amines in compounds undergoing metabolism can sometimes result in intramolecular reactions with metabolic intermediates. For example, the metabolism of a compound containing a pyrazinone ring led to the formation of two dihydro-imidazole and imidazolidine derivatives through intramolecular rearrangement (Subramanian et al., 2003).

AMG657417 contains a pyrrolidinyl moiety that was converted to a piperidine during biotransformation. The metabolism of the pyrrolidinyl moiety of AMG657417 can be compared with the metabolism of nicotine. Nicotine is metabolized to produce nornicotine via *N*-dealkylation or cotinine via oxidation of the α-carbon. The mechanism to form cotinine from nicotine was elucidated by several groups (Messina et al., 1997; Murphy, 1973; Yamazaki et al., 1999; Brandange and Lindblom, 1979; Gorrod and Hibberd, 1982). Two oxidation steps were involved in the biotransformation: oxidation to nicotine-$\Delta^{1'(5')}$-iminium ion by CYP, and oxidation of the iminium ion by cytosolic aldehyde oxidase. The iminium metabolic intermediate is generated in equilibrium with other species including a carbinolamine, an endocyclic enamine, and a ring-opened

aminoaldehyde intermediate (Sayre et al., 1997). These intermediates can form lactam and/or carboxylic acid, which are the routes of detoxification. Similar reaction mechanisms for AMG657417 are proposed in Figure 2. The initial oxidation is either dehydrogenation to the iminium species (a), or monooxygenation to the carbinolamine (b) which can decompose to the aldehyde (c). These species cannot be readily distinguished and probably exist in equilibrium. The presence of the exocyclic secondary amine serves to effectively trap the aldehyde (c) to form the six membered carbinolamine (d) which can undergo dehydration to form the iminium ion (e). In fact, species (a) to (e) could exist in equilibrium with the reaction driven to a single product via the reduction of the iminium (e) to form the product that was isolated and identified (M18). In order to interrogate this pathway, aldehyde and iminium intermediates were trapped using semicarbazide (Sladek, 1973) and cyanide (Gorrod et al., 1991), respectively. These conjugates of the intermediates were formed in NADPH-fortified incubations with recombinant CYP3A4, HLM, or S9 fraction.

Two semicarbazide conjugates (Ma1 and Ma2) were characterized by their different retention times and MS^3 spectra (Figure 5a and Figure 5b), indicating they were isomers. Conjugation of aldehyde intermediate (c) with semicarbazide and dehydration to an imine provides an opportunity for ring closure to either a pyrrolidine or piperidine analogous to that which occurs to form M18. These data support the formation of the proposed aldehyde intermediate (c) in the reaction.

Two cyanide conjugates (Mb1 and Mb2) were detected when AMG 657417 was incubated with HLM, S9 fraction, or CYP3A4. The two conjugates eluted at different retention times (Figure 6a) and had identical protonated molecules (*m/z* 463) and MS^2

fragmentation patterns (Figure 6b). These two structurally very similar forms could be positional isomers, or more likely two diastereomers resulting from the introduction of two chiral centers, one upon cyclization and the other upon cyanide adduction. A cyanide conjugate of a pyrrolidine iminium ion, structure (a) in Figure 2, was not detected, possibly because it is either not formed, or is more rapidly hydrated compared to its reaction with cyanide. This would not be the case for the piperidine iminium ion, structure (b) in Figure 2, since it was trapped with cyanide.

Since M18 cyanide conjugates were observed in the incubation of AMG657417 with HLM and KCN, enzyme(s) in the liver microsomes could play a role in the formation of M18. Chemical inhibition with ketoconazole (1 μM) was performed to assess the involvement of CYP3A in the oxidation. The product formation (M18) in human S9 was 94% inhibited by ketoconazole, suggesting that the oxidation of AMG657417 was almost exclusively catalyzed by CYP3A. Thus, almost all the intermediate metabolites formed (e.g., aldehyde and iminium from the Schiff reaction) were the chemically derived products following CYP3A oxidation, and once formed, the imimium ion can be further reduced to M18 by enzyme(s) in the cytosol.

In conclusion, the mechanism of the biotransformation from the five-membered pyrrolidinyl moiety of AMG657417 to the six-membered piperidinyl ring of M18 is proposed to have proceeded as shown in Figure 2. CYP3A was largely involved in the α-carbon oxidation of the pyrrolidinyl ring, and the presence of reductases in the cytosol appears to be essential for the formation of the final metabolite M18. The successful trapping of the reaction intermediates with semicarbazide and cyanide supports the proposed reaction mechanisms.

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Authorship Contributions

Participated in research design: Faye Hsieh, Magang Shou

Conducted experiments: Yuping Chen

Performed data analysis: Faye Hsieh, Yuping Chen, Gary L. Skiles

Wrote or contributed to the writing of this manuscript: Faye Hsieh, Yuping Chen, Gary L. Skiles, Magang Shou, Dean Hickman

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Legends for Figures

Figure 1. Biotransformation of AMG657417 to metabolite M18 (**I**) in human hepatocytes and liver S9 fraction.

Figure 2. Proposed reaction mechanisms for the conversion of pyrrolidinyl moiety on AMG657417 to piperidinyl product (M18).

Figure 3. Product ion mass spectra and fragmentations of **a)** AMG657417 and **b) I** obtained from collision-induced-dissociation (CID) of *m/z* 438.

Figure 4. Extracted ion chromatogram at *m/z* 511 of semicarbazide conjugates formed in incubation of AMG657417 and semicarbazide with NADPH-fortified human S9 fraction.

Figure 5. The MS^2 (*m/z* 511), MS^3 (*m/z* 511 → 494) and MS^3 (*m/z* 511 → 436) spectra of a) Ma1, the pyrrolidine semicarbazide conjugate, and b) Ma2, the piperidine semicarbazide conjugate.

Figure 6. Two cyanide conjugates formed in the incubation of AMG657417 and KCN with NADPH-fortified human S9 fraction: **a)** extracted ion chromatogram at *m/z* 463 displayed two cyanide conjugates (Mb1 and Mb2), and **b)** Mb1 and Mb2 have identical product ion spectra (CID on *m/z* 463) with a fragment at *m/z* 436 resulting from the neutral loss of HCN.

Figure 1.

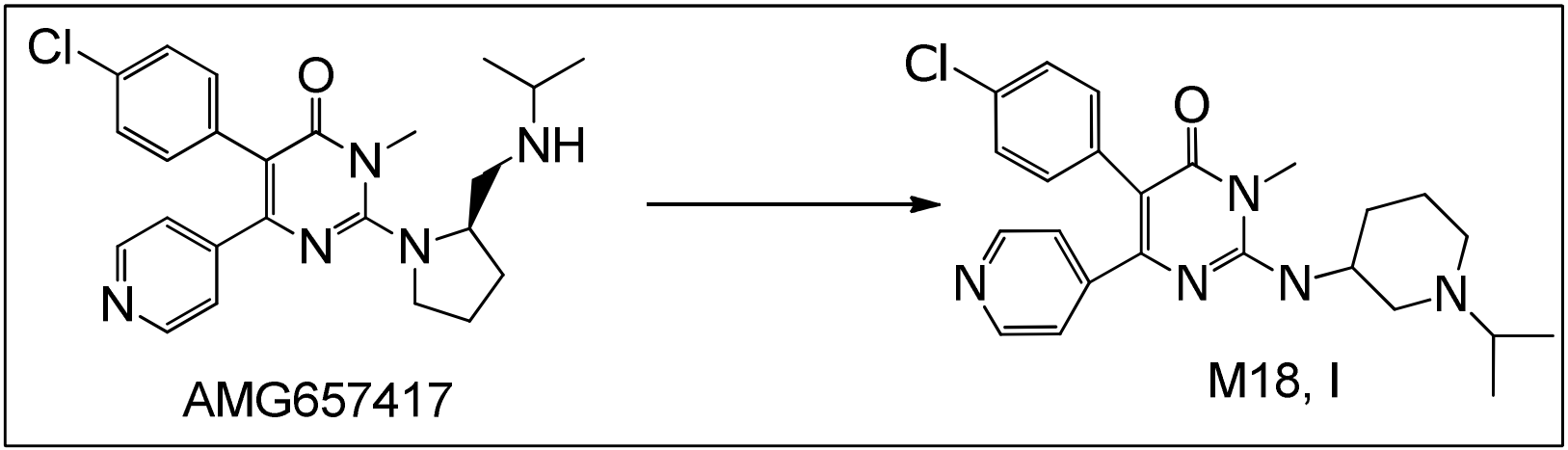

Figure 2.

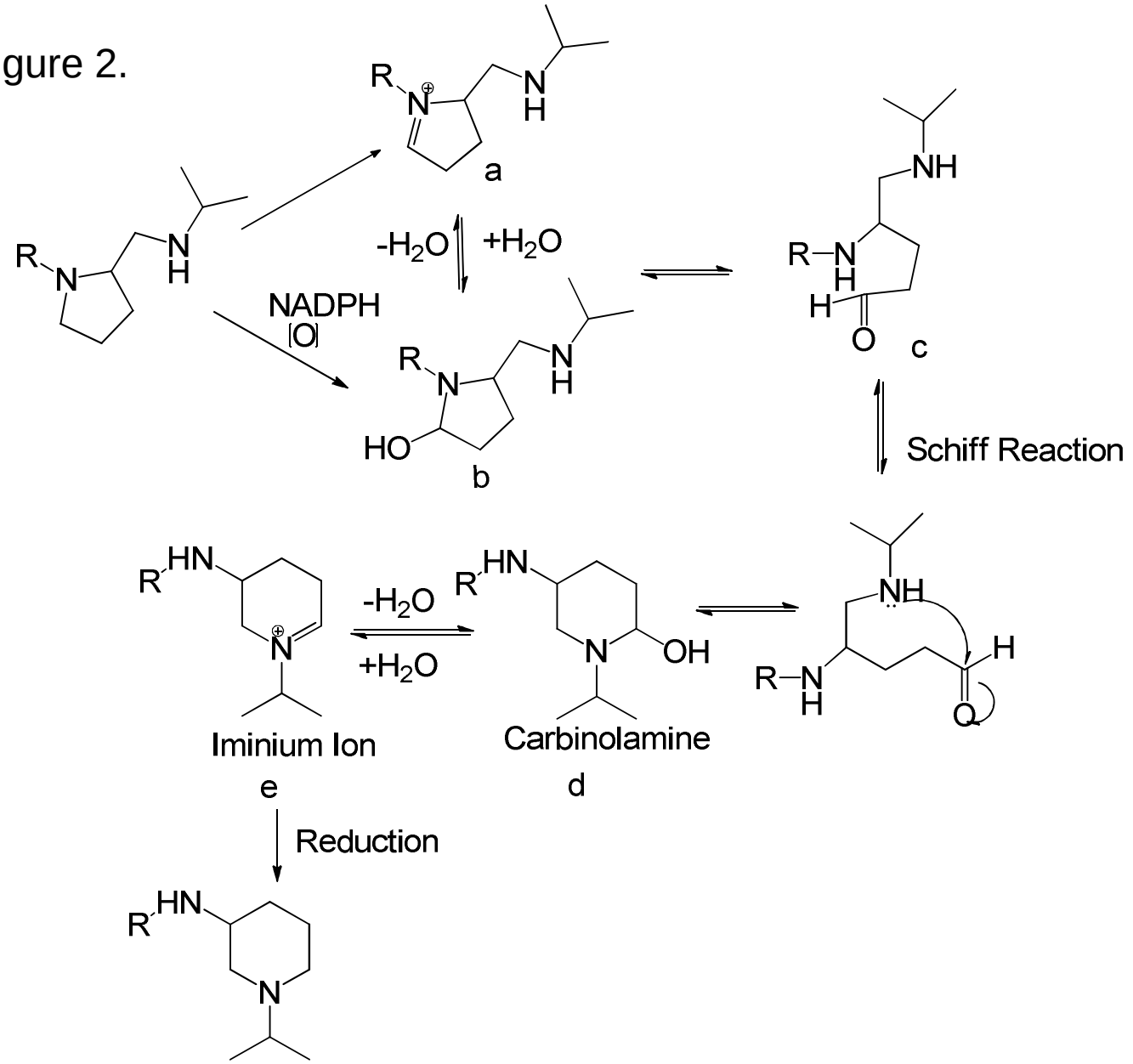

Figure 3.
a)

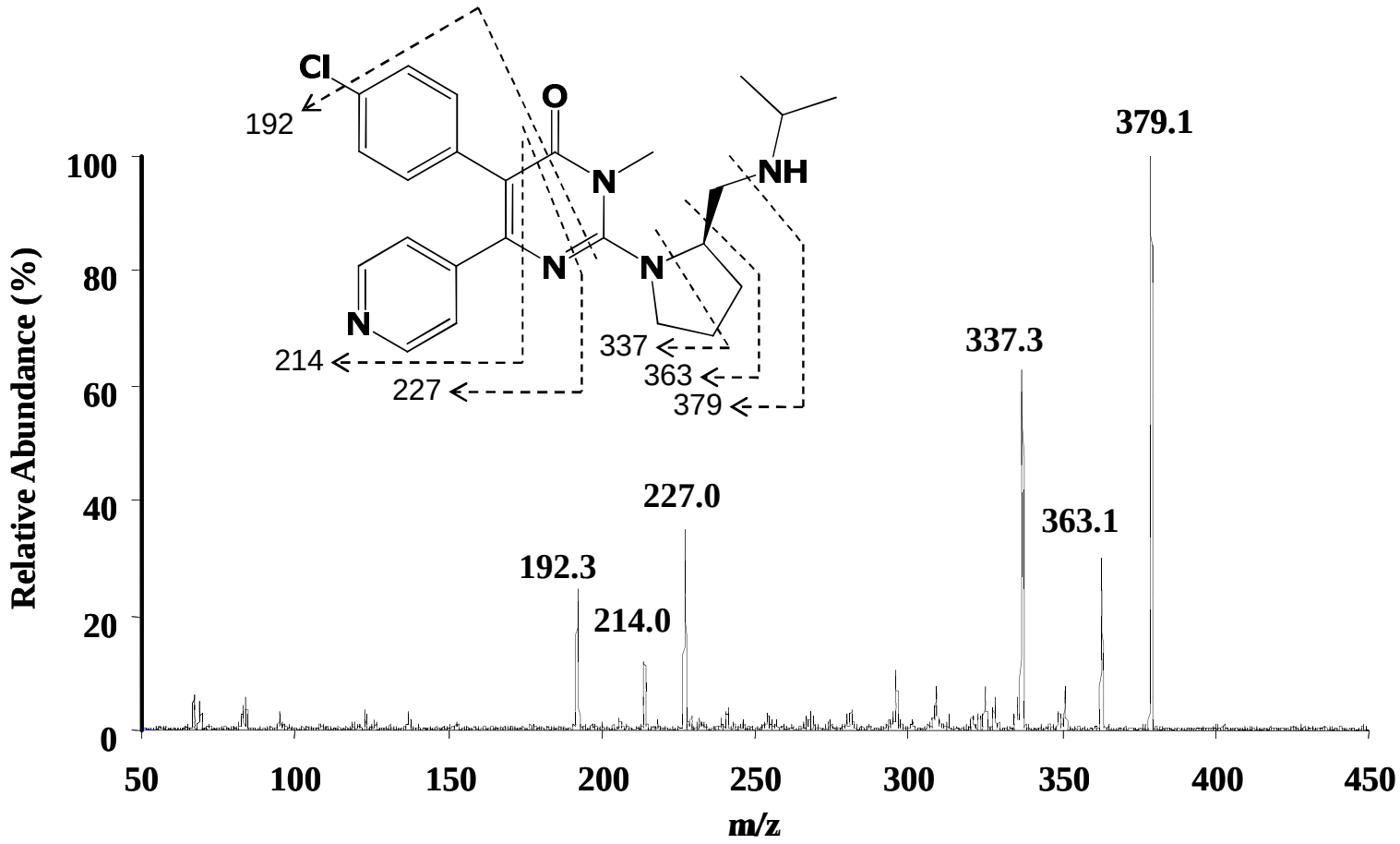

Figure 3.
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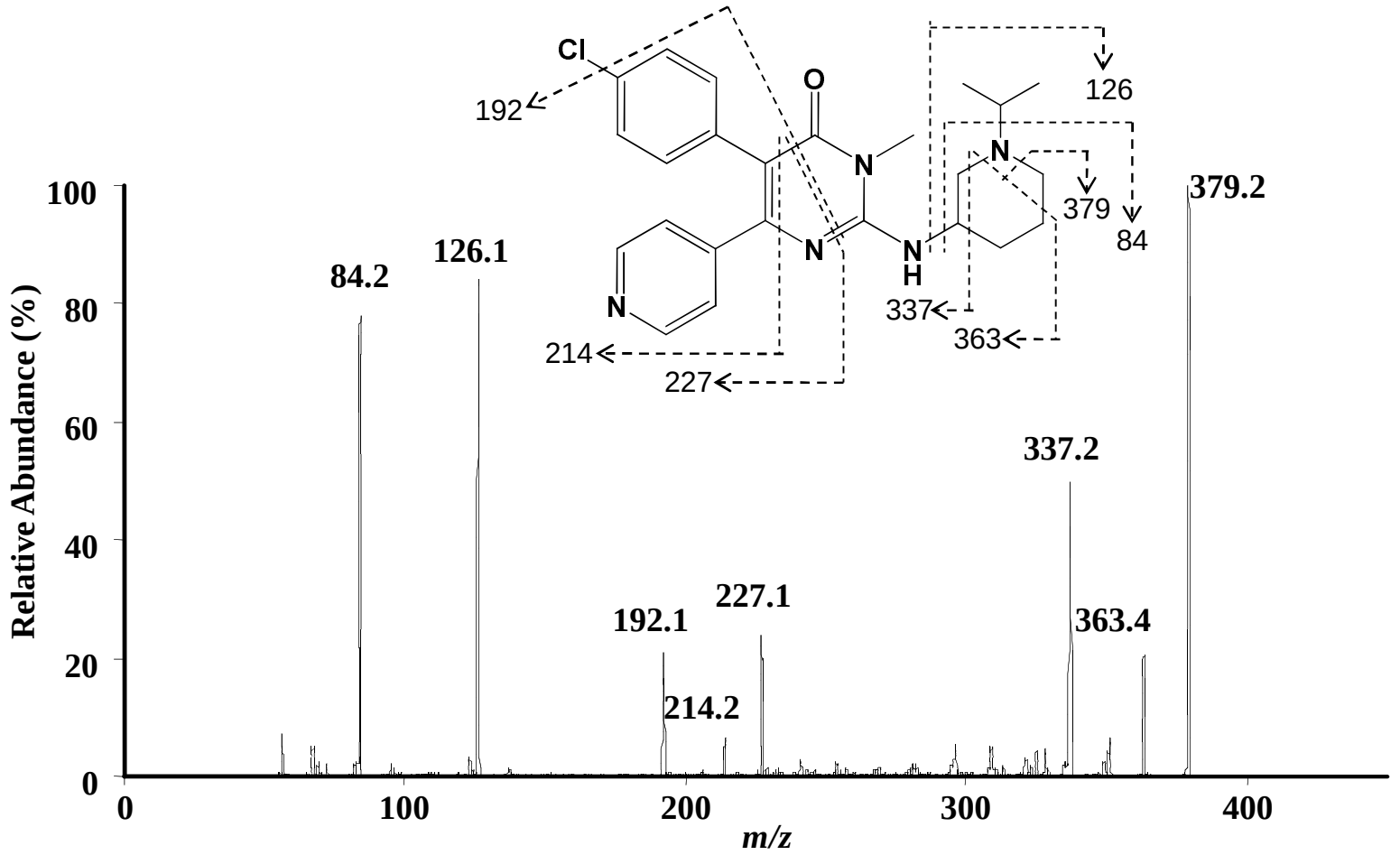

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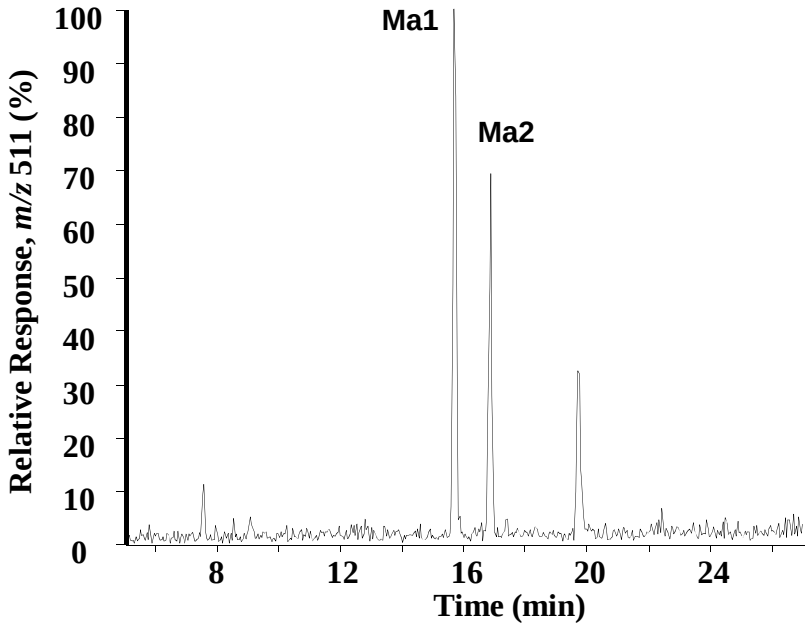

Figure 5.

a)

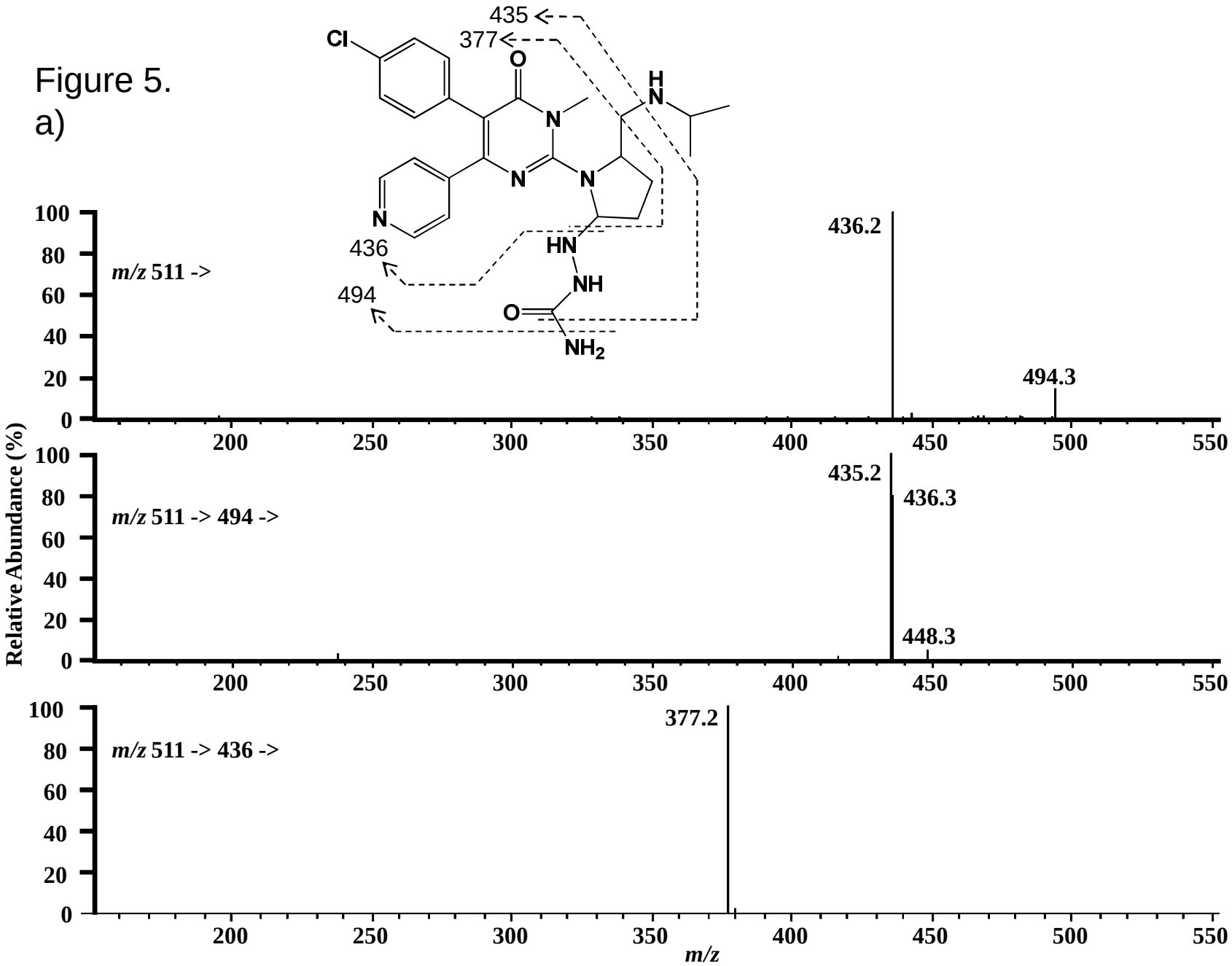

Figure 5.
b)

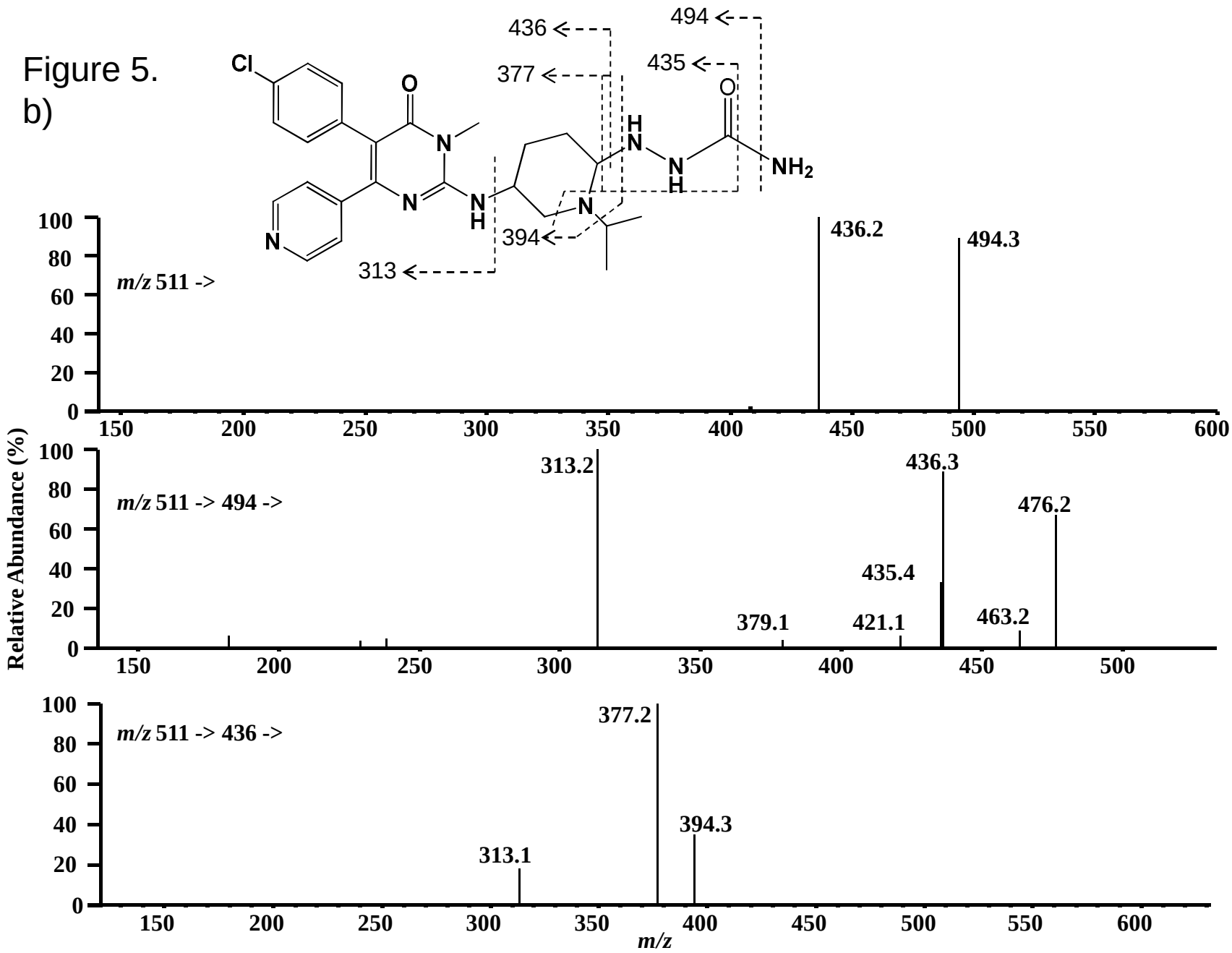

Figure 6.