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Org. Process Res. Dev., **Just Accepted Manuscript** • DOI: 10.1021/acs.oprd.5b00312 • Publication Date (Web): 03 Dec 2015

Just Accepted

ACS Publications

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Applications of $TiCl_3$ as A Diagnostic Reagent for the Detection of Nitro and N-oxide Containing Compounds as Potentially Mutagenic Impurities (PMIs) Using Ultra-High Performance Liquid Chromatography Coupled with High Resolution Mass Spectrometry

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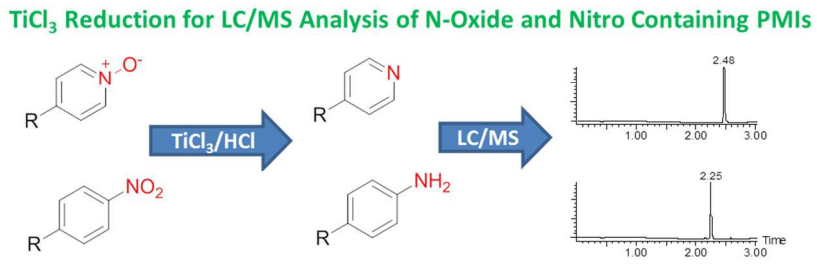
Table of Contents Graphic



ABSTRACT

The ICH has strict guidelines for limiting the presence of potentially mutagenic impurities (PMIs) in marketed drugs. Therefore, it is important to fully characterize and quantitate all possible PMIs that could arise during the process of synthesizing and developing a drug. Two important and prevalent examples of PMIs are compounds containing N-oxide and nitro functional groups. $TiCl_3$ derivatization is an established method for determining the presence or absence of N-oxide metabolites by reduction to the corresponding amine. In this study we demonstrate a novel application of $TiCl_3$ reduction combined with high resolution UHPLC/HRMS to analyze PMIs. The results indicate that a variety of N-oxide and nitro containing compounds can be readily characterized by this facile platform method. In addition, we show that this chemical derivatization method can be utilized to enhance the ionization of nitro containing compounds for LC/MS analysis.

Keywords: PMI, UHPLC/HRMS, N-oxide, nitro, Titanium(III) Chloride, Reduction

INTRODUCTION

Residual impurities resulting from manufacturing and formulation, or from degradation of the active pharmaceutical ingredient (API), may be present in pharmaceutical products.[1-6] A subgroup of these impurities may present a potential for carcinogenic risk and therefore pose an additional safety concern to clinical subjects and patients[7-10]. Regulatory agencies have identified potentially mutagenic impurities (PMIs) as high priority in the drug development approval process. The ICH has recently issued a guidance document outlining how to assess and control PMIs in pharmaceuticals (ICH M7),[11] which outlines the goal of maintaining these impurities below an acceptable threshold of toxicological concern. Hence, robust analytical techniques are needed to detect PMIs at very low concentrations.

The use of ultra-high performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC/HRMS) is one of the most common analytical methods for the detection and characterization of PMIs.[12-13] Compounds containing nitro and N-oxide functionalities are often present as byproducts or impurities during the drug manufacturing process.[14] These moieties are two of the key structural alerting functional groups of PMIs.[15] However, some of these compounds are poorly ionized using electrospray ionization (ESI).[16] Thus, there is often need for extensive UHPLC/MS method development efforts in order to characterize these compounds.

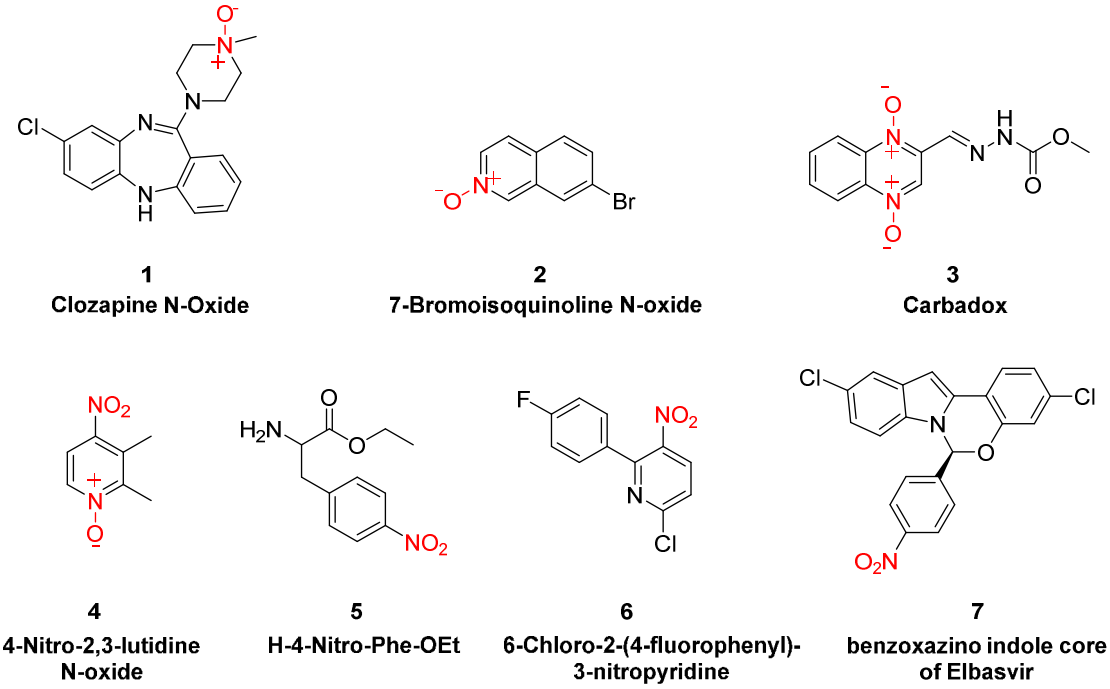
A variety of reagents have been reported in the literature for the reduction of *N*-oxides, including PCl_3, PPh_3, Raney Ni/H_2 or Pd/C, Fe/AcOH, Zn/aq NH_4Cl, $NaBH_4/AlCl_3$, NH_4COOH-Pd/C, AlI_3, $SnCl_2$/HCl, $TiCl_4$/NaI.[17] In comparison to the other treatments, Titanium (III) chloride ($TiCl_3$) derivatization has several advantages such as its ready availability, mild room

temperature reaction condition, high selectivity toward oxygenated species and fast reaction time.[18] The gram scale reduction of aliphatic and aromatic N-Oxides as well as aromatic nitro groups to the corresponding amines with $TiCl_3$ was extensively studied by Seaton and Somei et al.[19-20]

The utility of $TiCl_3$ was further expanded for analytical purposes as a reducing reagent for N-oxide containing drug metabolites. The use of HPLC in the analysis of N-oxides and sulfoxides was reported as early as 1980.[21] Analytical methods combining $TiCl_3$ reduction and LC/MS in drug metabolism studies had been established by 1997.[22] Kulanthaivel et. al. further expanded on this metabolite application, including the selectivity of $TiCl_3$ reduction for N-oxidation versus S-oxidation or C-hydroxylation, with a thorough investigation published in 2004.[23] However, the use of $TiCl_3$ as a diagnostic reagent in the detection of nitro and N-oxide containing PMIs has not been explored.

By accurate mass measurement alone, it can be unclear what functional groups give rise to any oxygen atoms present in the predicted chemical formula of an impurity. There could be N-oxide or nitro groups present that would be considered structural alerts for PMIs or there could be relatively less concerning carboxylic acids or alcohols in the structure of the impurity. Before undertaking extensive scale up, isolation, MSMS, or NMR efforts in order to provide a definitive structure, simple treatment with $TiCl_3$/HCl can provide quick insight into the presence or absence of N-oxide and nitro functional groups. In this study we report the use of commercially available $TiCl_3$/HCl reagent as a diagnostic reagent for the detection of several types of aliphatic and aromatic N-Oxides, quinoline N-Oxide, Quinoxaline N,N'-dioxide and nitros, as well as compounds with a combination of N-oxide and nitro groups (Scheme 1) using UHPLC/HRMS.

In addition, we have found this chemical derivatization method can be utilized to enhance the ionization of nitro containing compounds in LC/MS analysis.



Scheme 1. Representative N-oxide and nitro compounds chosen for the study

Table 1. Summary of the completion of reduction by Titanium(III) Chloride in 50% aqueous acetonitrile.

Tested Compound	Type of N-oxidation	Extent of reduction	By-product (wt%)
Clozapine N-Oxide (**1**)	aliphatic N-oxide	97%	N-Oxide (3%)
7-Bromoisoquinoline N-oxide (**2**)	quinoline N-oxide	94%*	chloroisoquinoline (6%)
Carbadox (**3**)	Aryl-N,N'-dioxide	94%	N-oxide (6%)
4-Nitro-2,3-lutidine N-oxide (**4**)	Aryl-NO_2, Aryl-NO	83%	N-hydroxylamine (17%)
H-4-Nitro-Phe-Oet (**5**)	Aryl-NO_2	>99%	N-hydroxylamine (<1%)
6-chloro-2-(4-fluorophenyl)-3-nitropyridine (**6**)	Aryl-NO_2	96%	N-hydroxylamine (4%)
Benzoxazino indole core of elbasvir (**7**)[25]	Aryl-NO_2	74%*	NO_2 (26%)

*required alternate solvent system for complete reduction.

RESULTS AND DISCUSSION

Development of a Platform Approach. Methanol, ethanol, and THF have each been reported as suitable reaction solvents for the reduction of N-oxides by $TiCl_3$.[18-23] In the present study, 50%

aqueous acetonitrile was found to be a simple and nearly universal reaction solvent that is easy to work with and more closely matches mobile phase conditions used for LC/MS analysis. The presence of water was crucial for the reaction solvent, particularly when working with nitro groups since an abundant proton source was required for complete reduction. Moreover, the addition of ammonium hydroxide followed by filtration were found to be important final steps in $TiCl_3$/HCl treatment. Ammonium hydroxide neutralizes any excess hydrogen chloride and converts all unreacted titanium chloride to titanium dioxide.[24] The precipitated titanium dioxide may then be removed by filtration. If these final steps were not taken, the presence of partially oxidized titanium reagent resulted in poor chromatography and a noisy background in the MS data. This approach was found to be universally applicable to the majority of analytes studied with a handful of easily addressed exceptions that will be discussed.

Reduction of N-oxides. N-oxide containing impurities are often formed as byproducts in oxidation reactions, and as discussed are important to identify as PMIs. Three N-oxide containing molecules were selected as model compounds to represent possible PMIs: compound **1** with an N-oxide of a tertiary aliphatic amine, compound **2** with a single aryl-N-oxide, and compound **3** with two aryl-N-oxides (Scheme 1). Each of the compounds tested was successfully reduced following treatment with $TiCl_3$/HCl and the results are summarized in Table 1. The reduction of the two aryl-N-oxides of compound **3** to the corresponding quinoxaline with 94% completion is shown in Figure 1. A characteristic shift in retention time combined with high resolution MS data confirmed the successful reduction of compound **3**. Retention of the main component shifted from 1.5 min for compound **3** to 1.8 min for the less polar reduced quinoxaline (compound **3a**). High resolution mass spectra confirmed the exact masses of both compound **3** and the reduced quinoxaline product (**3a**) with <5 mDa error. Additionally both the

unreacted compound **3** and the reduced quinoxaline (**3a**) underwent in-source fragmentation and lost a 32 Da methoxy group. When only HCl was added to the sample, no change in either retention or mass was observed indicating that $TiCl_3$ is necessary for reduction. Similar analysis was undertaken for compounds **1** and **2,** the results of which are summarized in Table 1.

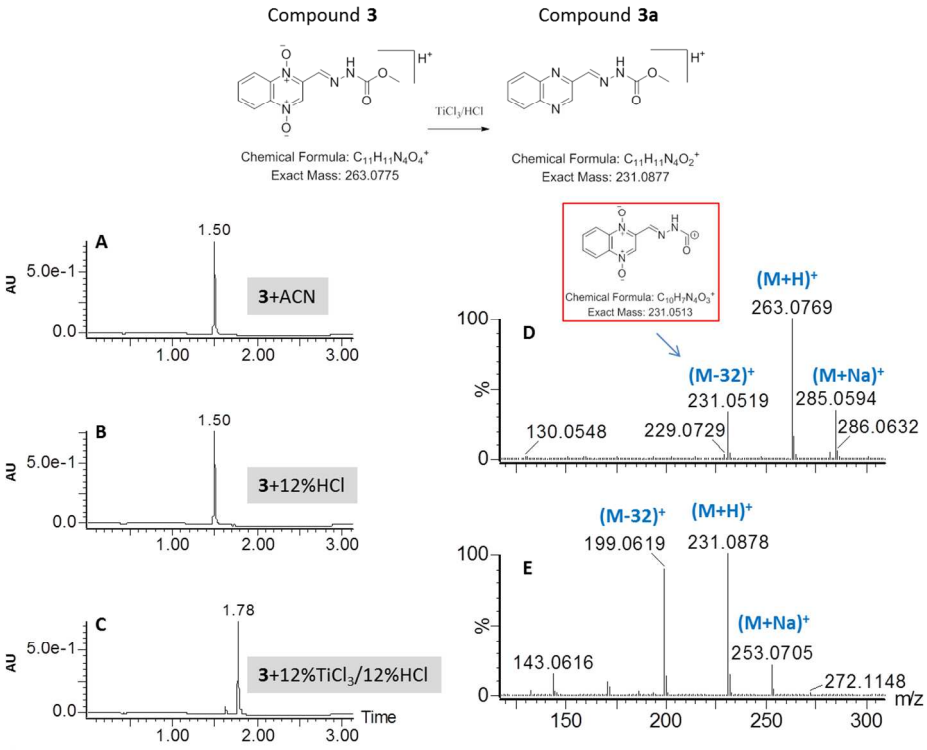


Figure 1. UHPLC/UV chromatograms at 254 nm of unreacted Compound **3** (A), HCl control (B) and $TiCl_3$/HCl treated sample (C). High resolution mass spectra of compound **3** (D) and reduced quinoxaline compound **3a** (E).

With our platform approach using 50% aqueous acetonitrile as the reaction solvent, compound **2** showed only 40% completion of reduction. However, when the reaction solvent was changed to 100% MeOH, >99% conversion to the reduced 7-bromoisoquinoline could be achieved. In this case, with sufficient detection limits 40% conversion would be adequate for compound identification, even if compound **2** was present as a low level impurity. However, a

simple switch of the reaction solvent can quickly achieve adequate conversion for accurate quantitation of the reduced species.

For N-oxide containing compounds similar to compounds **1-3**, simple $TiCl_3$ treatment could quickly determine whether or not a compound contains an N-oxide functional group that could be a structural alert. Upon $TiCl_3$ treatment of a suspected N-oxide containing PMI, the retention time of the peak of interest should shift and the accurate mass of the suspect compound would change by exactly the mass of one oxygen atom for each N-oxide group present. If these characteristic changes in retention time and accurate mass do not occur, then it would be unlikely that the suspect compound contains an N-oxide functional group.

Reduction of Nitro groups. As with N-oxide containing compounds, the presence of nitro containing PMIs is important to identify in the preparation of an API. Four compounds (**4-7**) each containing an aryl-nitro group were tested, with one of these compounds (**4**) also containing an aryl-N-oxide. Compounds **4-6** each showed >80% reduction with the major byproduct present being the N-hydroxylamine resulting from incomplete reduction of the nitro group (Table 1). Of particular note was compound **6.** Reduction of compound **6** to the corresponding aniline (compound **6a**) was achieved with 96% completion with 4% of the partially reduced N-hydroxylamine remaining (Figure 2). The retention time of the major component in each chromatogram shifted from 2.5 min for compound **6** to 2.3 min for the reduced aniline (compound **6a**). When only HCl was added to the sample, no change in either retention or mass was observed confirming that $TiCl_3$ is necessary for reduction. Prior to treatment with $TiCl_3$/HCl, the nitro containing compound **6** ionized poorly upon MS analysis with both (+)-ESI and (-)-ESI and would be difficult to identify by MS were it present as a low level impurity. Following reduction to the corresponding aniline, compound **6** displayed a 50 fold increase in ion

abundance by (+)-ESI (Figure 2) as indicated by the increase in XIC peak area from 38 for *m/z* 253 (compound **6**) to 2932 for *m/z* 223 (compound **6a**). The lower ion abundance for compound **6** was partly the result of the electron withdrawing nature of the aryl-nitro group which renders the pyridine nitrogen less basic and therefore less easily protonated upon analysis by (+)-ESI. This also contributes to the earlier retention time of the reduced aniline as it should be more basic than its nitro containing counterpart and therefore more likely to be charged – and hence earlier eluting – in the presence of acidic mobile phase.

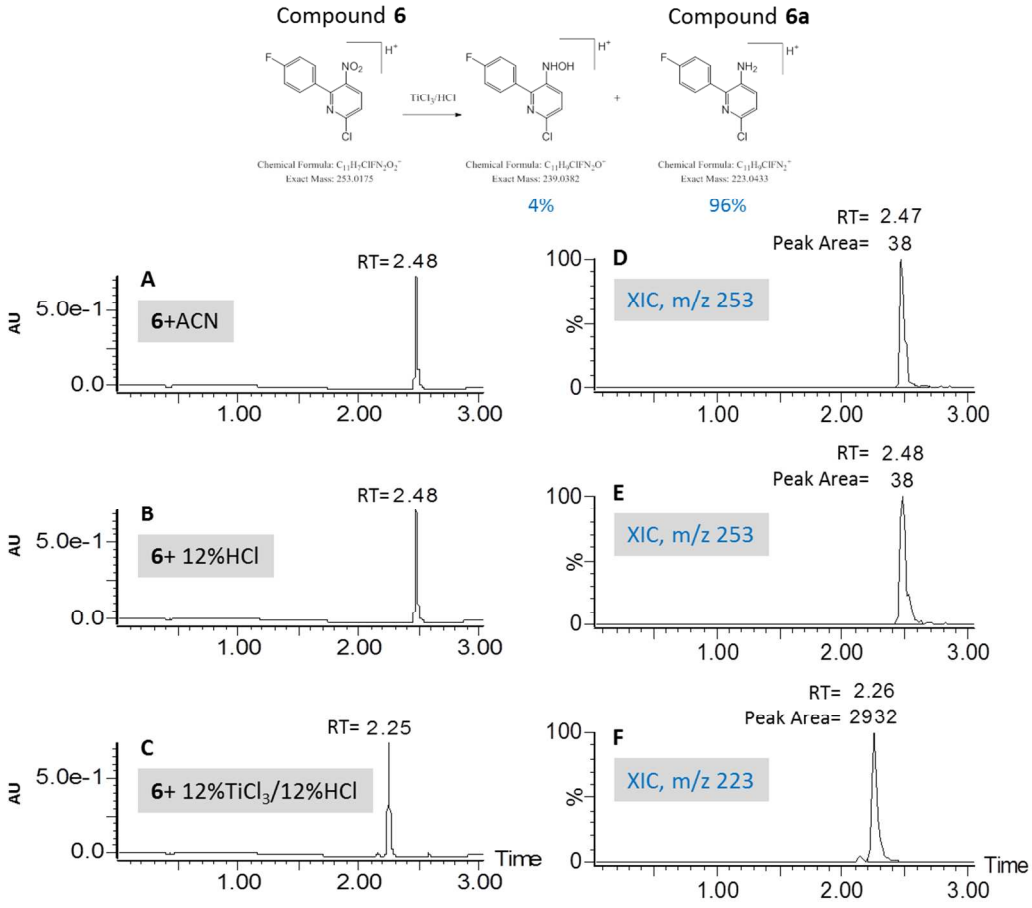


Figure 2. UHPLC/UV chromatograms at 254 nm of unreacted compound **6** (A), HCl control (B) and $TiCl_3$/HCl treated sample (C). MS selected-ion chromatograms for compound **6** with the target mass of *m/z* 253 (D, E) and compound **6a** with the target mass *m/z* 223 (F).

The one compound in this study for which complete reduction of the aryl-nitro group was not achieved with the standard approach (50% aqueous acetonitrile) was compound **7,** the benzoxazino indole core of Elbasvir.[25] This was due to the poor solubility of compound **7** in our acetonitrile/water solvent system. However, in a chloroform/methanol/water solvent system, 74% conversion to the corresponding aniline was achieved (Figure 3). Compound **7** was first dissolved in chloroform (1 mg/mL) and diluted with methanol/water (50/50) to 0.1 mg/mL and treated with 10 µL of $TiCl_3$/HCl reagent. Successful reduction to the corresponding aniline was confirmed by high resolution MS with <2 mDa error. As with Compound **6**, the ion abundance of the reduced aniline (compound **7a**) by (+)-ESI increases >80 fold over the corresponding nitro containing compound (XIC peak area increases from 127 to 10760) thus facilitating identification and quantitation by MS.

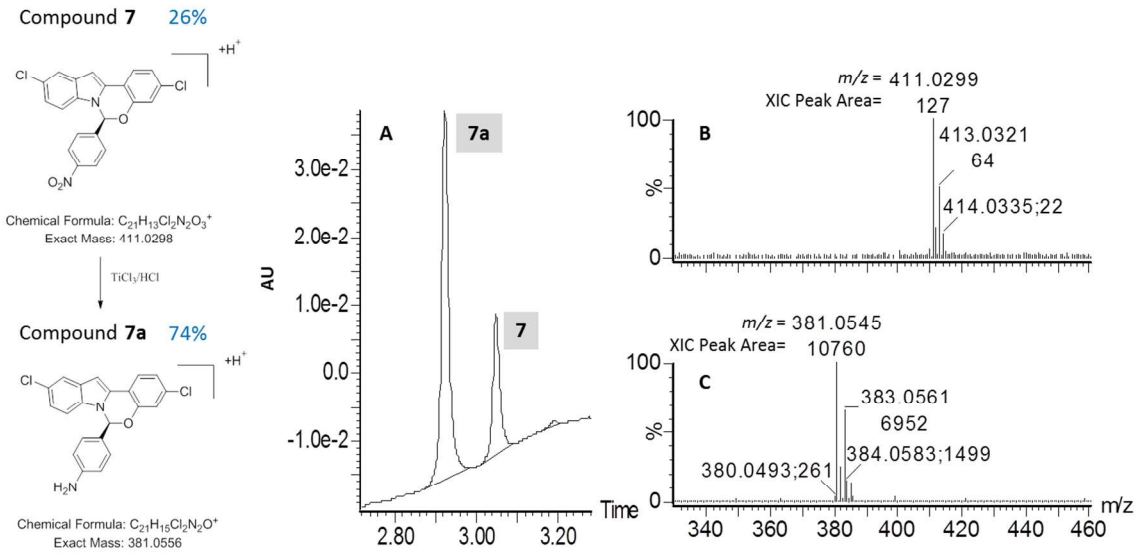


Figure 3. UHPLC/UV chromatogram at 254 nm for the $TiCl_3$ treated sample (A), and high resolution mass spectra of compound **7** (B) and the reduced aniline compound **7a** (C)

For nitro containing compounds similar to compounds **4-7**, $TiCl_3$ reduction will both aid in the confirmation of the presence of the structural alerting nitro functional group as well as

increase the limits of detection and quantitation for LC/MS analysis for such compounds through reduction to the corresponding aniline with increased ionization efficiency. Accurate quantitation is of particular importance for PMIs, so the increased ionization efficiency achieved by $TiCl_3$ treatment is of vital importance for the quantitation of low levels of nitro containing PMIs.

CONCLUSION

We have developed a broadly applicable platform method for the characterization of nitro and N-oxide containing PMIs through reduction with a commercially available $TiCl_3$/HCl reagent followed by UHPLC/HRMS analysis. We have further demonstrated that in some cases minor changes to the reaction solvent can lead to significant improvements to the extent of conversion to the reduced product. This simple method has proven invaluable in the identification of PMIs since with it the presence or absence of N-oxide and nitro functional groups that are considered structural alerts can be quickly determined. Additionally, for poorly ionizing compounds containing aryl-nitro groups, the reduction of the nitro groups to their corresponding anilines results in an increase in ionization efficiency with ESI-MS thus aiding in the identification and quantitation of these potentially mutagenic impurities by UHPLC/HRMS.

EXPERIMEMTAL

Chemicals and reagents. 7-Bromoisoquinoline N-oxide was purchased from Alfa Aesar (Ward Hill, MA). 6-chloro-2-(4-fluorophenyl)-3-nitropyridine was purchased from Fisher Scientific (Pittsburgh, PA). H-4-Nitro-Phe-OEt.HCl was purchased from Bachem America Inc. (Torrance, CA). Clozapine N-Oxide, 4-Nitro-2,3-lutidine N-oxide, Carbadox (Vomitoxin), and Titanium (III) chloride (ca. 12 wt. % solution in 12 wt. % hydrochloric acid) were purchased from Sigma

Aldrich (St. Louis, MO). All solvents used for LC/UV/MS analysis were of OPTIMA LC/MS grade purchased from Fisher Scientific (Pittsburgh, PA).

Sample Preparation for $TiCl_3$ Reduction. Stock solutions of the representative N-oxide and nitro compounds were prepared at 0.5 mM (ca. 0.1~0.2 mg/mL) in aqueous acetonitrile (50% in water) . For a typical $TiCl_3$ reduction experiment, an aliquot of 1000 µL of N-oxide stock solution was treated with 5 µL of $TiCl_3$/HCl (4 mol equivalent of $TiCl_3$). After the reaction mixture was vortexed at room temperature for 1 min, 40 µL of 5N ammonium hydroxide was added and the sample was votexed for one additional minute to precipitate titanium (as titanium dioxide). A 500 µL aliquot was transferred to a filter vial (Thomson SINGLE StEP™ Filter Vials 0.45 µm PTFE, Thomson Instrument Company, Oceanside, CA) for LC/MS analysis. Similarly, the nitro containing compounds were treated with 10 µL of $TiCl_3$/HCl because the reduction of a nitro group to an amine group is a 6-electron process.[26] Two control samples were also prepared by mixing an aliquot of 1000 µL stock solution with 5 µL acetonitrile and 5 µL 12% HCl, respectively.

UHPLC/HRMS analysis. LC/MS analysis was performed on a Waters Acquity UPLC system, consisting of a binary pump, a sample manager, a TUV detector and a Waters Synapt G1 Mass Spectrometer (Waters, Milford, MA) under positive or negative ESI conditions. The output signal was monitored and processed using MassLynx software designed by Waters (Milford, MA).

The separation was carried out on a Waters HSS T3 column (2.1 x 50 mm, 1.8 µm particle size). The mobile phase consisted of 0.1% Formic Acid in both Water (mobile phase A) and Acetonitrile (mobile phase B). The injection volume was 2 µL. Analytes were eluted using a

gradient method consisting of an initial hold at 5% mobile phase B for 0.5 min, followed by a linear gradient to 99% B over 2 min, then a hold at 99% B for 0.5 min. The flow rate was 0.4 mL/min, and the column temperature was set at 40°C. UV detection was set at 254 nm.

The eluent was introduced directly into the mass spectrometer by electrospray. Source temperature and desolvation temperature were set at 130 °C and 350 °C, respectively. Nitrogen was used as both cone gas (40 liters/h) and desolvation gas (600 liters/h), and argon was used as collision gas. The capillary voltage was set to 3 KV. The cone voltages in a range from 10 to 20 V were applied. Leucine enkephalin was used as the lock mass (m/z of 556.2772) for accurate mass calibration and was introduced using the LockSpray interface at 10 μL/min at a concentration of 0.5 mg/mL in 50% aqueous acetonitrile containing 0.1% formic acid. In mass spectrometry scanning, data were acquired in centroid mode from *m/z* 100 to 1000. For MSMS fragmentation of target ions, collision energies ranging from 6 to 20 V were applied.

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ACKNOWLEDGMENT

The authors would like to thank Drs. Zhihong Ge, Caroline McGregor, Christopher J. Welch and Michael H. Kress for their support on this project.

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