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TITLE PAGE

Unusual Biotransformation Reactions of Drugs and Drug Candidates

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RUNNING TITLE PAGE

Running Title: Unusual Biotransformation Reactions

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Abbreviations:

ADC, antibody-drug conjugates; ADME, absorption, distribution, metabolism and excretion; AI, artificial intelligence;AO, Aldehyde oxidase; BACE1, β-site amyloid precursor protein-cleaving enzyme; ChK1, check point kinase 1; cIM, cyclic ion mobility; DBD-H, 4-(N,N-Dimethyl-amino-sulfonyl)-7-hydrazino-2,1,3-benzoxadiazole; DDI, drug-drug interaction;

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ERK, extracellular signal-regulated kinase; $5HT_6$, serotonin receptor subtype 6; MAO, monoamine oxidase; MAPK, mitogen-activated protein kinase; mARC, mitochondrial amidoxime reducing component; 6-MNA, 6-methoxy-2-naphtylacetic acid; MPS, microphysiological systems; NAT, *N*-acetyltransferase; P450, cytochrome P450; ProTac, proteolysis targeting chimera/ProTaC; UGT, UDP-glucuronosyltransferase.

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ABSTRACT

Detailed assessment of the fate of drugs in non-clinical test species and humans is essential to ensure the safety and efficacy of medicines in patients. In this context, biotransformation of drugs and drug candidates has been an area of keen interest over many decades in the pharmaceutical industry as well as academia. Although many of the enzymes and biotransformation pathways involved in the metabolism of xenobiotics and more specifically drugs have been well-characterized, each drug molecule is unique and constitutes specific challenges for the biotransformation scientist. In this mini-review written for the Special Issue on the occasion of the 50th Anniversary celebration of DMD and to celebrate contributions of Prof. F. Peter Guengerich, one of the pioneers of the drug metabolism field, recently reported "unusual" biotransformation reactions are presented. Scientific and technological advances in the "toolbox" of the biotransformation scientists are summarized. As the pharmaceutical industry continues to explore therapeutic modalities different from the traditional small molecule drugs, the new challenges confronting the biotransformation scientist as well as future opportunities are discussed.

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SIGNIFICANCE STATEMENT

For the biotransformation scientists, it is essential to share and be aware of unexpected biotransformation reactions so that they can increase their confidence in predicting metabolites of drugs in humans to ensure the safety and efficacy of these metabolites before the medicines reach large number of patients. The purpose of this review is to highlight recent observations of “unusual” metabolites so that the scientists working in the area of drug metabolism can strengthen their readiness in expecting the unexpected.

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INTRODUCTION

The recognition of bioconversion of xenobiotics goes back approximately 100 years, and these early studies in animals and humans had mainly focused on small molecules.(Bachmann and Bickel, 1985; Guengerich, 2021) Metabolism of drugs started to gain attention in 1950s with particular focus on detoxication (Handler and Perlzweig, 1945; Brodie et al., 1958) and drug-drug interactions (DDI).(Remmer, 1959) In early 1960s, cytochrome P450s (P450s) were discovered as drug metabolizing enzymes followed by other non-P450 oxidoreductases.(Omura and Sato, 1962; Guengerich, 2022) Today, the majority of drugs on the market are known to be metabolized by P450s, and aldehyde oxidase (AO), UDP-glucuronosyltransferases (UGTs), sulfotransferase, *N*-acetyltransferases (NATs) are other important contributors.(Evans and Relling, 1999; Saravanakumar et al., 2019) One hundred years after the recognition of drug metabolism, biotransformation studies have become an integral part of drug discovery and development studies leading to an immense expansion of the collective knowledge of the scientists in the area. Before, giving examples of some of these biotransformation reactions, it is important to emphasize the importance of metabolism studies in bringing safe and efficacious medicines to patients.

Biotransformation studies in drug discovery and development

During drug discovery and development, it is essential to study and understand the metabolism of drugs and drug candidate to assess the safety (Schadt et al., 2018) and potency of drug metabolites, (Fura et al., 2004; de Jesus, João Paulo Almirão et al., 2021) assess the DDI potential of metabolites, (Fu et al., 2022) optimize pharmacokinetic properties via modulating metabolic pathways, (Broccatelli et al., 2019) and to minimize formation of undesirable metabolites such as reactive metabolites.(Thompson et al., 2012; Brink et al., 2017)

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Metabolite identification and/or profiling studies carried out during lead optimization and earlier stages of drug discovery programmes can be instrumental in designing new chemical entities with improved pharmacokinetic properties. (Cerny et al., 2020; Shanu-Wilson et al., 2020) These studies are commonly complemented by metabolite trapping experiments to assess the formation of undesired reactive metabolites which due to their unstable nature cannot be detected in the absence of trapping agents (Ma and Subramanian, 2006; Inoue et al., 2015) and potentially can lead to adverse drug reactions in patients.(Thompson et al., 2016) Based on the information obtained from such trapping studies, the drug design teams can optimize the structure of drug candidates to block the pathways leading to reactive metabolite formation and/or introduce "soft spots" on the molecule to redirect the metabolic pathways towards the formation of stable metabolites.(Kalgutkar, 2020)

For the safety testing of metabolites, it is important to generate sufficient information during both preclinical and clinical phases to increase confidence in the assessment of human (in particular circulating) metabolites and ensure the appropriate testing of these metabolites in preclinical toxicology studies as detailed in the regulatory guidelines.(FDA, 2020; ICH, 2013) Although *in vitro* and preclinical *in vivo* studies provide significant guidance with respect to potential human metabolites, it is not uncommon to come across surprises with respect to the formation of disproportionate human metabolites during the clinical studies which may necessitate the synthesis of such metabolites and their safety testing in preclinical toxicology studies at adequate exposure levels. (Schadt et al., 2018; Zheng et al., 2018; Asano et al., 2022)

Methodologies to study drug metabolism

Use of preclinical species is common to study metabolism of new chemical entities, however, it is important to consider the species differences in enzymatic pathways which may result in

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misleading information in terms of metabolites formed in humans.(Baillie and Rettie, 2011) Therefore, over the many years, efforts have focused on the development of *in vitro* systems with the ultimate aim of increasing the confidence in predicting human metabolites, identifying the enzymes involved in the metabolism of drugs and minimizing animal testing. In the recent years, various advances have been made in developing such in vitro systems for application to metabolite profiling and identification studies.(Docci et al., 2019) Although it is not within the scope of this review to provide a comprehensive listing of such *in vitro* systems, several examples are briefly mentioned below, nevertheless.

Several *in vitro systems* which can maintain metabolic activity for an extended period of time have been made available to study the metabolism of in particular low clearance drug candidates. Hμrel® coculture system (Hultman et al., 2016), HepatoPac® micropatterned coculture system (Ballard et al., 2020) are two examples of such systems which have been successfully applied to study drug metabolism. In addition to these 2D systems, 3D liver spheroids have been assessed for their utility in biotransformation studies (Pinheiro et al., 2017) providing the advantage of maintained enzymatic activity for long periods of time (>2 weeks). (Kanebratt et al., 2021) These long-term incubations offer the possibility of simulating steady-state conditions in humans via “multiple dosing” in the system as well as the opportunity to study low-turnover compounds and to detect secondary metabolites. Utilization of 3D liver spheroids in combination with microphysiological systems (MPS) is an alternative approach creating an even more physiological relevant model with a circulating medium mimicking the blood flow. (Foster et al., 2019; Cox et al., 2022) In these organ-on-a-chip systems, combination of multiple compartments representative of different organs can be applied to metabolism studies with the aim of assessing formation and elimination pathways of different metabolites. (Kim et al., 2015; McAleer et al., 2019) Although majority of the *in vitro* models focus on the hepatic metabolism, there are *in vitro* systems being developed for

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the study of extrahepatic and in particular intestinal metabolism such as cryopreserved enterocytes (Ho et al., 2017), permeabilized cryopreserved human enterocytes (MetMax®) (Li et al., 2018) and more recently, a duodenum intestine-chip model.(Kasendra et al., 2020)

When summarizing methodologies in the toolbox of the biotransformation scientist, it will not be possible to proceed without mentioning isotope labelling even though this is not a new approach. Stable isotope or radioisotope labelling of drugs and drug candidates is a common methodology to aid in comprehensive understanding of the metabolic pathways. Drugs labelled with radioisotopes make the detection of metabolites which are not "visible" by other detection methods possible and provide quantitative information. (Isin et al., 2012) On the other hand, stable isotope labelling can be valuable in detecting unexpected metabolites as well as understanding the detailed mechanism of the biotransformation reactions and several examples presented in this review utilize one or both of these labelling approaches (*vide ultra*).

Mass spectrometry has been utilized in biotransformation studies for more than 50 years and with the introduction of liquid chromatography into the system, LC-MS has become the standard analytical tool for the analysis of samples from metabolism studies (Wen and Zhu, 2015) and there is continuous development directed towards improving the sensitivity and efficiency in detecting metabolites as well as facilitating structural identification. Cyclic ion mobility (cIM) mass spectrometry applied to the resolution of isomeric acylglucuronides, (Higton et al., 2021), and on-line coupling of electromembrane extraction with MS to study drug metabolism in liver spheroids (Skottvoll et al., 2021) are two examples of advances recently reported in this area. It is important to remember that LC-MS can also be further coupled with NMR to enable unambiguous characterization of metabolites in an efficient manner. (Gathungu et al., 2020)

Importance of *in silico* prediction tools (Kirchmair et al., 2015; Tyzack and Kirchmair, 2019) and automated (or semi-automated) metabolite identification packages should not be underestimated in aiding the biotransformation scientists in their metabolite identification studies.(Pähler and Brink, 2013; Ahlqvist et al., 2015) On the point of the biotransformation scientists, as the available tools and technologies available evolve and advance, the role biotransformation scientists play in drug discovery and development is and will likely to remain essential, a point which was recently elaborated on by Kramlinger *et al.(Kramlinger et al., 2022)*

Unusual Biotransformation Reactions

Thanks to the extensive work carried out by many scientists over the last 70 years in particular, our understanding of involvement of enzymes in the metabolism of drugs, drug candidates and xenobiotics at large has expanded immensely. Despite of this substantial available knowledge, scientists working in the field of drug metabolism continue to report unusual observations leading to unexpected metabolites or involvement of enzymes that are not expected to be involved in the metabolism of drugs with certain chemical motifs. In order to increase awareness and alert the drug metabolism scientist for such unexpected metabolites, we compiled previously published (and in some cases unpublished) unusual observations and shared with the scientific community. A brief summary of previous compilations as well as a selection of recent examples are presented below.

BRIEF HISTORICAL PERSPECTIVE

Although covering the entire 200 years of drug metabolism is certainly out of scope of this review, it is important to remind that one of the first metabolite identification experiments has been performed by Justus von Liebig in 1829.(Liebig, 1829) He was able to isolate hippuric acid from the urine of the horses. The intermediacy of acyl CoA conjugate of benzoic acid

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was not known at the time, but Prof. von Liebig nevertheless succeeded in demonstrating that the bioconversion occurs in vivo (Fig. 1). Arguably, this bioconversion might have been considered unusual at the time.

More than 150 years after this metabolite identification study, I had the privilege of co-authoring several compilations of unusual biotransformation reactions catalyzed by P450 and non-P450 enzymes together with Prof. F. Peter Guengerich who is one of the founding fathers of drug metabolism. Even though, we referred to these biotransformation reactions as "unusual", for the majority of the cases, the initial step is rather a simple one catalyzed by a well-known drug metabolizing enzyme, such as P450s. However, subsequent chemical steps result in the formation of an unexpected (hence the term unusual) final metabolite product. In this part of the review, selected examples from our previous compilations as well as first treatment of the subject by Prof. Guengerich are presented.(Guengerich, 2001; Isin and Guengerich, 2007; Guengerich and Isin, 2014)

Chemically driven intramolecular rearrangement reactions may lead to ring expansions (Figure 2A)(Mutlib et al., 2000), ring contractions (Fig. 2B),(Yin et al., 2004) and even new ring formations(Fig. 2C (Reilly et al., 2003) and Supplemental Fig. 1(Kamel et al., 2010)). To this group of reactions leading to unusual metabolites, dimerizations can certainly be added as well (Fig. 2D).(Dalvie and O'Connell, 2004)

In drug metabolism, it is common to see *O*- and *N*-dealkylation reactions catalyzed mainly by P450 mediated hydroxylation of the carbon alpha to the heteroatom.(Guengerich, 2018) However, due to the stable nature of the C-C bond, *C*-dealkylations are rather rare.(Bolleddula and Chowdhury, 2015) C-dealkylation of LC15-0133, a dipeptidyl peptidases IV inhibitor (Figure 3A)(Yoo et al., 2008) and aplidine (Fig. 3B), a marine natural

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product being evaluated for its antitumor and antiviral properties(Brandon et al., 2005; Brandon et al., 2007) are two such examples of *C*-dealkylation reactions.

Although oxidative metabolism is the most common biotransformation pathway for the marketed drugs, facilitating the elimination of the drug molecule from the body by increasing hydrophilicity,(Josephy et al., 2005) there are also rare examples of reductive metabolism of drugs. Aldehyde oxidase has been shown to reduce insecticide imidacloprid in laboratory animals (Fig. 4A),(Dick et al., 2005) and *N*-hydroxylamine reductase system has been proposed to be involved in the reduction of thrombin inhibitor ximelagatran (Fig. 4B).(Gustafsson et al., 2004; Andersson et al., 2005) and subsequently mitochondrial amidoxime reducing component (mARC) has been identified as the missing component of the reducing system.(Havemeyer et al., 2006; Ott et al., 2015) Examples of more recent reduction reactions are given later in this review.

Before moving onto more recent examples, it is important to point out conjugation reactions may also lead to unusual metabolites. Conversion of proton pump inhibitor omeprazole to pyrdinylmethyl sulfenic acid and the imidazolyl GSH conjugate has been proposed to be initiated by a nonenzymatic reaction with GSH (Fig. 5A).(Weidolf and Covey, 1992)

An acyl CoA conjugate has been reported to be involved in the metabolic conversion of (*R*)-flunaxoprofen to (*S*)-flunoxaprofen-1-*O*-β-glucuronide. In this example, an epimerase enzyme catalyzes the chiral inversion following the formation of the acyl-CoA thioester (Fig. 5B).(Grillo et al., 2010)

Metabolism of the drug candidate AZD6610 also involves the intermediacy of an Acyl CoA-thioester. In this particular case, in analogy to fatty acid metabolism, a 2-carbon chain shortening takes place resulting in the formation of the carboxylic acid metabolite (Fig. 5C).(Guengerich and Isin, 2014)

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KEY RECENT ADVANCES

Since the publication of our previous reviews on the subject, many other unusual metabolites have been reported in the literature and some selected examples are described below. Some of the approaches and technologies used to isolate and characterize these unusual metabolites are also mentioned for some but not all of the examples to avoid redundancy.

Cyclizations, dimerizations, isomerizations

Gu et al. reported the cyclization of AZD7325, a $GABA_{A\alpha2,3}$ receptor modulator resulting in a long-circulating metabolite (Fig. 6). (Gu et al., 2018) α-Carbon oxidation of the AZD7325 followed by dehydration is proposed to lead to an unstable intermediate which undergoes oxidative cyclization followed by aromatization to form metabolite M9. It is interesting to note that M9 together with several other metabolites were either not detected or detected as minor metabolites in plasma samples following a single dose administration to humans, whereas these metabolites were observed as major metabolites after repeated dosing. In this study, the authors utilized collision-induced dissociation as an ionization technique in a linear ion trap as part of their LC-MS/MS set-up used for metabolite identification.Another interesting cyclization reaction involves the glutathione adduct of vildagliptin, a dipeptidyl peptidase-4 inhibitor (Villhauer et al., 2003) which has been reported to cause idiosyncratic liver injury in one patient in Japan (Kurita et al., 2014). The proposed mechanism for the formation of thiazoline containing metabolite M464 involves the non-enzymatic chemical nucleophilic attack of GSH to the nitrile moiety followed by hydrolysis and cyclization of the GSH adduct (Fig. 7).(Mizuno et al., 2019) Another related metabolite M407, corresponding cysteine analogue of M464, has also been observed. These two metabolites are detected in rat urine, feces and bile and authors speculate that vildagliptin can react covalently with cysteine

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residues of proteins in human which might have played a role in the previously reported case of liver injury.

In our previous reviews, we had included several dimerization reactions initiated by P450 mediated radical formation. Another such dimerization reaction has been reported recently by Takahashi *et al.* for GDC-0994, an extracellular signal-regulated kinase (ERK) 1 and 2 inhibitor. (Ren et al., 2015) The resulting homodimers formed via C-C (M13) and C-N (M14) coupling are detected in bile and feces obtained from the *in vivo* rat studies. Based on their elegant computational work, authors propose that the dimer formation takes place via tandem N-H abstraction in the P450 3A4 active site followed by radical coupling (Fig. 8).(Takahashi et al., 2020). In this work, a propriety system (BMO Production Kit, HepatoChem Inc., Beverly, MA) utilizing organometallic catalysts are used to generate the metabolites of interest. After isolation of metabolites, in addition to one dimensional ^{1}H and ^{13}C NMR spectroscopy, two-dimensional correlation spectroscopy, heteronuclear single quantum correlation spectroscopy, heteronuclear multiple bond correlation spectroscopy, and rotating frame nuclear Overhauser effect spectroscopy were employed at room temperature as well as at 335K for the unambiguous characterization of metabolites. The increase in temperature helped to obtain well resolved NMR spectra, as both dimeric metabolites resulted in broad resonances due to restricted bond rotation at room temperature.A rather complex pathway has been proposed for the biotransformation of GDC-0575, a check point kinase 1 (ChK1) inhibitor. The first step of the metabolism of GDC-0575, is as in many other cases, an oxidation catalyzed by P450 enzymes. The resulting epoxide undergoes a cyclization reaction to lead to intermediate 1 which in turn is either oxidized further to give metabolite M12 or react with another molecule of GDC-0575 to give dimeric metabolite M17 (Fig. 9). For the sake of simplicity, the details of the pathway are not shown below and the readers are encouraged to consult the original manuscript.(Zhang et al., 2022) As the incubations with

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microsomes and expressed P450s did not yield sufficient material for characterization of metabolites of interest, authors utilized a chemical degradation approach to generate M12 and M17 insufficient quantities for structural identification by NMR.

Mitragynine is an alkaloid component of the herbal supplement derived from the leaves of the *Mitragyna speciocas* tree commonly known as kratom.(Hassan et al., 2013) One of the hydroxylated metabolites of mitragynine is 7-OH mitragynine which is known to have analgesics effects and proposed to contribute to the addictive properties of kratom. Kamble *et al.* has studied the plasma stability and metabolism of 7-OH mitragynine in an effort to understand better the pharmacological effects of kratom in humans. (Kamble et al., 2020) In human plasma, it was shown that 7-OH mitragynine is converted to a metabolite referred by the authors as the mitragynine pseudoindoxyl (Fig. 10). This metabolite was generated from incubations of 7-OH mitragynine with human plasma followed by isolation and purification using preparative thing layer chromatography and HPLC for further characterization. The mechanism is proposed to be analogues to a semipinacol rearrangement, previously reported to take place for mitragynine in fungi (Zarembo et al., 1974), however, the nature of the plasma enzymes involved in this reaction are not known.

Reductions

As mentioned above, although oxidative pathways are responsible for the metabolism of majority of drugs, several examples of reductive drug metabolism have been reported in the recent the years. One such example is the reductive cleavage of sulfonamide drug candidate SAM-760, a serotonin receptor subtype 6 ($5HT_6$) receptor antagonist being developed for the treatment of Alzheimer's disease. (Fullerton et al., 2018) Formation of benzene sulfinic acid (confirmed by comparison with an authentic standard) as a major metabolite of SAM-760 in human hepatocytes has been attributed potentially to a thiol-mediated reductive cleavage of

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the aryl sulfonamide moiety due to the presence of GSH or other thiols present in the incubation (Fig. 11). Possibility of nucleophilic displacement of the aryl moiety was ruled out based on the absence of any GSH or thiol adducts. (Sawant-Basak et al., 2018)

Gut microbiota can play an important role in particular in the reductive metabolism of drugs. (Dhurjad et al., 2022) Several examples of these reductions have been reviewed previously (Guo et al., 2020) and a recent example was reported by Guo *et al.* for tacrolimus, (Guo et al., 2019) a commonly used immunosuppressant for kidney transplant recipients. Metabolite M1 formed by the reduction of the ketone moiety (Fig. 12) was detected in stool samples from healthy adults as well kidney transplant recipients and was assessed to be much less potent than tacrolimus. Authors based on their bacterial screening panel concluded that most *Clostridiales* bacteria are able to metabolize tacrolimus extensively and this reductive metabolism in the gut may be responsible for the low and variable oral bioavailability of tacrolimus. The authors generated M1 from incubations with *F. prausnitzii* (also belonging to *Clostridiales* order) cells. In addition to mass spectrometry and NMR spectroscopy, infrared spectroscopy was utilized in this study, in particular, to confirm that M1 is a carbonyl reduction product of tacrolimus.Involvement of gut microbiota in the reductive metabolism was also reported for LY3202626, a β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) inhibitor being assessed as a potential treatment for early Alzheimer's disease.(McKinzie et al., 2021) LY3202626 is converted to M2 which together with M16 are the most abundant metabolites in feces and present as the only source of radioactivity in the feces after 480h following the administration of LY3202626 (Fig. 13). Metabolite M16 was shown to be formed upon reduction of M2 in fecal homogenates under anaerobic conditions and it is proposed to be reabsorbed leading to enterohepatic recirculation. M16 is reoxidized by aldehyde oxidase to M2 in the liver which may explain the observed slow excretion of radioactivity in the human mass balance, excretion and metabolism study.(Katyayan et al.,

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2020) Due to the involvement of aldehyde oxidase in the formation M12 from M16, M16 was incubated with human liver cytosol for isolation of M2 for NMR analysis.Another rather uncommon reductive pathway involves the mARC (Havemeyer et al., 2011) and it has recently been shown that mARC system mentioned earlier in this review can catalyze the reduction of hydroxamic acid moiety to the corresponding amide for several drugs. (Ginsel et al., 2018) The reduction of vorinostat, a histone deacetylase inhibitor, (Duvic and Vu, 2007; Ramalingam et al., 2007) is one of the examples for this reductive metabolism pathway (Fig. 14).

Other unusual biotransformation reactions

Recently, Matsumoto *et al.* has reported an example of a rarely observed C-C bond cleavage via a Baeyer-Villiger oxidation as a key step in the conversion of non-steroidal anti inflammatory prodrug nabumetone to its active metabolite 6-methoxy-2-naphtylacetic acid (6-MNA). In their study, authors showed that FMO5 is involved in the biotransformation of the 3-OH nabumetone to the 6-MNA via the formation of the intermediate aldehyde (6-MN-CHO). The initial oxidative step to the α-hydroxy metabolite (3-OH-NAB) was catalyzed by P450s 2B6, 2C18 and 3A4 (Fig. 15).(Matsumoto et al., 2021) In order to confirm the intermediacy of 6-MN-CHO, the authors performed a trapping experiment by incubating 3-OH-NAB with human FMO5 followed by addition of a fluorescence labelling reagent, 4-(N,N-Dimethyl-amino-sulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H). The DBD-H derivative of 3-OH-NAB was detected using an HPLC system coupled to a fluorescence detector.Incorporation of an oxetane moiety has been shown to improve the overall pharmacokinetic as well as physicochemical properties of AZD1979 (Johansson et al., 2016) a melanin-concentrating hormone receptor 1 antagonist. Although the metabolites of AZD1979 reported are not unusual structurally, the involvement of epoxide hydrolase (Li et al., 2016) and GST (Li et al., 2019) in the formation of the two metabolites leading to oxetane

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and azetidine ring opening, respectively, is rather unexpected (Fig. 16). In this work, rat S9 liver fractions were chosen as the biocatalytic system for the synthesis of M12 for further characterization as it was shown by UPLC-HRMS that same GSH adducts was formed both in rat liver S9 fractions and human hepatocytes.During their Phase I studies with pimasertib, an inhibitor of MEK1 and 2 signaling protein of the mitogen-activated protein kinase (MAPK) pathway, Scheible *et al.* detected and reported a total of 14 different metabolites in plasma, urine, and feces samples upon administration of the ^{14}C labelled pimasertib to humans.(Scheible et al., 2017) One of these metabolites, M554, a phosphoethanolamine conjugate of pimasertib, which is detected as a major metabolite in plasma and urine, is of particular interest due to its novel structure (Fig. 17). The authors succeeded in identifying the structure of M554 via NMR spectroscopy after isolation and purification from *in vitro* incubations with HepaRG cells as well as comparison with the chemically synthesized authentic standard of the proposed structure. Although the exact mechanism of formation of M554 is not known, one of the proposed pathwaysis the engagement of pimasertib in lipid metabolism, more specifically the Kennedy pathway (Gibellini and Smith, 2010; Ghodke et al., 2022) due to presence propanediol moiety which is structurally similar to glycerol.(Schneider and Vance, 1979)

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CURRENT CHALLENGES AND KNOWLEDGE GAPS

For many years, drug discovery efforts hence the biotransformation studies have focused on "traditional" orally bioavailable small molecules that happen to fall into the chemical space described by Lipinski's rule of 5.(Lipinski et al., 1997) However, as the diversity of targets of interest to the pharmaceutical industry expanded, it has become essential to expand both the chemical space (Doak et al., 2014) and diversity of the modalities to address the targets previously thought to be undruggable.(Wu et al., 2014; Huang and Dixit, 2016)

Expansion of drug modalities beyond "traditional" small molecules requires the development of alternate *in vitro* systems to study the absorption, distribution, metabolism and excretion (ADME) properties of such molecules as well as consideration of enzymatic pathways which can be rather different than the ones involved in the metabolism of small molecules. These modalities such as peptides and peptidomimetics, oligonucleotide-based therapeutics, proteins, antibodies as well antibody-drug conjugates (ADC) are metabolized (or catabolized) by proteases, peptidases, or nucleases. Recent publications have described various approaches for studying the biotransformation of protein therapeutics,(Schadt et al., 2019) antisense oligonucleotides, *N*-acetylgalactosamine conjugated small interfering RNA(McDougall et al., 2022) (Robin McDougal) and ADCs.(Bolleddula et al., 2020; Cai et al., 2020) Admittedly, the resulting products from the degradation of these large molecule modalities are rather predictable, however, such biotransformation studies require different *in vitro* systems, analytical approaches and a knowledge of enzymes and biotransformation pathways rather different than the "traditional" small molecules and hence may be considered as unusual in the context of this review. For example, all of the 10 ASOs approved by FDA to date have been reported to be degraded primarily by endonucleases and exonucleases in the bloodstream and the target cells.(Migliorati et al., 2022) Fomivirsen. a first-generation ASO developed as an antiviral agent was reported to be metabolized or rather degraded in the retina via the

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cleavage of phosphodiester bonds by exonucleases.(Geary et al., 2002) Mipomersen is a second generation ASO developed for the treatment of familial hypercholesterolemia and has increased resistance towards degradation by nucleases compared to first generation ASOs. Metabolism of mipomersen involves degradation initially by endonucleases followed by exonucleases, and the metabolites were reported to be excreted in the urine.(Crooke and Geary, 2013) Endonucleases and exonucleases have been reported to be involved in the metabolism of eluforsen, developed for the chronic inhalation treatment of cystic fibrosis patients. Interestingly, however, additional metabolites resulting from the oxidation of the phosphodiester bonds of eluforsen have been also observed in mouse lung samples from *in vivo* studies. (Kim et al., 2019)

To the list of these alternative modalities we can also add targeted protein degraders (proteolysis targeting chimera/ProTaC) which have rather different physicochemical properties than traditional small molecules.(Pike et al., 2020)

As part of the efforts to optimize the pharmacokinetic profile of small molecule drug candidates and minimize victim drug-drug interaction potential, the metabolic pathways of more drug candidates are being shifted towards non P450 enzymes such as aldehyde oxidases (AO). Although the metabolites formed by AO are predictable in many of the cases, difficulties in predicting human pharmacokinetics of drugs being predominantly metabolized by AOs has led the researchers to focus on improving the understanding of drug metabolism mediated by these enzymes. In addition to AO, monoamine oxidase (MAO) and xanthine oxidoreductase are other non-P450 enzymes that need to be considered in the oxidative metabolism of drugs. (Rendić et al., 2022) Although known for a long time, the importance of microbiota involvement in drug metabolism is also gaining more recognition, and efforts are directed towards developing *in vitro* systems and understanding better the involvement of gut bacteria in the metabolism of drugs.(Kang et al., 2013; Colotti and Rinaldi, 2020; Yu et al.,

2017) In a recent publication, authors reported their studies on human gut microbiotal metabolites of resveratrol. (Iglesias-Aguirre et al., 2022) In this work, stool samples from healthy volunteers were collected, and fecal suspensions were prepared. The compounds of interest were incubated in fecal cultures under anoxic conditions, and samples were analyzed using ULPC-MS and GC-MS. The results of metabolite identification studies from the *in vitro* system confirmed the results from the *in vivo* studies.

Progress in artificial intelligence (AI) driven predictive approaches heavily relies on the availability of good quality, well-curated, reusable data and capturing biotransformation pathways and metabolite information in a structurally searchable manner is not trivial. Implementing customized corporate biotransformation databases is crucial in maximizing the value that can be harvested from biotransformation studies carried out over the many years in a pharmaceutical company.(Iegre et al., 2016)

PERSPECTIVE ON FUTURE DIRECTIONS

As exemplified above, despite the knowledge accumulated over the years, metabolism of drug molecules continues to challenge the biotransformation scientists with the formation of unexpected metabolites. Despite of the fact that the model systems, analytical technologies, and software tools evolve facilitating the detection and identification of metabolites, the experienced biotransformation scientists are expected to remain essential for elucidation of previously unobserved metabolites and metabolic pathways. As the pharmaceutical industry diversifies their drug discovery and development programmes to alternative modalities, the focus is shifting towards the biotransformation studies of these modalities where the metabolites can be rather predictable but unusual when compared to small molecule drugs.

In a drug discovery and development programme, metabolite identification studies mainly focus on predicting and understanding the drug metabolism in healthy volunteers. However, the disease state as well as the maturation is known to have an impact on the drug metabolizing enzymes and hence the metabolism of drugs. As the pharmaceutical industry continues to put effort on personalized medicine, being able to assess and predict the metabolism in a certain patient population gains importance, one important example being the paediatric population.(van den Anker et al., 2018; van Groen et al., 2021)

Although very valuable information can be obtained from metabolite identification studies, this value can be further increased by combining with other information and providing context. Mass spectrometry imaging in particular (Granborg et al., 2022) and multi-modal imaging in the broader sense are areas which are already developing rapidly and expected to be applied more frequently in the context of metabolite identification studies. Such a multi-modal approach makes it possible to get the most information from a single study or even a single sample by combining molecular information on a drug molecule, its metabolites as well

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as endogenous molecules together with anatomical and functional information on a particular tissue or organ of interest.(Dannhorn et al., 2020; Vermeulen et al., 2022) To be able to maximize the value from the large amounts of data generated, workflows taking advantage of deep learning approaches are being developed.(Race et al., 2021) A recent publication by Strittmatter *et al.* clearly demonstrates application and the potential impact of multi-modal imaging on drug discovery and development. (Strittmatter et al., 2022) In this work, intratumor distribution of gemcitabine, used as a treatment for pancreatic cancer, and its metabolites were studied in a mouse model of pancreatic cancer. Mass spectrometry imaging data showing the distribution of gemcitabine and its metabolites in the tumour are spatially overlayed with imaging mass cytometry, multiplex immunofluorescence microscopy, and hematoxylin and eosin staining images. The outcome from this multi-modal imaging approach allowed the assessment of the effect of the drug and the metabolites in relation to the tumour microenvironment.Finally, metabolites can be useful starting points for the generation of new chemical entities and diversifying the chemical space.(Romero et al., 2021; Charlton and Hayes, 2022) Therefore, another area that is expected to continue to grow will be biocatalytic approaches utilizing in particular engineered bacterial enzymes for the synthesis of new molecules which are difficult to make via chemical synthesis.(Thomson et al., 2022) It is also important to note the contribution of biocatalysis to green chemistry efforts.

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CONCLUSIONS

In this review, the current and potential future toolbox and the challenges of the biotransformation scientists in metabolite identification studies during drug discovery and development was discussed. Several previously compiled as well as recently reported unusual biotransformation reactions resulting in the formation of unexpected metabolites are presented. As can be seen in almost all the examples, the metabolism of drugs and drug candidates is initiated by a well-known drug metabolizing enzymes and pathway. However, understanding and elucidating the subsequent chemical and enzymatic steps is key in constructing a complete picture of the biotransformation pathways of the drug. In particular for the early career biotransformation scientists, identification of unusual metabolites and elucidating the pathways leading to these metabolites constitute a significant challenge. Published case studies shared with the scientific community certainly help to increase awareness and prepares the biotransformation scientists to think beyond the expected or in other words common metabolites. In addition, developing a solid organic chemistry knowledge and being well-versed in “arrow pushing” is essential when searching for the unexpected metabolites. Advanced analytical technologies and software tools as described above can be of significant assistance but only when these tools coupled to the intuition and creativity of the biotransformation scientist, novel discoveries can be made. It is also very important for the pharmaceutical companies to train scientists by organizing customized training courses where the biotransformation scientists have the opportunity to go through case studies in detail. Finally, it is the hope of the author that the biotransformation scientists working in the area of drug metabolism will find this review useful in their daily work and the examples presented will help them tackle the challenges they face.

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AUTHORSHIP CONTRIBUTION

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FIGURE LEGENDS

Figure 1. In vivo conversion of hippuric acid to benzoic acid in horses first reported by Justus Von Liebig in 1829.

Figure 2. Examples of ring formation, expansion and contraction reactions summarized in previous compilations A. Ring expansion B. Ring contraction C. New Ring Formation D. Dimerization.

Figure 3. *C*-dealkylation of A. LC15-0133 B. Aplidine.

Figure 4. Examples of reductive metabolism A. AO mediated reduction of neonicotinoid B. *N*-hydroxylamine reductase mediated reduction of ximelagatran.

Figure 5. Conjugation reactions leading to unexpected metabolites A. Conversion of omeprazole to pyrdinymethylsulfenic acid and imidazolyl GSH conjugate B. Conversion of (*R*)-flunoxaprofen to (*S*)-flunoxaprofen-1-*O*-β-glucuronide C. Chain shortening of AZD6610.

Figure 6. α-carbon oxidation of AZD7325 resulting in the formation of a cyclized metabolite M9.

Figure 7. Formation of cyclized metabolite M464 from vildagliptin initiated by non-enzymatic reaction with GSH.

Figure 8. P450 mediated dimerization of GDC-0994.

Figure 9. Metabolic pathways of GDC-0575 resulting in the formation of oxidized metabolite M12 and dimeric metabolite M17.

Figure 10. Oxidative metabolism of 7-OH mitragynine followed by a semipinacol-like rearrangement resulting in the formation of a mitragynine pseudoindoxyl metabolite.

Figure 11. Biotransformation of 3-OH nabumetone leading to C-C bond cleavage via a Baeyer-Villiger oxidation.

Figure 12. Metabolic pathways of AZD1979 catalyzed by epoxide hydrolase and GSH.

Figure 13. Reductive cleavage reaction leading to the formation of benzene sulfenic acid from SAM-760.

Figure 14. Reductive metabolism of tacrolimus resulting in the formation of hydroxy metabolite M1.

Figure 15. Reduction of LY3202626 mediated by gut microbiota

Figure 16. Reduction of vorinostat hydroxamic acid moiety to the corresponding amide metabolite by mitochondrial amidoxime reducing component.

Figure 17. Involvement of pimasertib in lipid metabolism pathway resulting in the formation of phosphoethanolamine conjugate M554.

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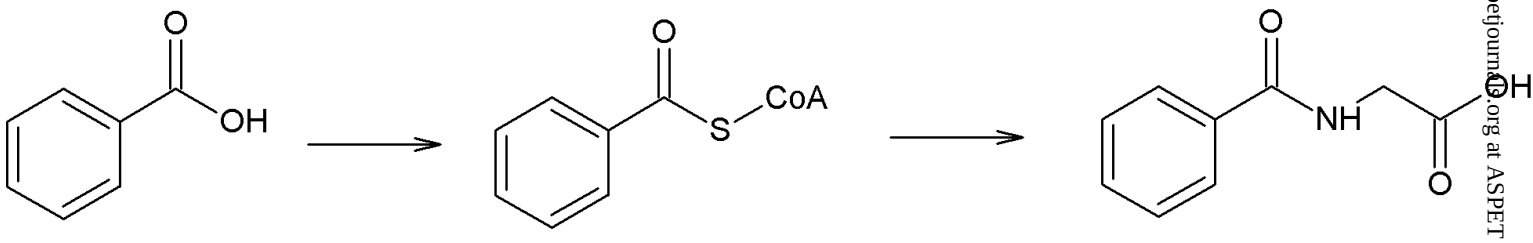
Figure 1



Benzoic acid

Hippuric acid

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Figure 2

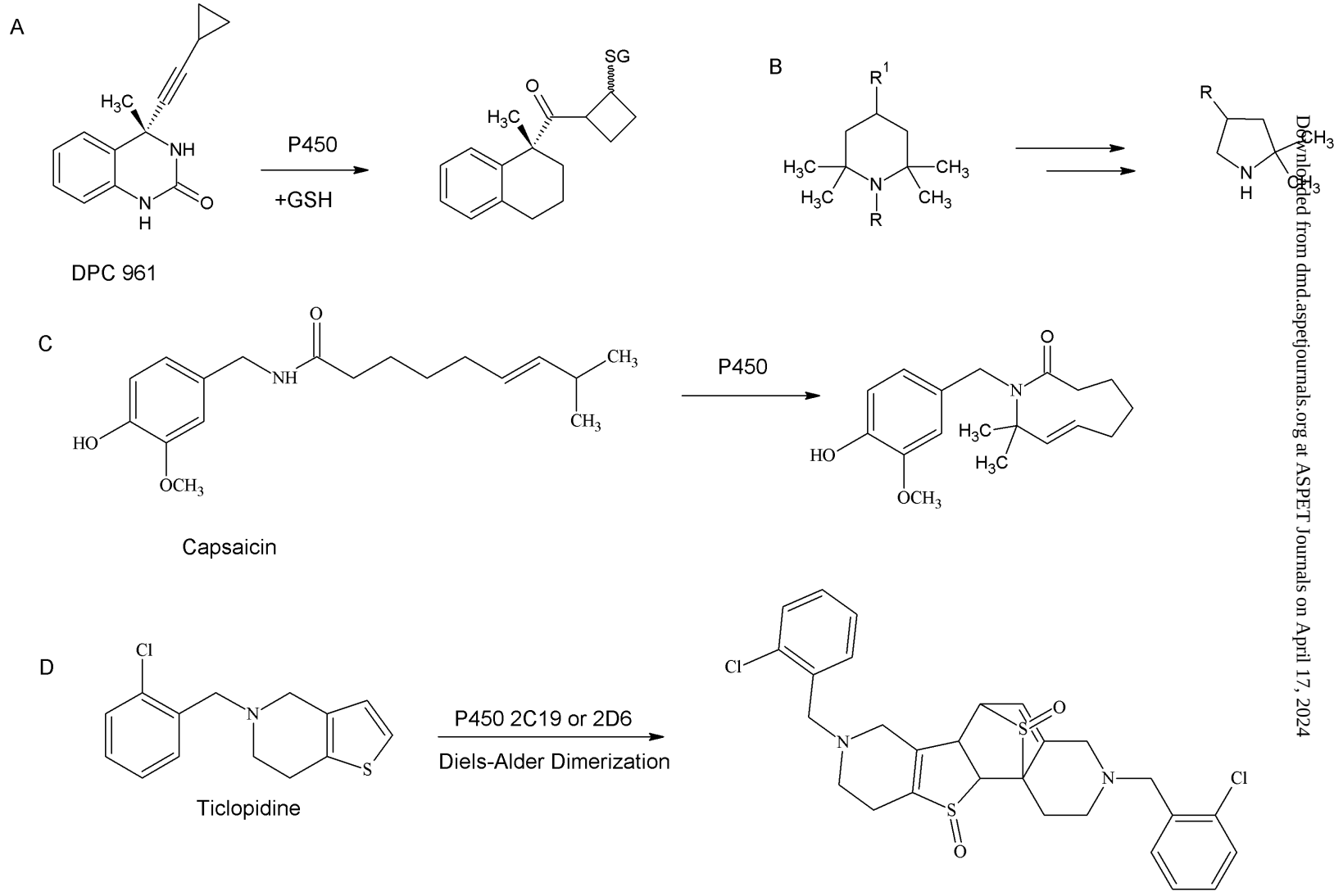

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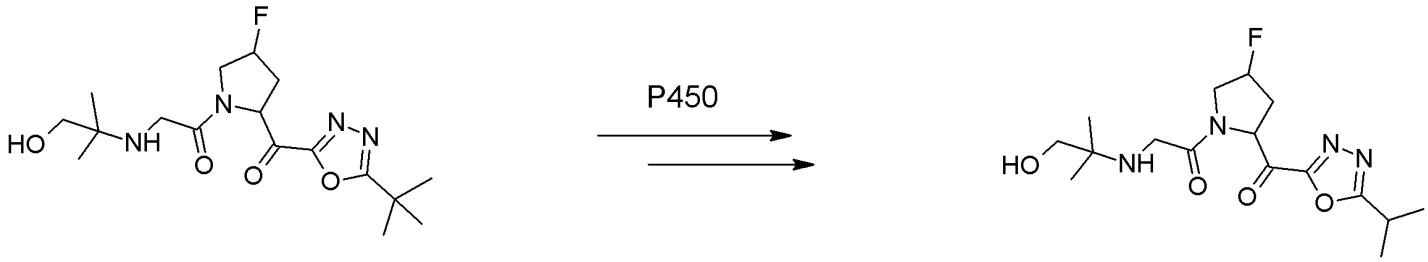
Figure 3

A



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B

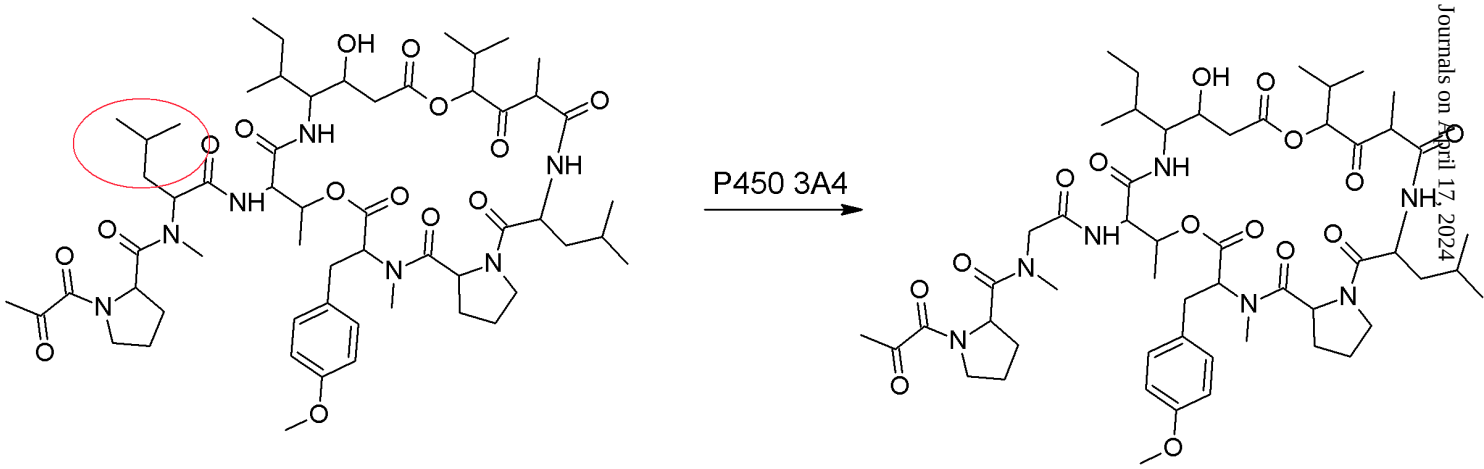

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Figure 4

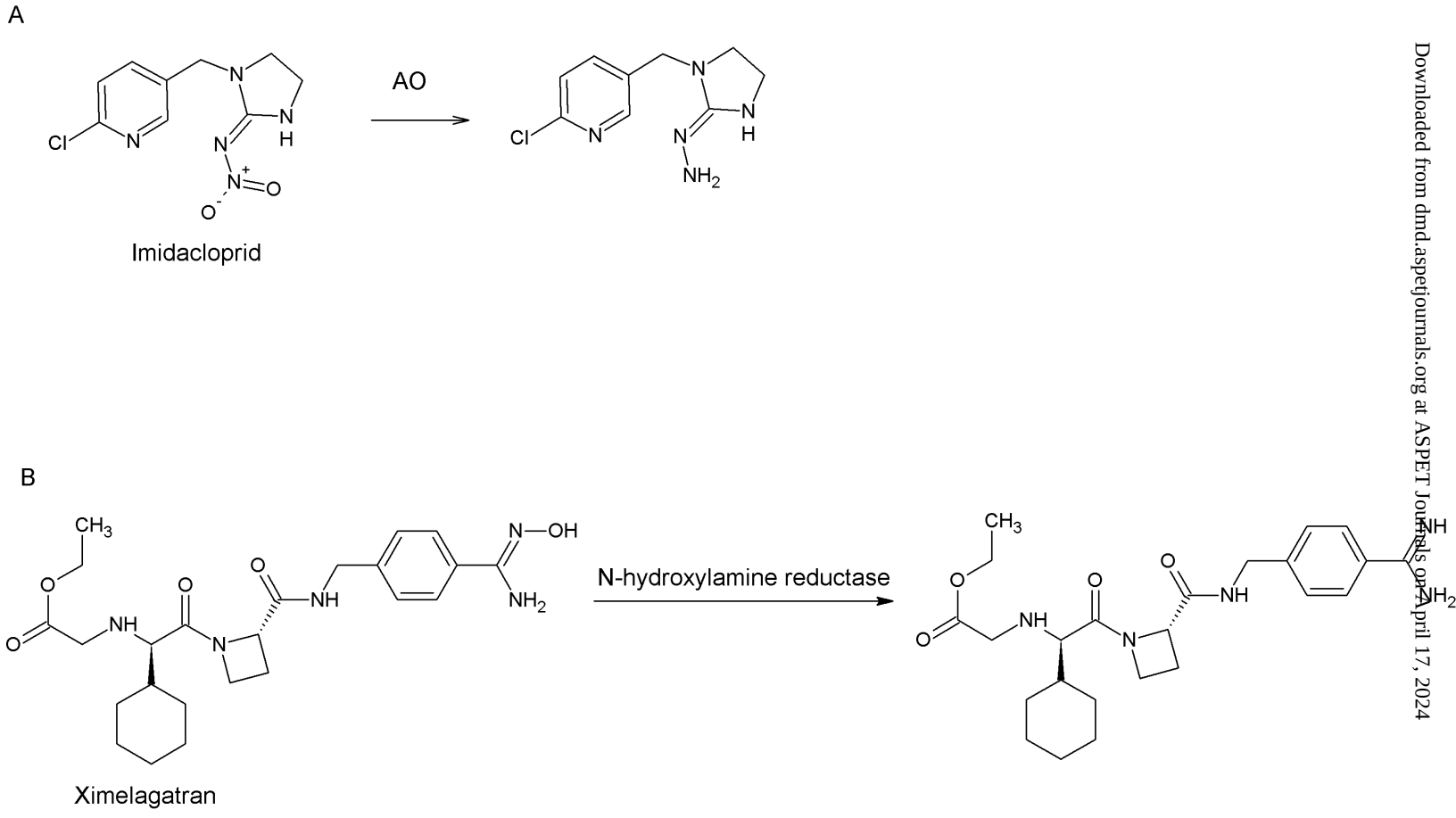

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Figure 5

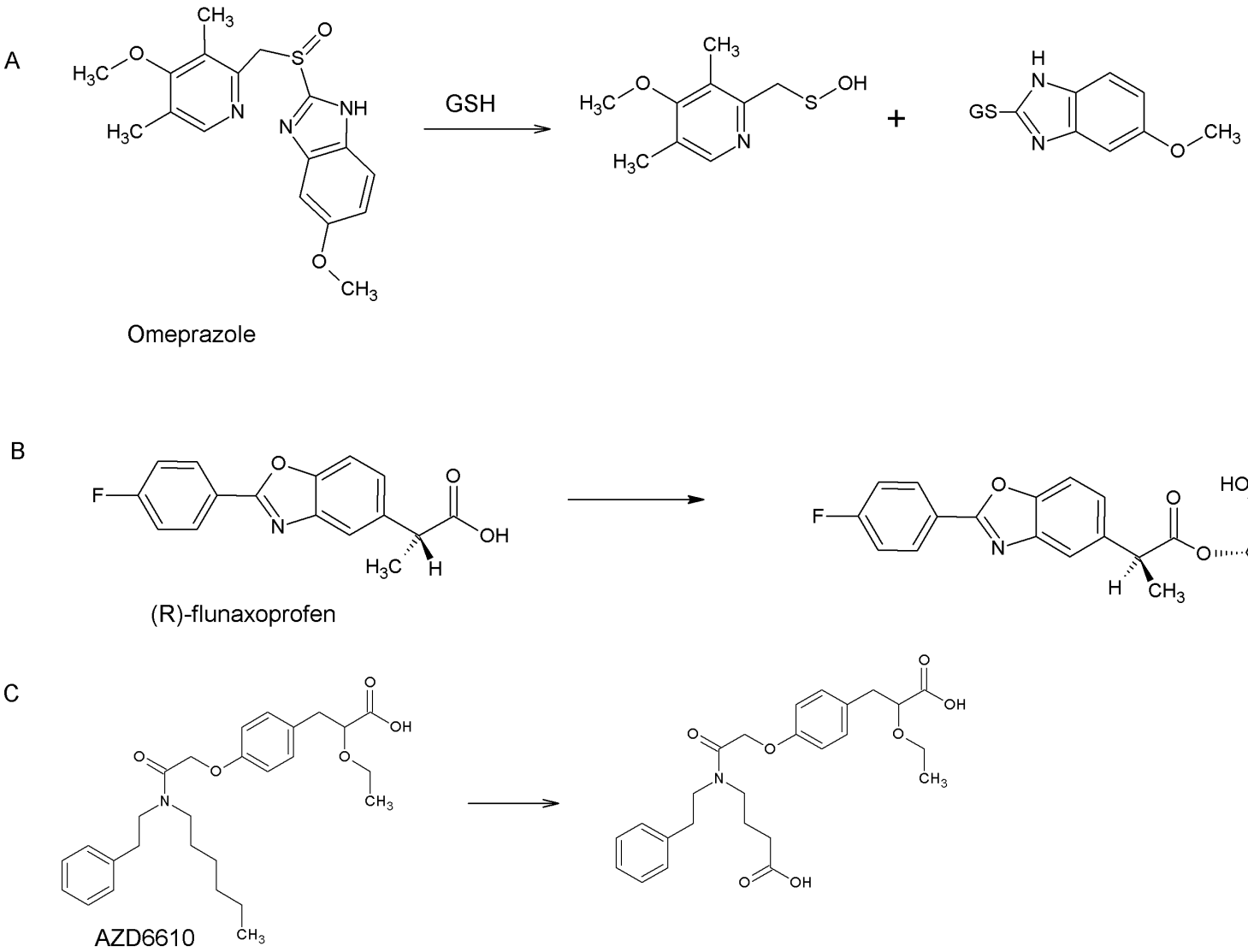

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Figure 6

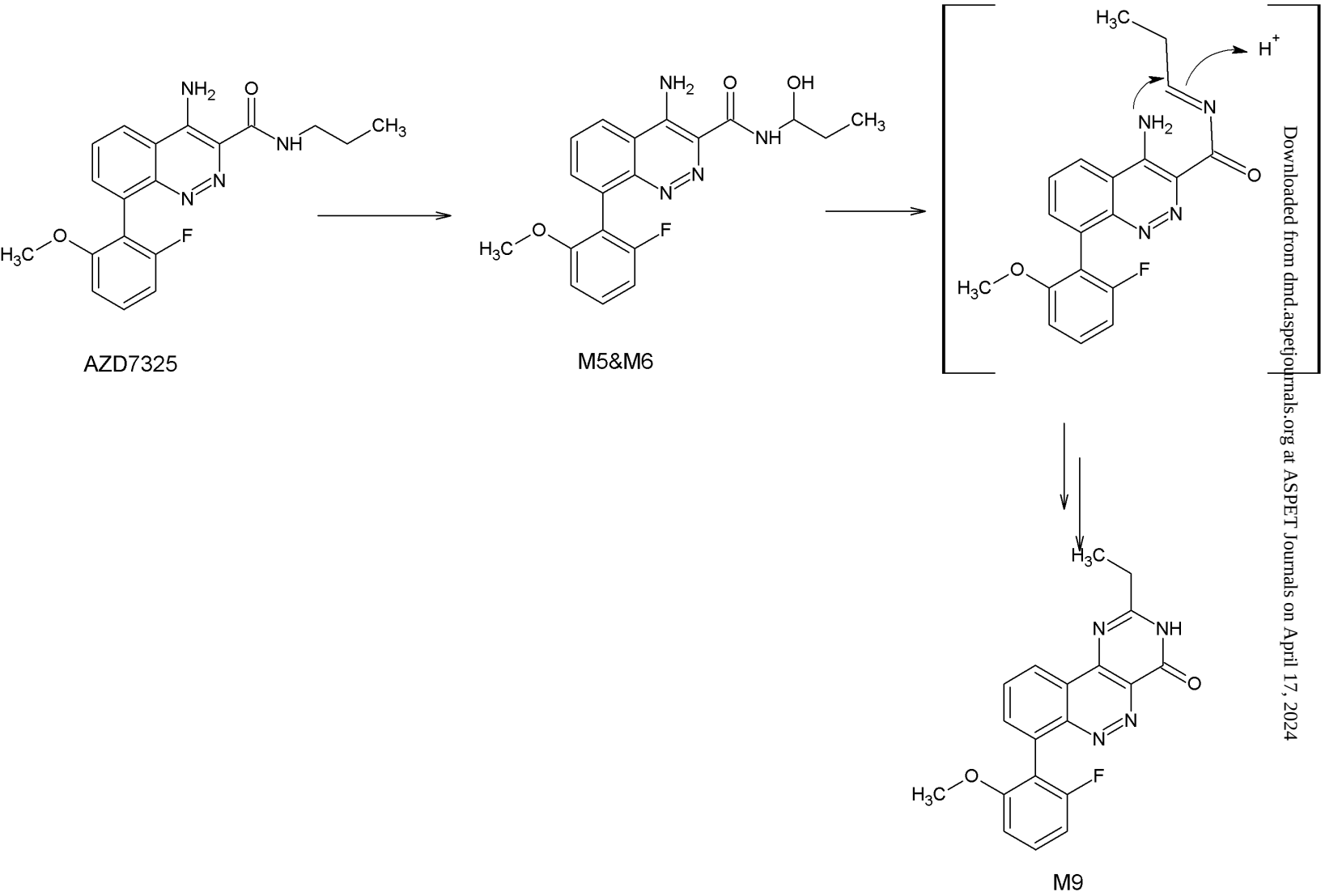

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Figure 7

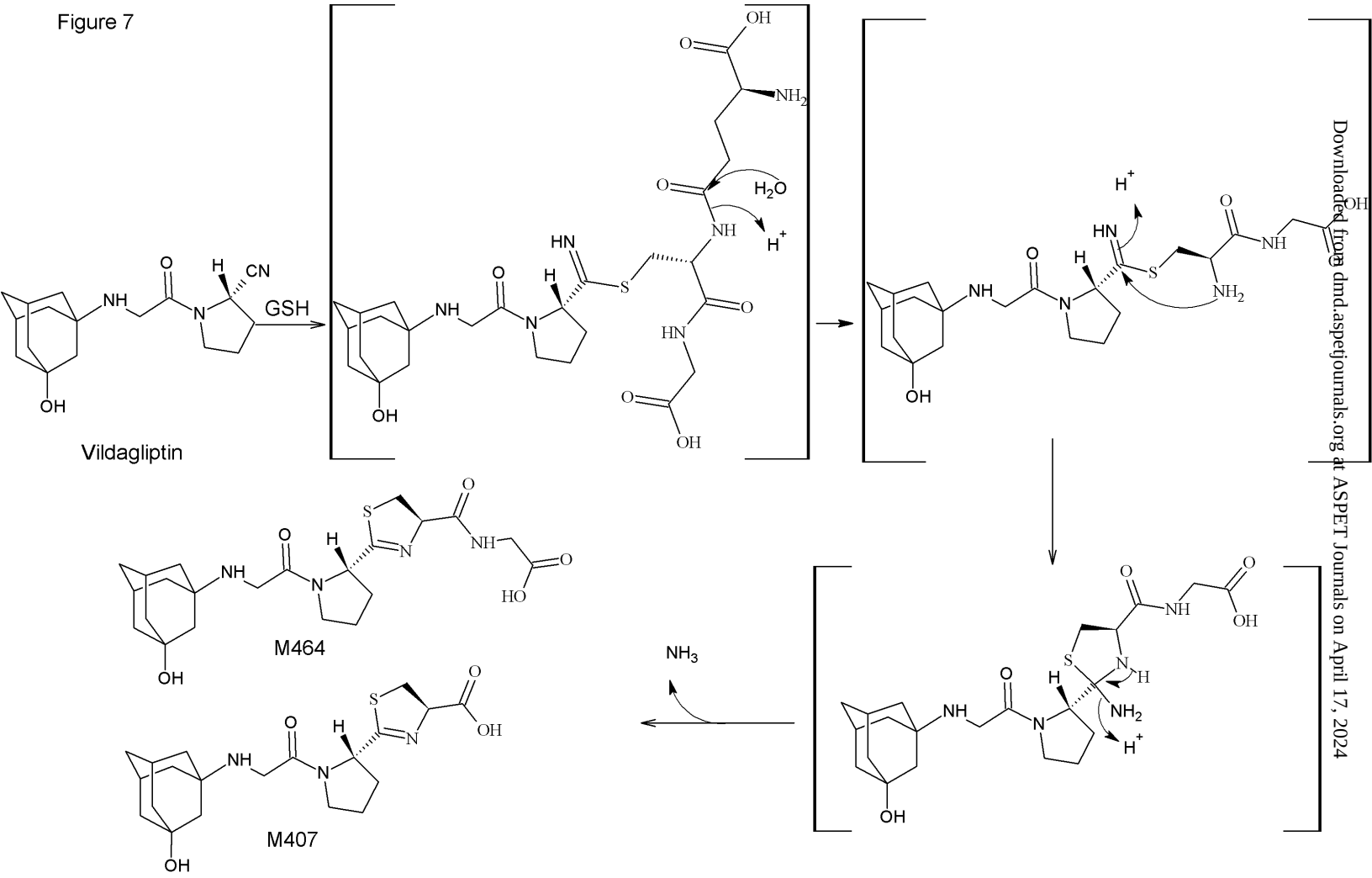

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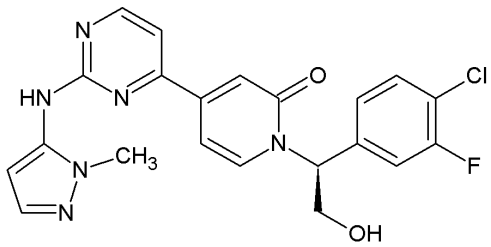
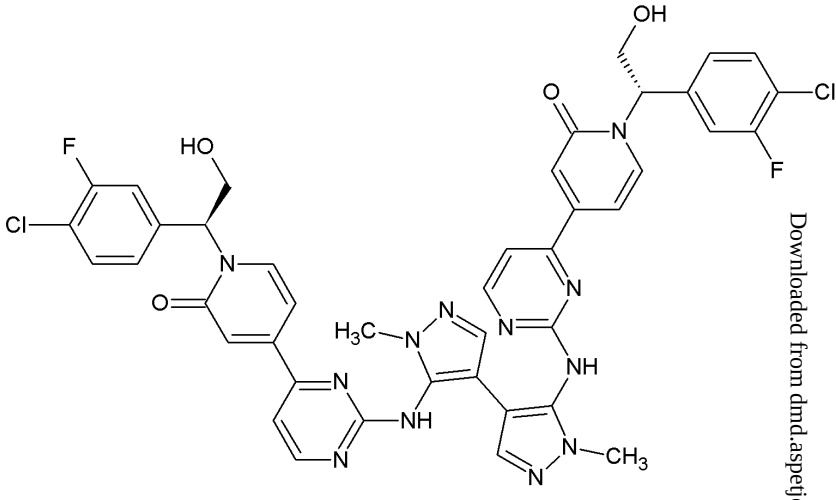
Figure 8



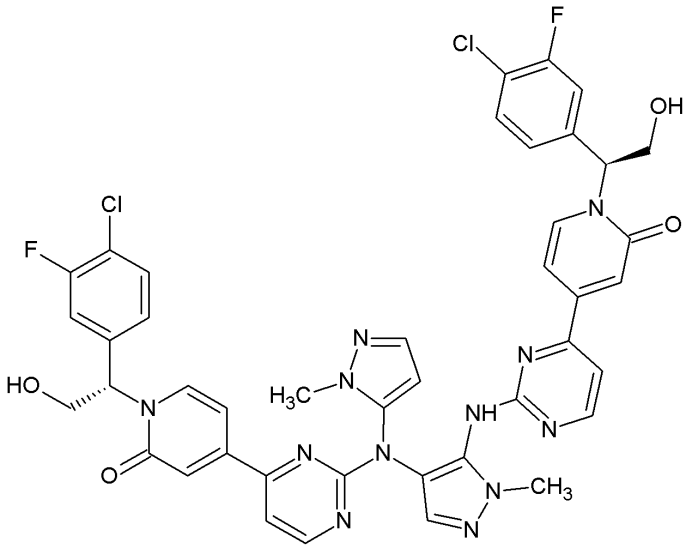
GDC-0994

C-C Coupling



M13

C-N Coupling



M14

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Figure 9

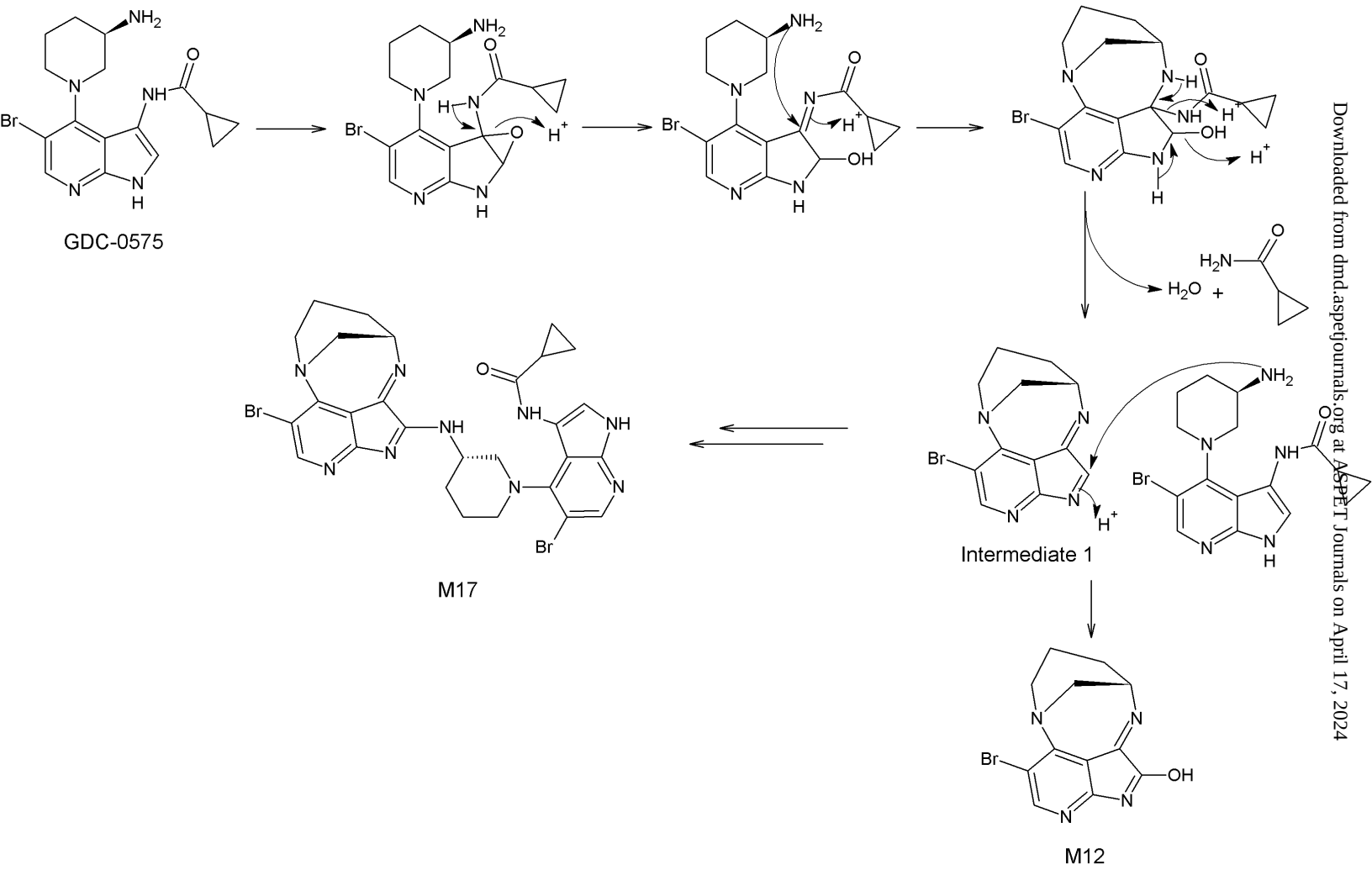

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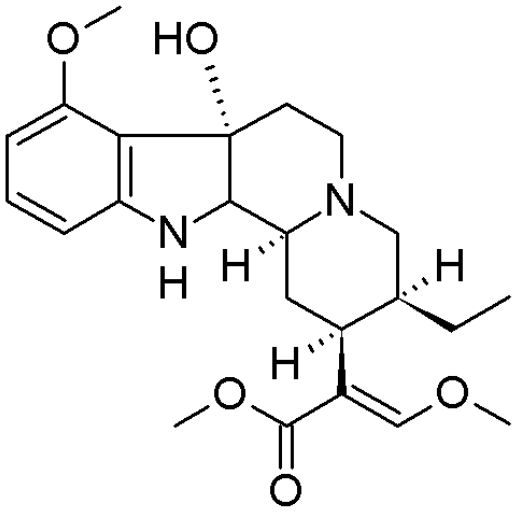
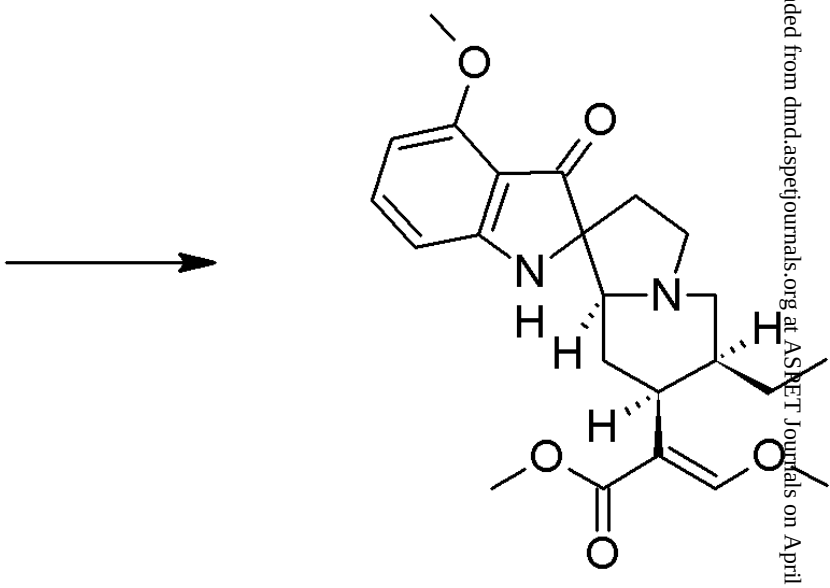
Figure 10



7-OH mitragynine



Mitragynine
pseudoindoxyl

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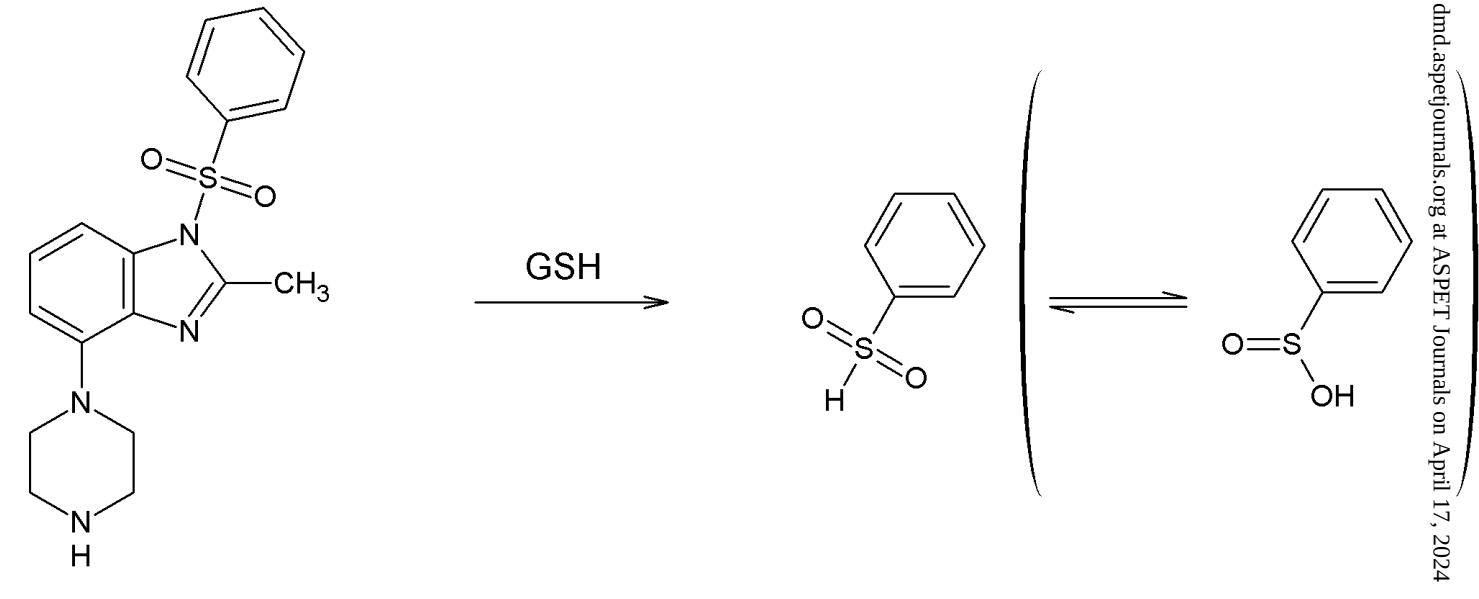
Figure 11



SAM-760

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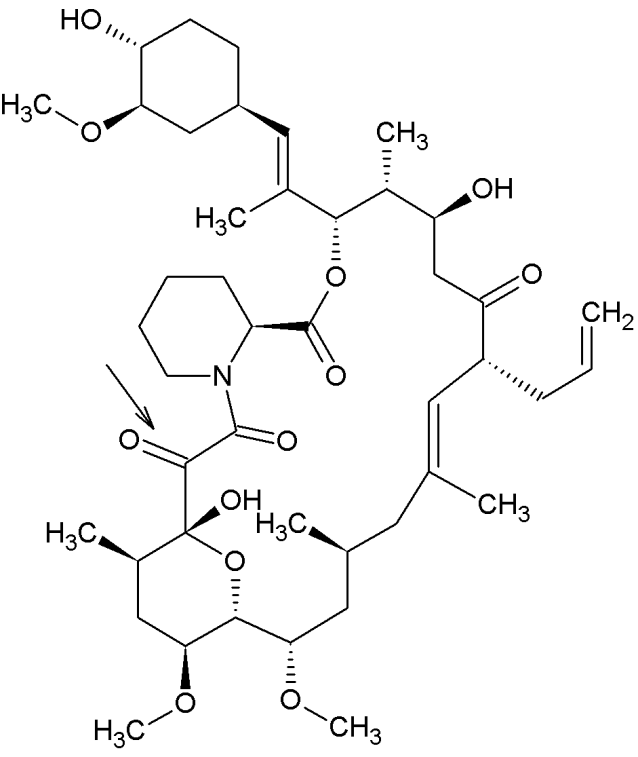
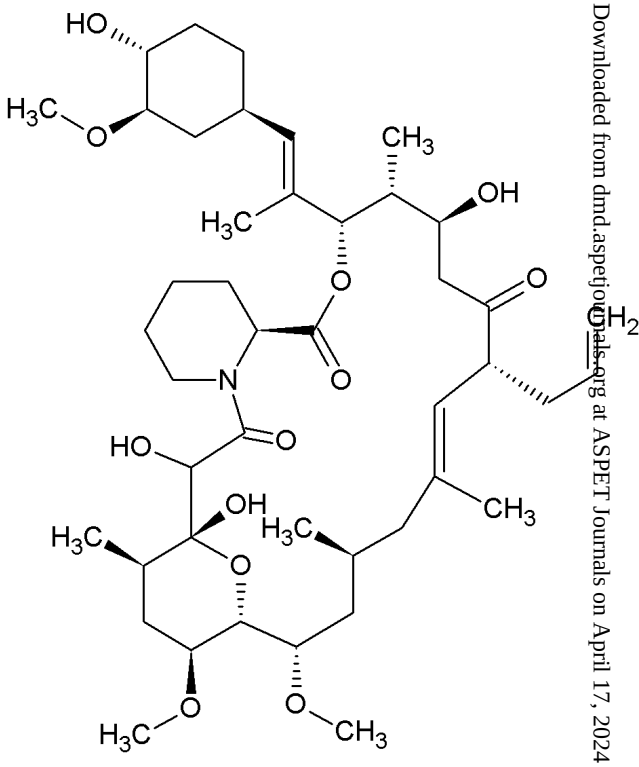
Figure 12



Tacrolimus



M1

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Figure 13

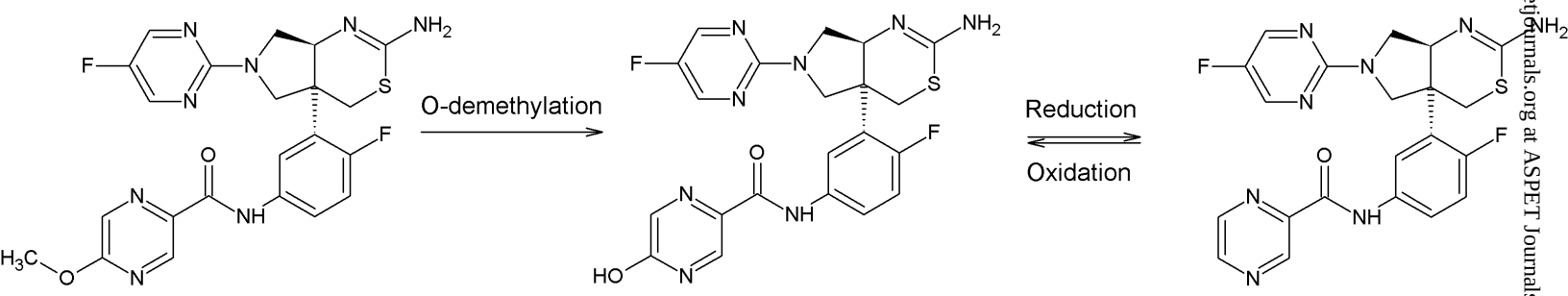

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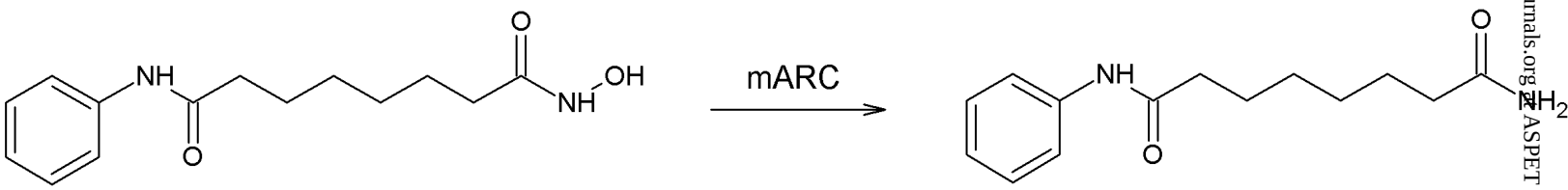
Figure 14



Vorinostat

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Figure 15

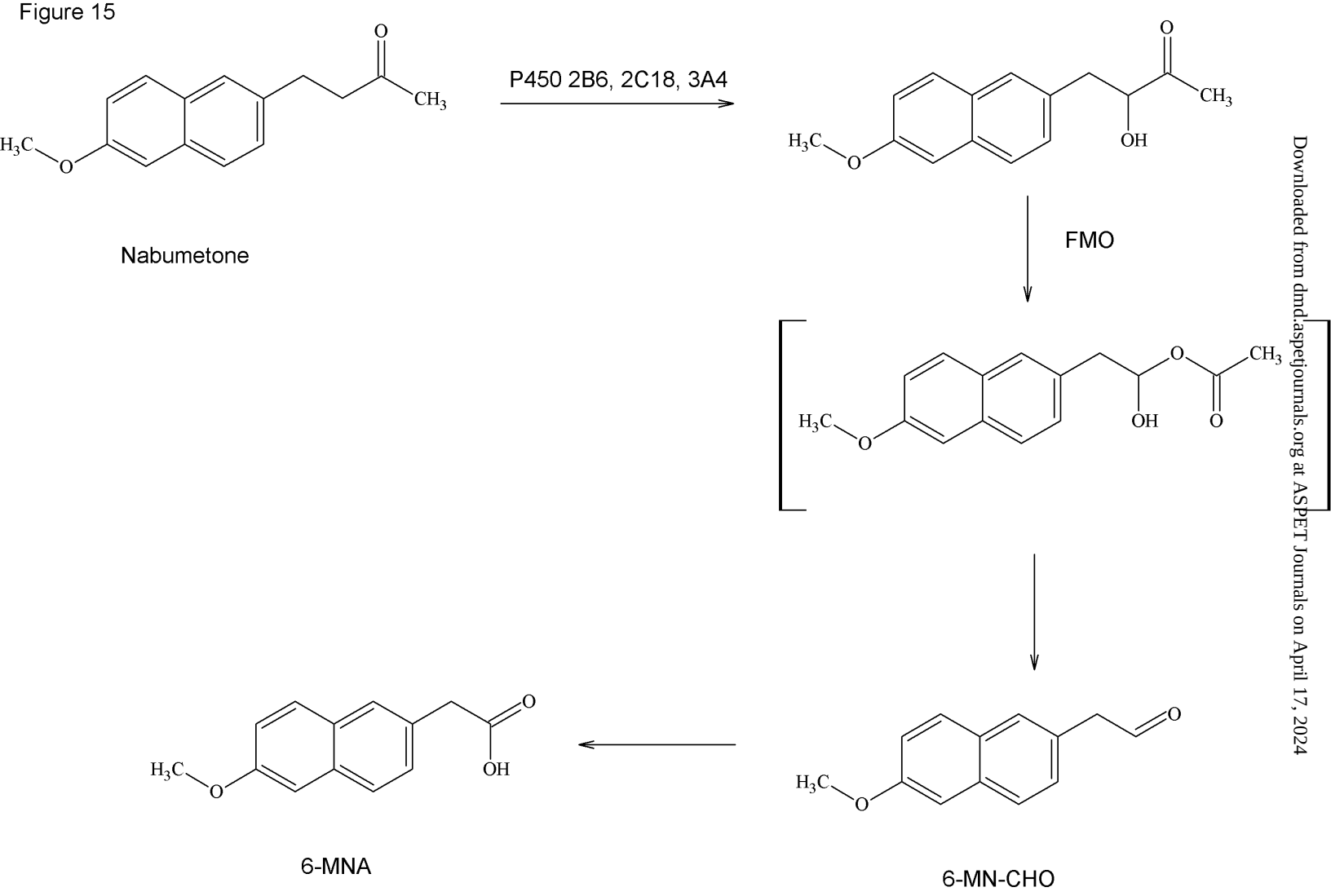

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Figure 16

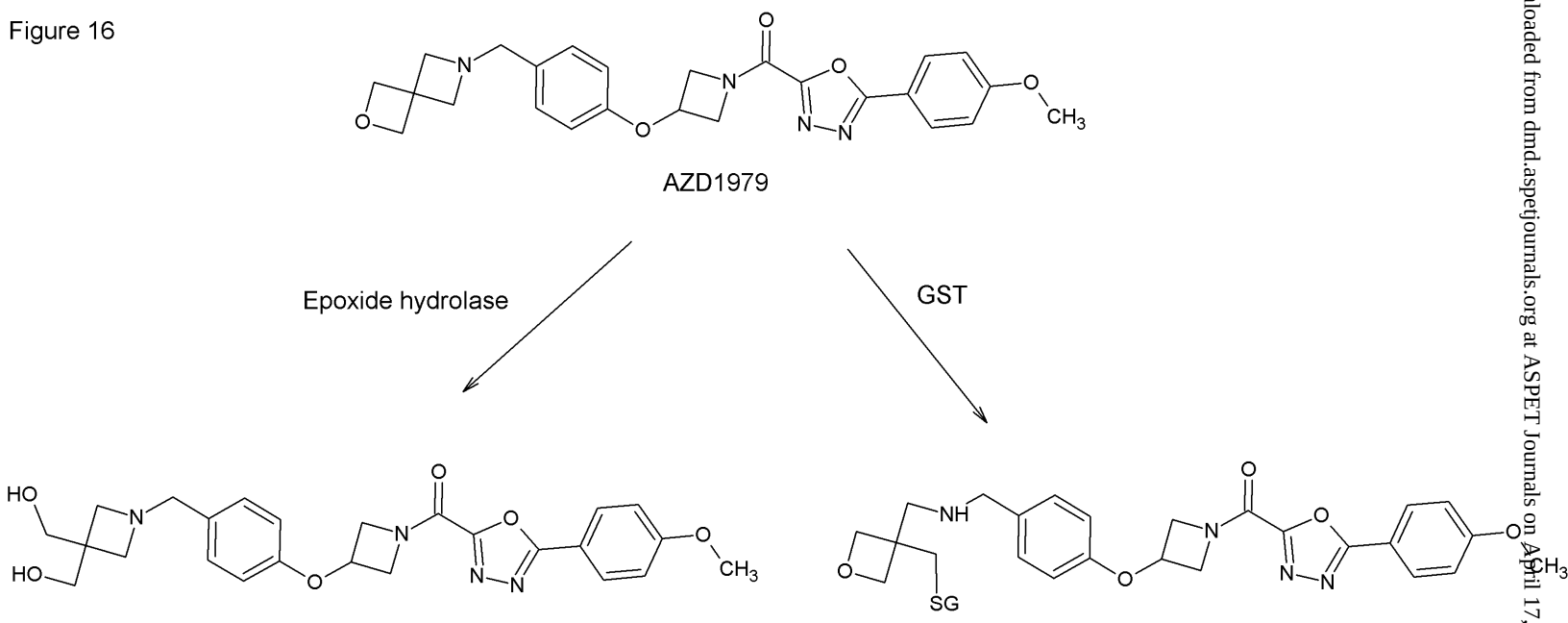

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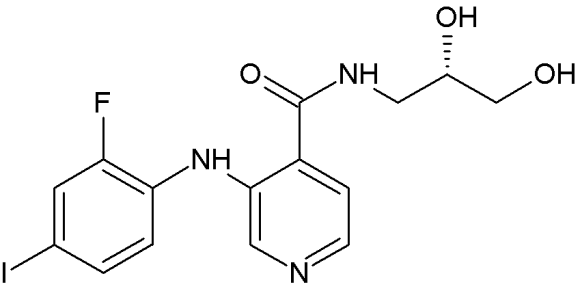
Figure 17



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