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Escherichia coli overexpressing a Baeyer-Villiger monooxygenase from *Acinetobacter radioresistens* become resistant to Imipenem

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*Running title: *Baeyer-Villiger monooxygenase and antibiotic resistance*

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ABSTRACT

Antimicrobial resistance is a global issue currently resulting in fatalities of hundreds of thousands a year worldwide. Data present in literature illustrate the emergence of many bacterial species that display resistance to known antibiotics; *Acinetobacter* spp. is a good example of this. We report here that *Acinetobacter radioresistens* has a Baeyer-Villiger monooxygenase (Ar-BVMO) with 100% amino acid sequence identity to Ethionamide monooxygenase of the MultiDrug-Resistant (MDR) *Acinetobacter baumannii*. Both enzymes are phylogenetically only distantly related to other canonical bacterial BVMO proteins. Ar-BVMO is not only capable of oxidizing two anticancer drugs metabolized by human FMO3, Danusertib and Tozasertib, but can also oxidize other synthetic drugs such as Imipenem. The latter is a member of carbapenems, a clinically important antibiotic family used in the treatment of MDR bacterial infections. Susceptibility tests performed by the Kirby-Bauer disk diffusion method demonstrate that Imipenem sensitive *E. coli* BL21 cells overexpressing Ar-BVMO become resistant to this antibiotic. Agar disc diffusion assay proves that when Imipenem reacts with Ar-BVMO, it loses its antibiotic property. Moreover, NADPH consumption assay with the purified Ar-BVMO demonstrates that this antibiotic is indeed a substrate and its product is identified by LC-MS as a BV oxidation product of the carbonyl moiety of the β-lactam ring. This is the first report of an antibiotic-inactivating BVMO enzyme that whilst mediating its usual BV oxidation, is also operating by an unprecedented mechanism of carbapenem resistance.

INTRODUCTION

Bacteria classified within the genus *Acinetobacter* play an increasingly important role in pathogenesis of human diseases. Among members of this genus, *Acinetobacter baumannii* is the most frequently isolated species from humans (found in endotracheal aspirate, blood, peri-anal and wound secrets) where it is known to manifest multidrug-resistance (MDR) (1). *Acinetobacter* species are widely distributed in nature and can be found in soil, water, sewage, and in variety of foodstuffs (1-2). They are present in the hospital environment especially intensive care units (3-4) as they are inhabitants of healthy human skin as part of normal flora and can be isolated from dry surfaces and equipment and, they can easily survive for many days or weeks, even in dry conditions (1-2). Increasing recovery of the MDR *A. baumannii* in nosocomial settings is a frightening reality (5,6) and the combination of its environmental resilience and its wide range of resistance determinants renders it a successful nosocomial pathogen (6). These MDR strains often spread to cause outbreaks throughout entire cities, countries, and continents (7-10). The importation of MDR strains from areas with high rates of antimicrobial resistance to areas with historically low rate has been demonstrated. United Kingdom and U.S. military and non-military personnel returning from operations in Iraq and Afghanistan harboured infections caused by multi-resistant *A. baumannii* (12-14). Resistance of *A. baumannii* to the carbapenem Imipenem, which is the drug of choice for treating serious infections caused by this species, leads to difficult-to-treat nosocomial infections (7,15-16). Carbapenem resistance results mostly from the expression of acquired carbapenem-hydrolyzing oxacillinases in *A. baumannii*. The blaOXA-23 gene that confers drug resistance in *A. baumannii*, codes for the oxacillinase OXA-23, one of the five Ambler class D β-lactamases (17). Outbreaks of OXA-23 producing *A. baumannii* have been reported worldwide and may represent an emerging threat (18). Poirel and co-workers found that *Acinetobacter radioresistens*, a commensal bacterial species that is identified on the skin of hospitalized and healthy patients is the source of the *bla*OXA-23 gene (19).

Recently we have identified a novel Baeyer-Villiger monooxygenase (Ar-BVMO) in *A.*

radioresistens strain S13 (20). Baeyer-Villiger monooxygenases (BVMOs) are flavin-containing enzymes that mediate specific Baeyer-Villiger oxidations on the carbonyl moiety of substrates. Ketones are converted into the corresponding esters and lactones by the Baeyer-Villiger reaction. BVMO enzymes are abundant in bacterial, fungal and plant genomes but they are absent in animal and human genomes. All characterized BVMOs contain a flavin cofactor that is crucial for catalysis while NADH or NADPH is required as electron donor. Most reported BVMOs are soluble proteins in contrast to many other monooxygenase systems that are often found to be membrane bound. BVMOs are divided into three different types according to their general characteristics (21). Type I enzymes contain a tightly bound FAD cofactor, are NADPH-dependent, possess two Rossmann folds for dinucleotide binding and a conserved Baeyer-Villiger motif, a fingerprint sequence (FXGXXXHXXXW(P/D)) involved in catalysis (22). Type II enzymes do not present the Baeyer-Villiger sequence motif, utilize FMN as coenzyme and NADH as co-substrate whereas type 0 use FAD and NAD(P)H and lack the BVMO fingerprint motif (23). A heme-containing BVMO has also been reported belonging to the cytochrome P450 superfamily (24). Earlier studies had already suggested Baeyer-Villiger activity of other eukaryotic cytochrome P450 enzymes (25). This finding indicates that during evolution several different enzymes have evolved into Baeyer-Villiger monooxygenases. Most biochemical and biocatalytic studies have been performed with Type I BVMOs (23). This is partly due to the fact that they represent relatively uncomplicated monooxygenase systems. These monooxygenases are typically soluble and composed of only one component. Expression systems have been developed for a number of Type I BVMOs, while no recombinant expression has been reported for Type II. All these enzymes are known to perform various catalytic activities on different compounds even partially sharing their substrate profile. BVMOs are also known to perform oxygenation on heteroatom-containing compounds. The first evidence of soft nucleophilic containing substrate oxidation was reported with CHMO from *Acinetobacter* sp. that showed 4-tolyl ethyl sulfide conversion to the corresponding (S)-sulfoxide with a modest enantioselectivity (26). BVMOs may also have medical relevance as in the case of Ethionamide monooxygenase (EtaA), a Type I BVMO capable of converting a range of ketones into the corresponding esters (27). Except for catalyzing

Antimicrobial Agents and Chemotherapy

Baeyer-Villiger oxidations, the enzyme is also able to oxidize the sulfide moieties of several antitubercular thioamide drugs (27-28). The oxidized drugs appear to be highly toxic to the mycobacteria, indicating that Ethionamide monooxygenase acts as a prodrug activator.

Phylogenetic analysis placed the sequence of Ar-BVMO together with *Mycobacterium tuberculosis* EtaA (20) with only a distant relation to the other known class I BVMO proteins. *In vitro* experiments carried out with the purified enzyme confirmed that this novel BVMO is indeed capable of typical Baeyer-Villiger reactions as well as oxidation of the prodrug Ethionamide (20). Ortiz de Monellano's group (28) demonstrated that Ethionamide is also oxidized by human flavin-containing monooxygenase 3 (FMO3). Human FMO enzymes are a superfamily of flavoprotein monooxygenases involved in the detoxification of a wide range of xenobiotics containing a soft nucleophile, usually nitrogen or sulfur (29). Since Ar-BVMO has been shown to oxidize Ethionamide, we wondered whether this enzyme could play a role in detoxification of xenobiotics, recognizing synthetic compounds such as drugs, typically recognized by human FMO3. We took into consideration two synthetic drugs, Danusertib and Tozasertib, shown to be N-oxygenated by human FMO3 (30-31) and certainly not present in the natural environment of this bacterium. Species belonging to *Acinetobacter* genus have been reported as one of the major super-bugs, resistant to antibiotic treatment (32). The presence in their genomes of genes coding for monooxygenases similar to those involved in drug metabolism in humans becomes highly relevant when tackling the threat they pose.

We report here that the overexpression of Ar-BVMO in *E. coli* renders it resistant to Imipenem and show that this enzyme whilst mediating its usual Baeyer-Villiger oxidation on the carbonyl moiety of the β-lactam ring, is also operating by an unprecedented mechanism of carbapenem resistance. In future, if under physiological conditions appreciable carbapenemase activity is attributed to this monooxygenase, it could represent a new target for drug design in the battle against carbapenems' resistance in *Acinetobacter*.

MATERIALS and METHODS

Bacterial strains

Escherichia coli strain BL21 (DE3) (Invitrogen) transformed with the expression vector plasmid pT7 harbouring the gene coding for *Acinetobacter radioresistens* S13 Baeyer-Villiger monooxygenase (Ar-BVMO) (20) and not transformed BL21 cells.

Reagents

FAD, acetonitrile, NADPH, Imipenem, methanol and salts were purchased from Sigma-Aldrich (Milan, Italy). Tozasertib and Danusertib were purchased from Aurogene (Rome, Italy).

Sequence retrieval, alignment and phylogenetic analysis

Amino acid sequences from completely sequenced genomes of proteobacteria were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov). BLAST (33) probing of database was performed with the BLASTP option of this program using default parameters. The ClustalW program (34) was used to perform pairwise and multiple amino acid sequence alignments.

Preliminary multiple alignments of amino acid sequences were generated with the program ClustalW using default gap penalties. The selection of characters eligible for the construction of phylogenetic tree was optimized by comparing entire sections of all the available BVMO alignments with comprehensive inventories of significant binary alignments obtained by BLAST probing of the protein database with representative bacterial sequences. The structure and organization of the BVMOs were deduced from the data present in GenBank from the microorganisms available in data banks. BLAST probing of the protein databases was performed with the BLASTP program choosing sequences with an E-value of 0.0, 100% query coverage and a threshold of 99% sequence identity. For construction of the phylogenetic tree, a representative of each class of BVMO was selected to demonstrate its relationship to the Ar-BVMO.

The phylogenetic tree was constructed using the Distance Matrix (DM) method using MEGA version 4 (35), after multiple sequence alignment and truncation to the same length. Distances according to the Kimura two-parameter model (36) and clustering with the neighbor-joining method (37) were determined using bootstrap values based on 1,000 replications.

Expression and purification of recombinant Ar-BVMO

The expression and purification of Ar-BVMO were performed as previously reported (20). After Ni-NTA affinity chromatography, the heterologous expression of Ar-BVMO yielded 8 mg of purified protein per litre of culture. The concentration of the pure Ar-BVMO was determined by spectroscopy with the peak absorbance at 450 nm and the extinction coefficient of free FAD □∑□□□ =11,300 M^{-1} cm^{-1}).

Ar-BVMO incubation with Tozasertib, and Danusertib

N-oxygenation of the anticancer drugs Danusertib and Tozasertib was evaluated by incubating a mixture of 0.3 µM of purified enzyme in 50 mM potassium phosphate buffer (pH 7.4), 0.5 mM NADPH and increasing amounts of substrates (6, 12.5, 25, 50, 75, 100, 150, 200, 300 and 400 µM) in a final volume of 200 µl. Incubations were carried out at 37°C for 15 min and were terminated by the addition of 100 µl of ice-cold methanol. The aqueous supernatant was centrifuged at 2,000x g for 10 min and was subjected to High Pressure Liquid Chromatography. Kinetic parameters related to the turnover were determined through Michaelis-Menten kinetics using SigmaPlot Software.

Analysis by High Pressure Liquid Chromatography (HPLC)

HPLC was performed on an Agilent quaternary pump HPLC system (Agilent Technologies) equipped with an analytical C18 column (4.6 x150 mm, 5 □m). The method to separate Tozasertib and "Tozasertib N-oxide" and Danusertib from "Danusertib N-oxide" used a mobile phase of 78% methanol and 22% distilled water at a flow rate of 1.0 ml/min. The formation of Tozasertib N-oxide was monitored at the wavelength of 250 nm while the Danusertib N-oxide product was monitored at 295 nm (30-31). The

kinetic analysis of N- or S-oxygenation was carried out using a nonlinear regression analysis program (SigmaPlot).

Susceptibility testing

Imipenem sensitivity of *E. coli* BL21 cells (i) transformed with pT7-Ar-BVMO induced with IPTG, (ii) not induced and, (iii) negative control- transformed with pT7 (carrying another gene (38)) induced with IPTG, was performed by disc diffusion method (39) according to the latest updated guidelines (2014) of the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) using standard antibiotic disks (Oxoid) and Mueller-Hinton agar (MHA) plates (Sigma). There were five replicates for each condition tested. Inhibition zones measured as mean diameter were categorized as 'susceptible' (S), 'intermediary' (I) and 'resistant' (R) according to EUCAST standards.

The effect of Ar-BVMO activity on the antimicrobial properties of Imipenem was subsequently assessed by a microbiological assay. Inactivation reaction contained 3 mM Imipenem, 2 mM NADPH and 100 µM of purified Ar-BVMO in 50 mM phosphate buffer pH 7.4, in a total volume of 0.5 ml. The reaction was allowed to proceed for 30 min at 37°C. A control reaction was performed in the same conditions described above without adding NADPH. Fifty µl aliquots of the two reactions were used for agar diffusion plate tests (40) in order to evaluate the effect of metabolized Imipenem on the growth of sensitive *E. coli* BL21 cells. MH agar plates were prepared using sterile MHA. Upon solidification of the agar medium, sensitive not transformed BL21 and transformed not induced BL21 cells were spread over the plate surface at a concentration of 0.5 MacFarland. Then, a well was made in the centre of each plate using a sterile Pasteur pipette. After adding 50 µl of the Ar-BVMO-Imipenem reaction with and without NADPH, an uninoculated agar plug larger than the well was placed over it to prevent volatilization of the enzymatic reaction into the headspace of the Petri dish (40). The diameter of the growth inhibition zone was measured after 24 hours from the application of the test substance at 37°C. There were five replicates for each condition tested.

Enzymatic activity assay

Enzyme activity was measured spectrophotometrically by monitoring the substrate-dependent decrease in NADPH concentration at 340 nm ($\sum_{340}$ nm = 6.22 mM^{-1} cm^{-1}). Reaction mixtures contained 50 mM Tris-HCl pH 8.0, 200 µM NADPH, and a range from 5 to 150 µM of Imipenem using air-saturated buffers. Steady state kinetic parameters were determined by fitting the initial rates, calculated from the first 200 s of the reaction, to the standard Michaelis-Menten equation using SigmaPlot program. Experiments were conducted in triplicates.

LC-MS/MS analysis

The chromatographic separations were run on a Phenomenex Luna C18 column, 150 × 2.0 mm, 3 µm particle size (Phenomenex, Torrance, CA, USA). Injection volume was 10 µL and flow rate 0.2 mL min^{-1}. Isocratic mobile phase composition was adopted: 5:95 acetonitrile/aqueous formic acid 0.05%. A LTQ - Orbitrap hybrid mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an atmospheric pressure interface and an ESI ion source was used. The LC column effluent was delivered into the ion source using nitrogen as sheath and auxiliary gas. The needle voltage was set at the 4.5 kV value. The heated capillary value was maintained at 270°C. The acquisition method used was previously optimized in the tuning sections for the analyte ion (capillary, magnetic lenses and collimating octapoles voltages) in order to achieve the maximum of sensitivity. Spectra were acquired in the positive HRMS and MS/MS mode, precursor ions 300 and 316, normalized collision energy 25 %, mass range 320-80 *m/z*.

RESULTS

Phylogenetic tree of the Ar-BVMO was inferred from the selected sequences by DM method as mentioned in Materials and Methods section. As shown in Figure 1, the clade comprising *Mycobacterium tuberculosis* EtaA, Ar-BVMO and *A. baumannii* EtaA belong to a separate group, distant from the other BVMO proteins. Furthermore, Ar-BVMO shares 100% amino acid sequence identity with the

Ethionamide monooxygenase of MDR *A. baumannii* strains (GenBank ID: gb|EXB35767.1|, gb|EXB73644.1|, gb|EXE14728.1| and gb|KCX39290.1|).

Previously in our laboratory we had tested different therapeutic drugs as possible substrates of this human hepatic enzyme including Danusertib and Tozasertib (29-30). Tozasertib (VX-680) and Danusertib (PHA-739358) are inhibitors of Aurora kinases which have been identified as a potential target in anticancer therapy. As a starting point, the possibility of these two inhibitors acting as substrates for Ar-BVMO enzyme was tested by investigating whether any enzymatic products could be detected. After each set of reactions at 37 ºC in the presence of NADPH, the product(s) was separated by HPLC and the data obtained are shown in Figure 2. As can be seen from in the figure, the expected N-oxides were detected with shorter retention times of 3.7 min for "Danusertib N-oxide" (Figure 2A) and 4.1 min for "Tozasertib N-oxide" (Figure 2C), in agreement with the previously published data by our group (30-31). The catalytic activity of Ar-BVMO towards two kinase inhibitors, Danusertib and Tozasertib, was investigated. Enzymatic reactions were carried out with the purified recombinant Ar-BVMO in the presence of NADPH and increasing amounts of each drug as reported in the experimental section. Kinetic parameters for the N-oxygenation for Danusertib (Figure 2B) and Tozasertib (Figure 2D) were determined by nonlinear regression analysis with a calculated K_M value of 352 ± 58 μM and 54.3 ± 8.3 μM, respectively. The corresponding V_{max} values of 8.1 and 7.4 nmol/min/mg of protein were also calculated for Danusertib and Tozasertib, respectively. These results confirm that the bacterial enzyme is indeed able to metabolize these two synthetic drugs.

Acquisition of Imipenem resistance by Escherichia coli overexpressing Ar-BVMO- Once it was proven that the bacterial Ar-BVMO is capable of metabolizing the synthetic drugs tested, our attention was turned to antibiotics as another class of synthetic drugs and directly relevant to the MDR effects seen in *Acinetobacter* spp. Initially, in order to understand whether the expression of Ar-BVMO in Imipenem sensitive *E. coli* BL21 cells could confer resistance to Imipenem (i.e. enzymatic reaction of Ar-BVMO with Imipenem resulting in a product no longer active as an antibiotic), a disc diffusion assay was performed following EUCAST guidelines (www.eucast.org). According to EUCAST clinical breakpoint

table v. 5.0 (valid from 2015/01/01) for *Enterobacteriaceae*, the zone diameter breakpoint for Imipenem is ≥22 mm for sensitivity and <16 mm for resistance, corresponding to MIC values of 2 and 8 mg/L of Imipenem, respectively, for sensitive and resistant *Enterobacteriaceae* including *E. coli*, which is the most important model organism of this family. As shown in Figure 3, the average diameter of the inhibition zone for *E. coli* BL21 cells (Figure 3 top panel-A), the same *E. coli* strain transformed with pT7-Ar-BVMO but not induced (Figure 3 top panel-B) and *E. coli* strain transformed with pT7 (negative control) IPTG-induced (Figure 3 top panel-C) was measured as 25.5 mm, 24.0 mm, and 28.0 mm, respectively. The results clearly show that the *E. coli* BL21 cells are sensitive to Imipenem (zone diameter breakpoint ≥22 mm) but there is no visible inhibition zone when *E. coli* BL21 cells are transformed and the expression of Ar-BVMO is induced by IPTG i.e. the cells became resistant to Imipenem (zone diameter breakpoint <16 mm), Figure 3 top panel-D. These data provided the preliminary but encouraging evidence that the over expression of Ar-BVMO in Imipenem sensitive cells may result in antibiotic resistance.

Ar-BVMO catalyses the inactivation of Imipenem- The effect of Ar-BVMO activity on the antimicrobial properties of Imipenem was further studied by a microbiological assay (41). The reaction product(s) of purified Ar-BVMO and Imipenem was loaded in a well in the MH agar plates where Imipenem sensitive BL21 cells and BL21 carrying pT7-Ar-BVMO whose gene expression was not induced, were grown at 37°C for 24 h. As shown in Figure 3 bottom panel-B and D Imipenem is not active after the reaction with Ar-BVMO since both not transformed and transformed but not induced BL21 exhibited an inhibition zone of 12.0 mm and 0.5 mm, respectively. When the enzymatic reaction without NADPH (negative control) was deposited in the agar well the diameter of the inhibition zone were 24.0 mm (Figure 3 bottom panel-A) and 24.5 mm (Figure 3 bottom panel-C) for not transformed BL21 and transformed but not induced BL21, respectively. These data also support the idea that the enzymatic reaction of Ar-BVMO converts Imipenem into an inactive compound no longer possessing its original antibacterial property.

Catalytic activity of purified Ar-BVMO in presence of Imipenem and identification of the products- Subsequent to the *in vivo* data, a spectroscopic method was used to measure the activity of the purified Ar-BVMO in the presence of Imipenem. In order to determine the steady state kinetic parameters for Ar-BVMO turnover, the NADPH consumption assay which has previously been described in literature as an efficient system to evaluate K_M (42), was used. This method relies on the spectrophotometric monitoring of the consumption of the co-substrate NADPH at 340 nm, in the presence of increasing concentrations of Imipenem. The experimental data from the NADPH consumption were fitted to a Michaelis-Menten curve used for the determination of a K_M value of 12.3 ± 0.8 µM (Figure 4) and a V_{max} value of 468.2 ± 9.6 nmol/min/mg of protein. These values are in the same range as those reported for known substrates of other BVMO enzymes (42) indicating that Imipenem could be a real substrate of Ar-BVMO.

In order to identify the product(s) of the Ar-BVMO reaction with Imipenem, LC-MS experiments were carried out. Imipenem incubation (100 µM) was performed in phosphate buffer (50 mM, pH 7.4) at room temperature and monitored by LC-high resolution mass spectrometry at different reaction times. Ar-BVMO and NADPH concentrations were 0.45 and 200 µM, respectively. The incubation mixture was simply ten-fold diluted using elution mobile phase and injected in the LC-MS instrument. Imipenem peak was eluted at 4.7 min. The main metabolite was detected as a double peak (5.0 and 5.5 min) due to a mixture of isobaric products. The accuracy of orbitrap high resolution mass determination allows to deduce that the elemental composition of the two compounds correspond to $C_{12}H_{18}N_3O_5S$ with 1.05 ppm of error (MH^+ ion at 316.0962 *m/z*). A classical Baeyer-Villiger asymmetric oxygen insertion can explain this finding as shown in Figure 5.

An analogous BV catalytical synthetic reaction was described regarding a similar lactam (bicyclo[3.2.0]hept-3-en-1-one) (43). In order to elucidate the structure, a MS/MS study was performed. High resolution MS/MS spectra of Imipenem and the corresponding Ar-BVMO oxidized products are shown in figures 6a and 6b, respectively. The MS^n study of Imipenem precursor ion (300.0867 *m/z*)

allows to hypothesize the fragmentation pathway shown in Figure 7. The main fragmentation pathways are the (consecutive) losses of water and carbon dioxide. The elimination of the amidino-ethylthio- lateral chain is noteworthy. High resolution mass defect data were fundamental to differentiate CO_2 and NH-CH-NH_2 losses, both with nominal mass of 44 Da but with different significant digits. The interpretation of MS/MS spectrum of Ar-BVMO metabolites is reported in Figure 8. A double CO_2 loss characterizes the spectrum. The intense ion due to the loss of the amidino-ethyilthio lateral chain confirms that oxygen insertion occurred on the lactamic ring. A possible intramolecular hydrogen bond justifies both the proposed formation of a new lactone cyclic structure and the chromatographic behavior of the two metabolites which are more retained than the parent compound on the reverse phase column. Successive losses of water, ammonia and ethylamidine molecules could explain all the obtained fragment ions. The same fragmentation pathway is valid in the interpretation of MS/MS spectrum of the ion corresponding to the asymmetrical isomer reported in Figure 5.

DISCUSSION

The World Health Organization (WHO) has recently classified antibiotic resistance as one of the three greatest threats to human health. The β-lactams are one of the most important and frequently used classes of antibiotics in medicine and are essential in the treatment of serious gram-negative infections. The past two decades have seen substantial increases in the utilization of carbapenems such as Imipenem and Meropenem. Predictably, this increase in carbapenem consumption has been accompanied by the emergence of carbapenem-resistant gram-negative pathogens (44). The initial and seemingly unstoppable success of antibiotics has been countered by an escalation of resistance mechanisms in bacteria with the emergence of many genera of bacteria that are resistant to all antibiotics (45-47). The genus *Acinetobacter* epitomizes this trend and deserves close attention. *Acinetobacter* spp. display mechanisms of resistance to all existing antibiotic classes as well as a prodigious capacity to acquire new determinants of resistance (3, 48-49).

Furthermore, *Acinetobacter* spp. appears to be well suited for genetic exchange and is among a unique class of gram-negative bacteria that are described as "naturally transformable" (50). The increasing recovery in the clinic of multidrug-resistant *A. baumannii* is a frightening reality (6). A previously published study describing the genome sequences of both susceptible (SDF) and resistant (AYE) isolates of *A. baumannii* has shed light on the abundance of resistance genes found in this organism (51). Fournier and co-workers identified a resistance island in AYE that contained a cluster of 45 resistance genes in the MDR isolate. Among the key resistance genes identified were those coding for β-lactamases, various aminoglycoside-modifying enzymes, and tetracycline efflux pumps. A significant fraction of the open reading frames were located in putative alien islands, indicating that the genome acquired a large amount of foreign DNA. More than 30 years ago it was demonstrated that *Acinetobacter* spp. could acquire antimicrobial resistance factors through conjugation of plasmids (52). The genome of *A. baumannii* not only contains a gene coding for an Ethionamide monooxygenase that phylogenetically is part of the same group as that of Ar-BVMO but also shares a 100% amino acid sequence identity with Ar-BVMO. As mentioned earlier, human FMO3, Ar-BVMO and *M. tuberculosis* EtaA can metabolize a common substrate, ethioamide. Based on this, we investigated the ability of Ar-BVMO to use as substrate two potent and selective Aurora kinase inhibitors, Danusertib and Tozasertib, used for the treatment of solid tumors and hematopoietic cancers and metabolized by human FMO3 in liver microsomes (53-54).

In vitro experiments with recombinant Ar-BVMO demonstrated that both Danusertib and Tozasertib are monooxygenated by Ar-BVMO with K_M values comparable to those obtained with recombinant human FMO3 (30). The reason why a prokaryotic flavin monooxygenase should metabolize substrates that are typically metabolized by the eukaryotic FMO enzymes is not clear. Since both proteins, together with EtaA belong to the same class of flavoproteins and are involved in detoxification processes, it can be speculated that during evolution some FMO proteins became BVMOs providing the acquiring organism new metabolic capabilities. This is not the first example of a bacterial FMO that shares the same substrate with a human flavoprotein. Chen and co-workers (55) showed that a FMO from *Methylocella silvestris* is

able to oxidize trimethylamine that is a typical substrate of eukaryotic FMO enzymes. It can be assumed that the *A. baumanni* enzyme would be able to monooxygenate the two anticancer drugs even if further experiments are required to confirm this. It could be hypothesized that genetic exchange between the progenitor (*A. radioresistens*) and its recipient of clinical relevance (*A. baumannii*), may have occurred in humans (19) leading to the BVMO acquisition by the latter species. Both *A. radioresistens* and *A. baumannii* are identified on the human skin, especially in hospitalized patients, it is therefore possible that BVMO gene exchange may have occurred at this location (4). In order to provide support for the hypothesis of lateral gene transfer, the genetic context of the Ar-BVMO (Accession number GG705134.1) was evaluated and compared to that of EtaA of *Acinetobacter baumannii* strains 1461402 (Accession number JEWP01000001) 230853 (Accession number JEWP01000002), 983759 (Accession number, JEX001000002); 263903-1 (Accession numberJMNM01000001). The genes flanking upstream and downstream of the Ar-BVMO were found to be not only identical but also in the same position as those of the four *A. baumannii* strains. In addition, the GC content (56) of the ethionamide monooxygenase genes of the four *A. baumannii* strains was compared with *A. radioresistens* genome content. The GC% was calculated using the Endmemo software (www.endmemo.com/bio/gc.php) and the values were 42.65% and 42.69% for *A. baumannii* Etha gene and Ar-BVMO of *A. radioresistens*, respectively. These data are not in support of a horizontal gene transfer unless a large portion of the genome has been transferred from *A. radioresistens* to *A. baumannii* and therefore a more in depth analysis is required which is outside the scope of this work.

The presence of a flavin monooxygenase in a MDR bacterium that has 100% sequence identity with Ar-BVMO (i.e. identical protein) with pro-drugs as substrates strongly suggests that the EtaA could be used by *A. baumannii* to metabolize/detoxify different types of synthetic drugs including antibiotics in exactly the same way as Ar-BVMO.

In this work we have demonstrated that sensitive *E. coli* cells overexpressing Ar-BVMO became resistant to the antibiotic Imipenem. NADPH consumption assay shows that Imipenem is a substrate of Ar-BVMO

and agar well diffusion assay proves that the enzyme destroys the antibacterial property of the antibiotic. Like all other β□lactams, Imipenem inhibits bacterial cell wall synthesis by binding to and inactivating penicillin binding proteins. Antibiotic resistance can occur via three general mechanisms: prevention of interaction of the drugs with target, efflux of the antibiotic from the cell, and direct destruction or modification of the compound. The most prevalent resistance mechanism against β-lactams is the production of β□lactamases. Among the β□lactamases, metalloenzymes are distinguished by having a zinc ion required for enzymatic activity (57). Sometimes referred to as carbapenemases, they are able to hydrolyze many β-lactam antibiotics including carbapenems (58). In the present work, for the first time, we demonstrate that a bacterial flavin monooxygenase is able to metabolize Imipenem in a way that it is no longer active by modification of the parent compound. Two molecules with evidence of the transformation of the lactam ring, according to Baeyer-Villiger oxygen insertion mechanism, were identified by LC-HRMS analysis. This is not the first report of a flavin monooxygenase catalysing antibiotic inactivation since Yang and co-workers have previously reported TetX, a flavin-dependent monooxygenase from bacteria of the genus *Bacteroides,* that regioselectively hydroxylates the tetracycline substrate (41). However, they could not capture the product and suggested that the resulting hydroxylated product was an unstable compound that underwent a non-enzymatic decomposition (41).

Future efforts will be focused on the screening of other carbapenem members and, if under physiological conditions appreciable carbapenemase activity is attributed to this monooxygenase enzyme, it could represent a new target for drug design in the battle against carbapenems' resistance in Acinetobacter.

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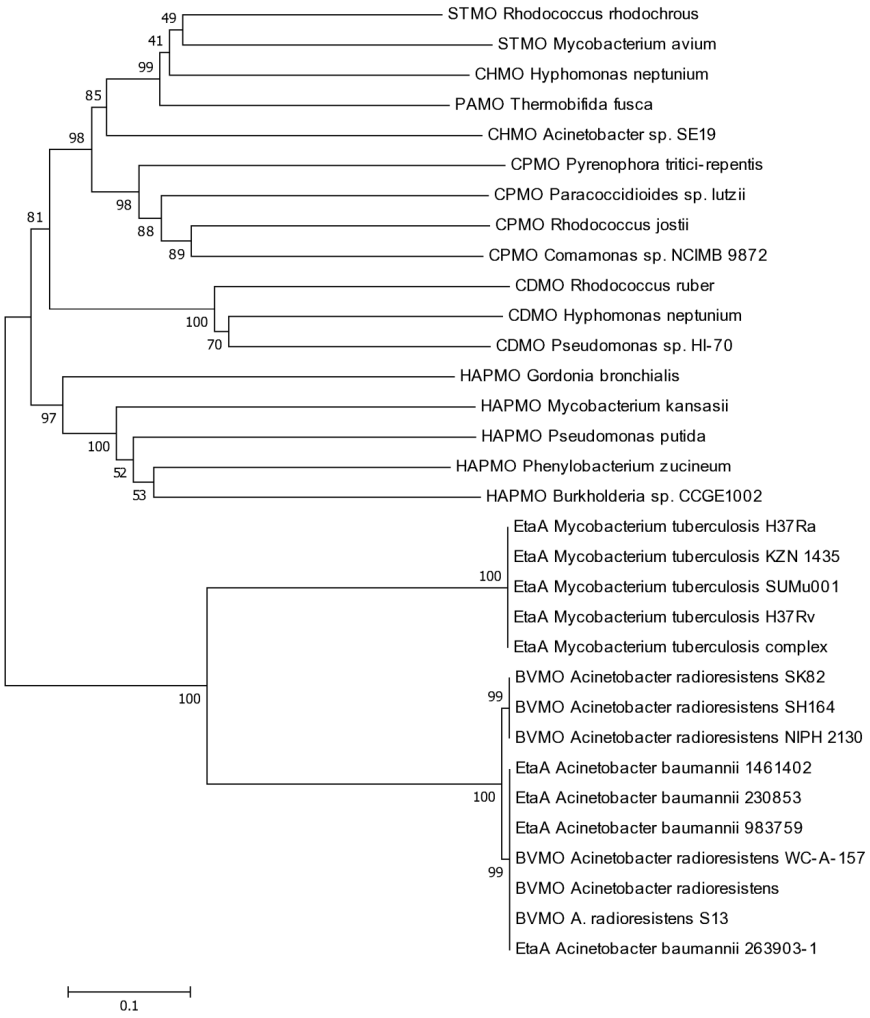

FIGURE 1. Phylogenetic position of Ar-BVMO in relation to the other BVMOs and *Acinetobacter baumannii* Ethionamide monooxygenase. Neighbor-joining consensus tree.

STMO *Rhodococcus rhodochrous*= steroid monooxygenase (BAA24454.1); STMO *Mycobacterium avium*= steroid monooxygenase (BAN31365.1); CHMO *Hyphomonas neptunium*= steroid monooxygenase (YP_760152.1); PAMO *Thermobifida fusca*= phenylacetone monooxygenase (Q47PU3.1); CHMO *Acinetobacter* sp. SE19= cyclohexanone monooxygenase (AF282240_5); CPMO *Pyrenophora treitici-repentis*= cyclopentanone monooxygenase (XP_001942142.1); CPMO *Paracoccidioides sp. lutzi*= cyclopentanone monooxygenase (XP_002792362.1); CPMO *Rhodococcus josti*= cyclopentanone monooxygenase (YP_703208.1); CPMO *Comamonas* sp. NCIMB 9872= cyclopentanone monooxygenase (BAC22652.1); CDMO *Rhodococcus ruber*= cyclododecanone monooxygenase (AAL14233.1); CDMO; *Hyphomonas neptunium* ATCC33707= cyclododecanone monooxygenase (ABI76430.1); CPDMO *Pseudomonas* sp. HI-70= cyclopentadecanone monooxygenase (BAE93346.1); HAPMO *Gordonia bronchialis*= hydroxyacetophenone monooxygenase (ACY20506.1); HAPMO *Mycobacterium kansasii*= hydroxyacetophenone monooxygenase (ZP_04749205.1); HAPMO *Pseudomonas putida*= hydroxyacetophenone monooxygenase (ACJ37423.1); HAPMO *Phenylobacterium zucineum*= hydroxyacetophenone monooxygenase (YP_002130647.1); HAPMO *Burkholderia* sp. CCGE1002= hydroxyacetophenone monooxygenase (ADG19710.1); EtaA *Mycobacterium tuberculosis* H37Ra= Ethionamide monooxygenase (YP_001285245.1); EtaA *Mycobacterium tuberculosis* KZN1435= Ethionamide monooxygenase (YP_003033907); EtaA *Mycobacterium tuberculosis* SUMu001= Ethionamide monooxygenase (ZP_07416557.1); EtaA *Mycobacterium tuberculosis* H37Rv= Ethionamide monooxygenase (NP_218371.1); EtaA *Mycobacterium* complex= Ethionamide monooxygenase (WP_003910007.1); BVMO *Acinetobacter radioresistens* SK82= Baeyer-Villiger monooxygenase (EET82318.1); BVMO *Acinetobacter radioresistens* SH164= Baeyer-Villiger monooxygenase (EEY85682.1); BVMO *Acinetobacter radioresistens* NIPH 2130= Baeyer-Villiger monooxygenase (ENV85773.1); EtaA *Acinetobacter baumannii* 1461402= Ethionamide monooxygenase (EXB35767.1); EtaA *Acinetobacter baumannii* 230853= Ethionamide monooxygenase (EXB73644.1); EtaA *Acinetobacter baumannii* 983759= Ethionamide monooxygenase (EXE14728.1); BVMO *Acinetobacter radioresistens* WC-A-157= Baeyer-Villiger monooxygenase (EJQ34398.1); BVMO *Acinetobacter radioresistens*= Baeyer-Villiger monooxygenase (ADF32068.2); BVMO *Acinetobacter radioresistens* S13= Baeyer-Villiger monooxygenase (GU145276.2); EtaA *Acinetobacter baumannii* 263903-1= Ethionamide monooxygenase (KCX39290.1).

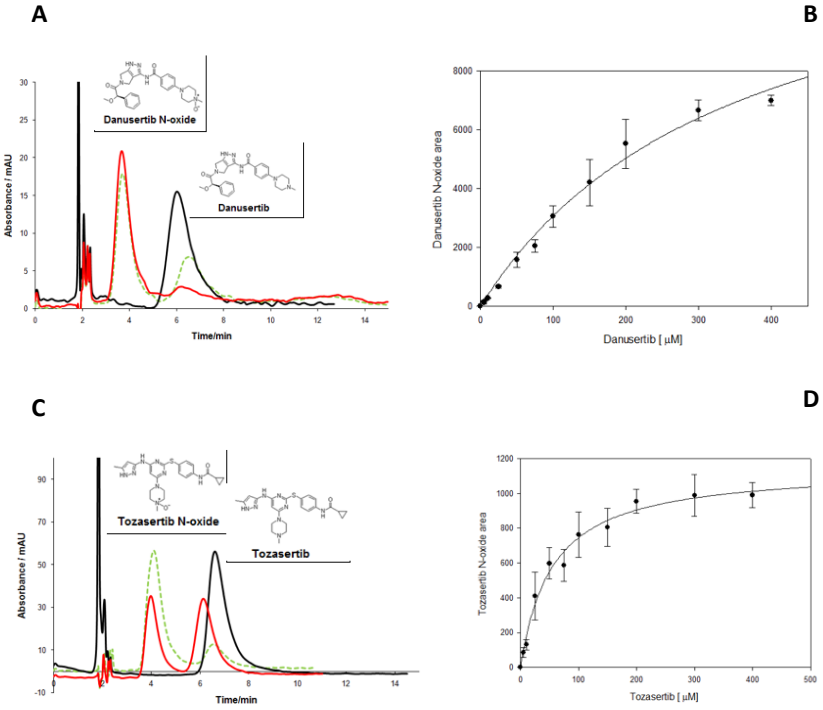

FIGURE 2. Danusertib and Tozasertib oxidation by purified recombinant Ar-BVMO. HPLC traces for Danusertib **(A)** and Tozasertib **(C)** conversion. The red trace shows Ar-BVMO reaction, the black trace shows the negative control without enzyme, the green trace shows the positive control with purified recombinant human FMO3. The retention times are 6.4 min for Danusertib and 3.9 min for the product "Danusertib N-oxide" and 6.5 min for Tozasertib and 4.4 min for the product "Tozasertib N-oxide". Michaelis Menten curve of the enzymatic "Danusertib N-oxide" (**B)** and "Tozasertib N-oxide" **(D)** product formed in the presence of increasing amount of Danusertib and Tozasertib, respectively.

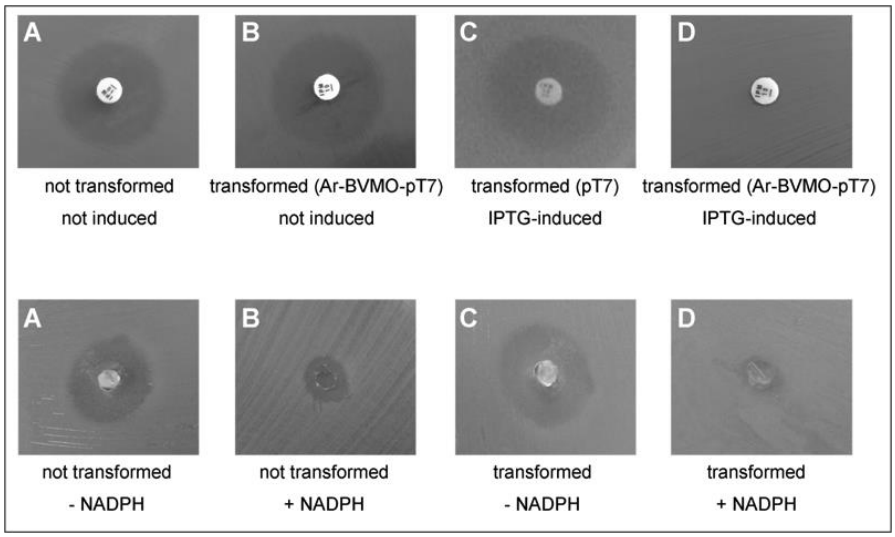

FIGURE 3. Imipenem antibiograms of *E. coli* BL21 cells against disk embedded Imipenem (Top panel). *E. coli* BL21 cells were plated on Muller Hinton agar plate at a concentration of 0.5 MacFarland and incubated overnight at 37°C. A) *E. coli* BL21; B) *E. coli* BL21 transformed with Ar-BVMO-pT7 but not induced; C) *E. coli* BL21 transformed with pT7 plasmid and induced with IPTG (negative control); D) *E. coli* BL21 transformed with pT7-Ar-BVMO and induced with IPTG.

Agar well diffusion assay (Bottom panel). Imipenem sensitive not transformed (A, B) and transformed but not induced BL21 cells (C, D) were spread over the plate surface of MHA at a concentration of 0.5 MacFarland. Fifty ml of the reaction product/s between Ar-BVMO and Imipenem were placed inside a well in the center of each plate. B, D: reaction with NADPH; A, C: reaction without NADPH.

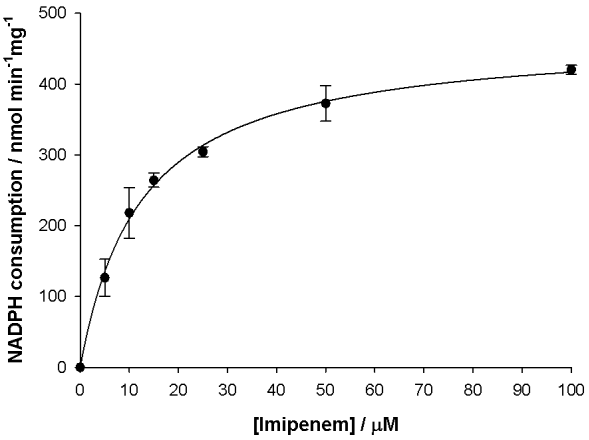

FIGURE 4. NADPH consumption assay for Ar-BVMO in the presence of Imipenem. Plot of enzymatic reaction velocity versus Imipenem concentration fitted to the Michaelis-Menten equation ($R^2 = 0.998$). Data are shown as mean ± SD of three different measurements.

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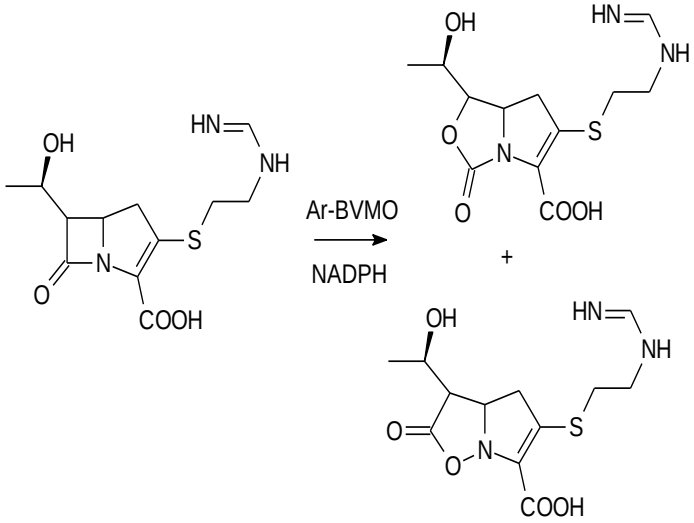

FIGURE 5. Asymmetric oxygen insertion in the carbapenem ring of imipenem according to the classical Baeyer-Villiger mechanism.

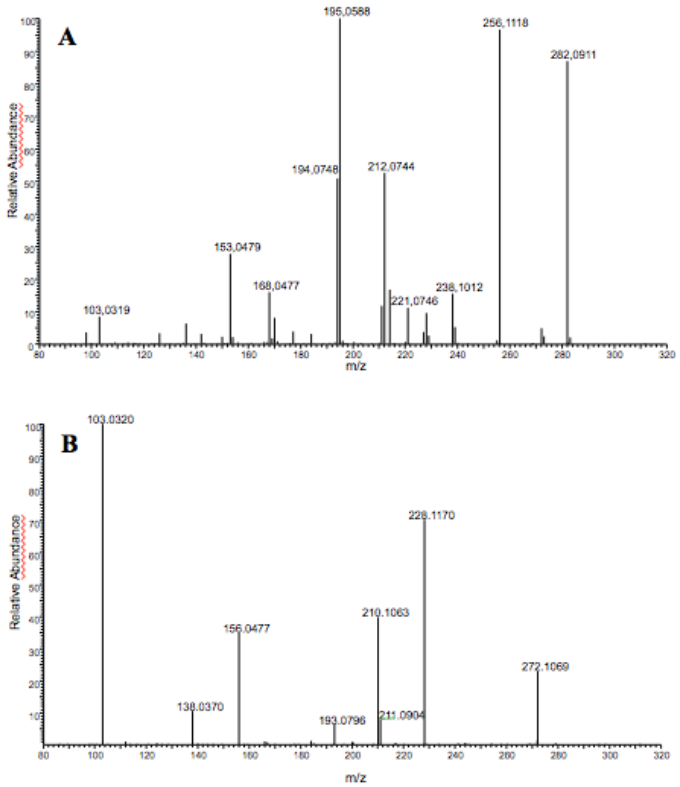

FIGURE 6. MS/MS high-resolution spectra of Imipenem and its metabolite. Spectrum of Imipenem 300.0987 m/z precursor ion (A) and spectrum of the first eluted M+O metabolite 316.0965 m/z (B) precursor ion acquired at 25% collision energy, in the range 80-320 m/z.

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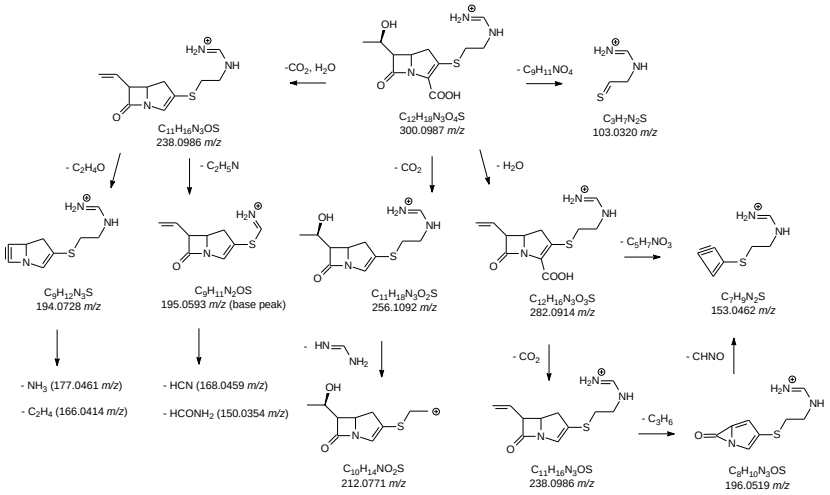

FIGURE 7. The hypothesized fragmentation pathway of Imipenem precursor ion (300.0867 m/z).

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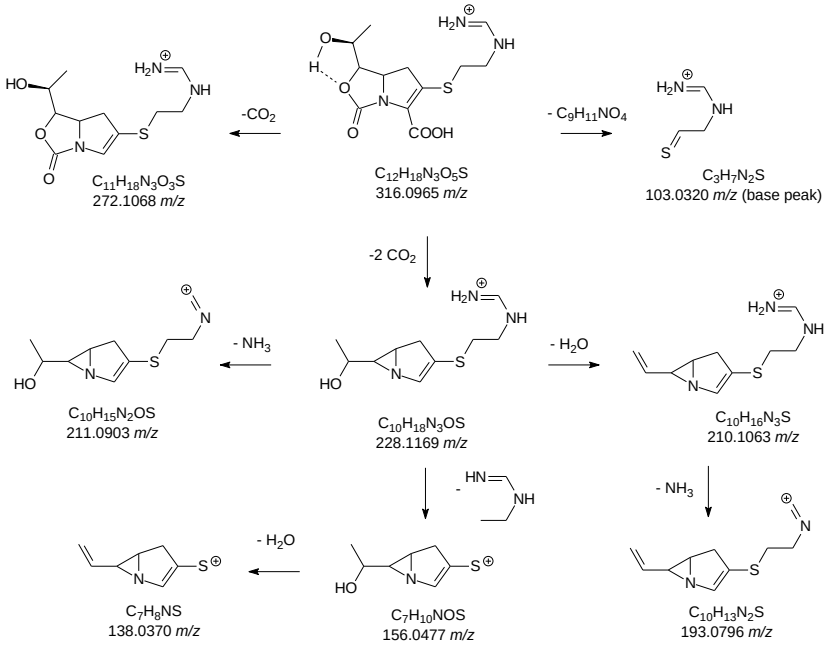

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FIGURE 8. The interpretation of MS/MS spectrum of Ar-BVMO metabolite.

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