Medicines for All Institute

Summary of Analytical Development Work on Synthesis of Ganaplacide

Report Prepared by:

Dr. Justina M. Burns
Dr. Ryan Littich
Dr. Daniel W. Cook

Contact: m4all@vcu.edu

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Executive Summary

This analytical development report (ADR) describes the results of the analytical method development efforts undertaken in conjunction with the synthetic process development of an advanced intermediate, 2-(4-fluorophenyl)-8,8-dimethyl-5,6,7,8-tetrahydroimidazo[1,2-a]pyrazine (**3.4**), in the synthesis of ganaplacide (GFN-001-GAN-PDR). Ganaplacide is a novel, broad acting antimalarial drug developed by Novartis in partnership with the Medicines for Malaria Venture (MMV). It is currently in stage III clinical trials as of September 2025.[1] Here, analytical methods developed by M4ALL are disclosed for in-process controls and final analysis of this advanced intermediate.

Published analytical methods were unavailable for M4ALL's synthesis of ganaplacide. Efforts were thus undertaken to develop a suite of methods capable of monitoring each reaction as well as characterizing the purity of the target compounds at each step of the synthesis. These efforts resulted in a primary method utilizing liquid chromatography with ultraviolet detection (LC-UV) for in-process controls and quantitative assays. Gas chromatography-mass spectrometry (GC-MS) was developed as a secondary method with alternative selectivity, particularly for regioisomers. Additional characterization methods include LC with evaporative light scattering detection (LC-ELSD) for counterion quantitation and gas chromatography with flame ionization detection (GC-FID) for solvents.

Medicines for All – Contributors to the Research and Report

Contributor	Title/Role
Dr. Ryan Littich	Chief Technology Officer [a]
Dr. Justina M. Burns	Assoc Dir of Analytical Chemistry (Project Lead)
Dr. Daniel W. Cook	Senior Analytical Chemist
Janie Wierzbicki	Assoc Dir of Project Management Office

[a] ADR review, revisions, additions

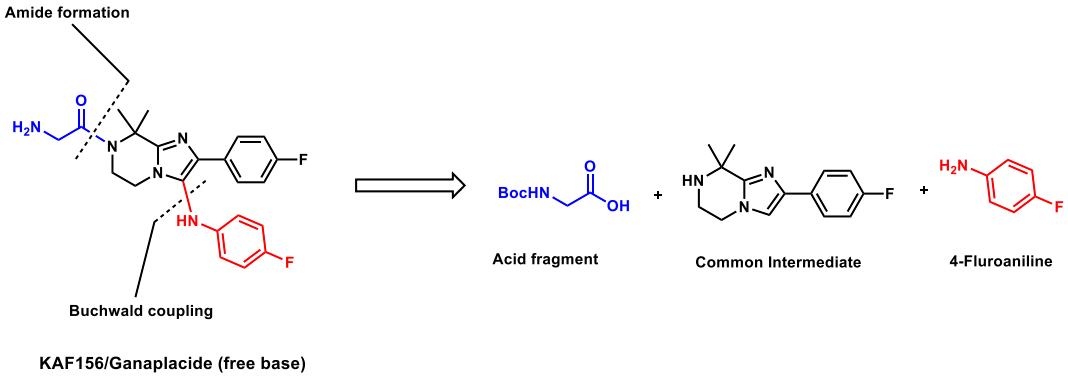
1 Contents

1 Introduction

Malaria remains one of the most devastating infectious diseases globally, with over 600,000 deaths annually, disproportionately affecting children under five years of age.[2] The emergence of resistance to artemisinin-based combination therapies (ACTs), the current frontline treatment, underscores the urgent need for novel antimalarial agents with distinct mechanisms of action and simplified dosing regimens.[3] Ganaplacide (development code KAF156), an imidazolopiperazine derivative, represents a promising next-generation antimalarial candidate developed by Novartis in collaboration with Medicines for Malaria Venture (MMV). Identified through high-throughput phenotypic screening of over two million compounds, ganaplacide exhibits potent activity against both *Plasmodium falciparum* and *Plasmodium vivax*, targeting multiple stages of the parasite lifecycle including liver, asexual blood, and sexual transmission stages.[2,4] Mechanistically, ganaplacide disrupts protein trafficking and folding within the parasite's endoplasmic reticulum, leading to ER expansion and impaired parasite viability.[5] Although its precise molecular target remains undefined, resistance has been associated with mutations in *PfCARL*, *PfUGT*, and *PfACT*, suggesting involvement in membrane protein trafficking and fatty acid transport.[4,5] Preclinical studies demonstrated ganaplacide's efficacy in vitro and in vivo, with favorable pharmacokinetics supporting once-daily dosing.[5] In combination with lumefantrine, a hemozoin formation inhibitor, ganaplacide has shown synergistic effects and robust activity against artemisinin-resistant strains.[5] This combination, formulated as a solid dispersion (SDF), has progressed through Phase 2 trials and is currently undergoing Phase 3 evaluation across multiple African countries.[3] Importantly, ganaplacide/lumefantrine therapy has demonstrated rapid parasite clearance (<48 hours), transmission-blocking potential, and chemopreventive efficacy against liver-stage infection.[4] These attributes position ganaplacide as a strong candidate to replace ACTs in regions burdened by multidrug-resistant malaria.

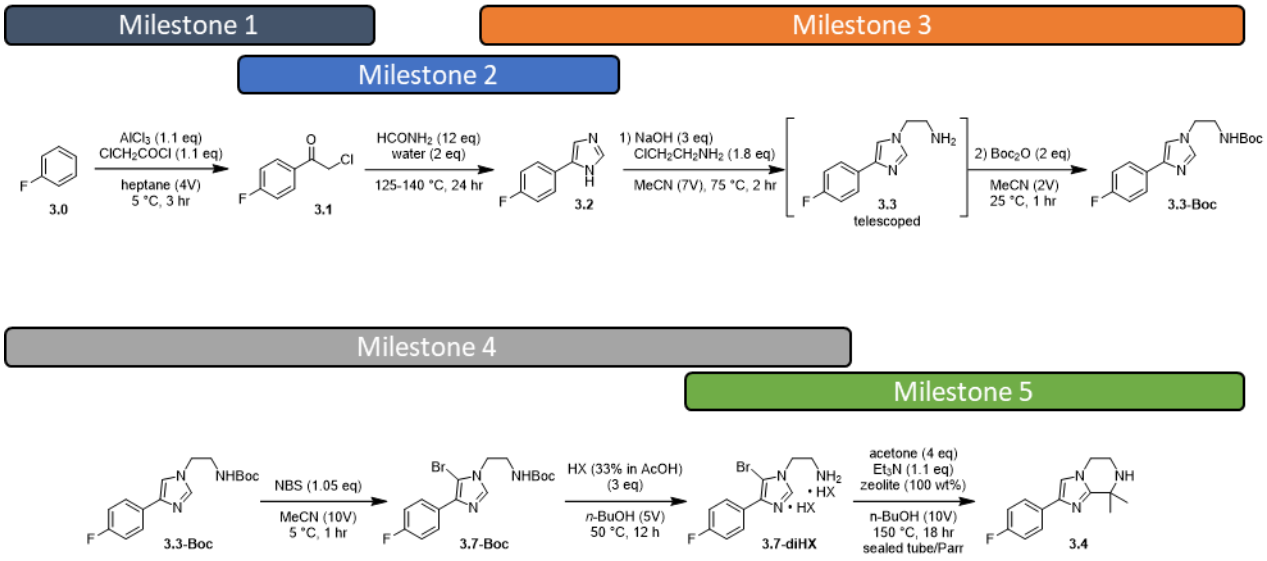
The publicly available synthetic route to ganaplacide published by Novartis suffers from several drawbacks which limit its cost-effectiveness and scalability to large-scale manufacturing[6,7]. This synthetic route uses a general retrosynthesis shown in Scheme 1.1. M4All's analysis has shown that the "common intermediate" is significant contributor to the overall costs of

ganaplacide. With this in mind, M4All chose to focus efforts on route scouting and optimization of this common intermediate molecule, **3.4**.



Scheme 1.1 Retrosynthetic disconnections for ganaplacide API and its simpler constituents for chemical synthesis.

The overall strategy proposed by M4All was divided into 5 milestones as shown in Scheme 1.2.



Scheme 1.2. Route to common intermediate **3.4**.

- **Milestone 1**: Milestone 1 is reported in the literature[8], starting with a Friedel-Crafts alkylation of the 4-flurobenzene **3.0**, and chloroacetyl chloride to generate **3.1**.
- **Milestone 2**: Cyclization of **3.1** to the imidazole, **3.2**

- **Milestone 3**: Telescoped alkylation of **3.2** using 2-chloroethylamine HCl to give **3.3** which was then protected with Boc_2O to give **3.3-Boc**.
- **Milestone 4**: Regioselective bromination of **3.3-Boc** using NBS to give **3.7-Boc** followed by acid mediated Boc-deprotection to give **3.7** as a salt.
- **Milestone 5**: The Pictet-Spengler reaction with simultaneous debromination of **3.7-diHBr** salt using acetone to give the common intermediate **3.4**

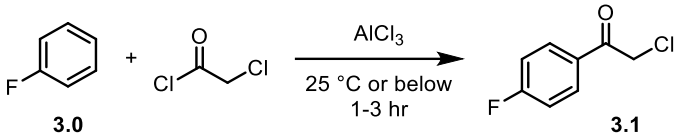
To facilitate the development of this process, M4All developed and implemented a suite of analytical methods for monitoring each reaction as well as characterizing the various intermediates, impurities (when possible) and the final product. The methods are described in the following sections and are summarized in the Appendix.

2 Results and Discussion

Liquid chromatography with ultraviolet detection (LC-UV) was used as the primary method for in-process controls as well as quantitative assays. As seen in the below sections, a single LC-UV method was developed for the entire synthesis. Gas chromatography with mass spectrometric detection (GC-MS) was used as a complimentary method for in-process controls as it provided alternative selectivity for many of the compounds. Additional methods were used for specific analyses and are described below.

2.1 3.0 to 3.1 (Milestone 1)

Friedel Crafts acylation was employed for the transformation of **3.0** to **3.1** (Scheme 2.1.1). While this common reaction is most frequently performed in dichloromethane, the US Environmental Protection Agency has severely restricted its use in both industrial and academic settings.[9] After a screening of various solvents, heptane was selected.



Scheme 2.1.1. Milestone 1 - Friedel Crafts Acylation

2.1.1 Pharmacopoeia Methods for 3.1

Neither compendial methods nor monographs from the United States Pharmacopoeia and/or the European Pharmacopeia are available for **3.1**.

2.1.2 Method Development for 3.1

2.1.2.1 In Process and Assay Analysis for 3.1

LC-UV was developed as the primary chromatographic method for monitoring reaction progress and assay of the final product (**3.1**). Chromatographic separation was achieved using an Agilent Zorbax Eclipse XDB-C18 column (4.6 x 250 mm, 5 µm) at a temperature of 30 °C. The flow rate was 2 mL/min with an injection volume of 1.5 µL. A binary mobile phase gradient elution was used with mobile phases of 25 mM potassium phosphate buffer at pH 6.2 (A) and acetonitrile (B). Initial conditions were set to 30% B for 4.5 min followed by a ramp over 2.5 min to 70% B. This was held for 4.5 min, total run time 12 min. A diode array detector was used with 254 nm selected as the primary detection wavelength. The full method is included in Appendix 3.1 (LCUV_GAN_ComInt_v1). Figure 2.1.2.1.1 is a representative chromatogram for the starting material and product analyzed in the current method.

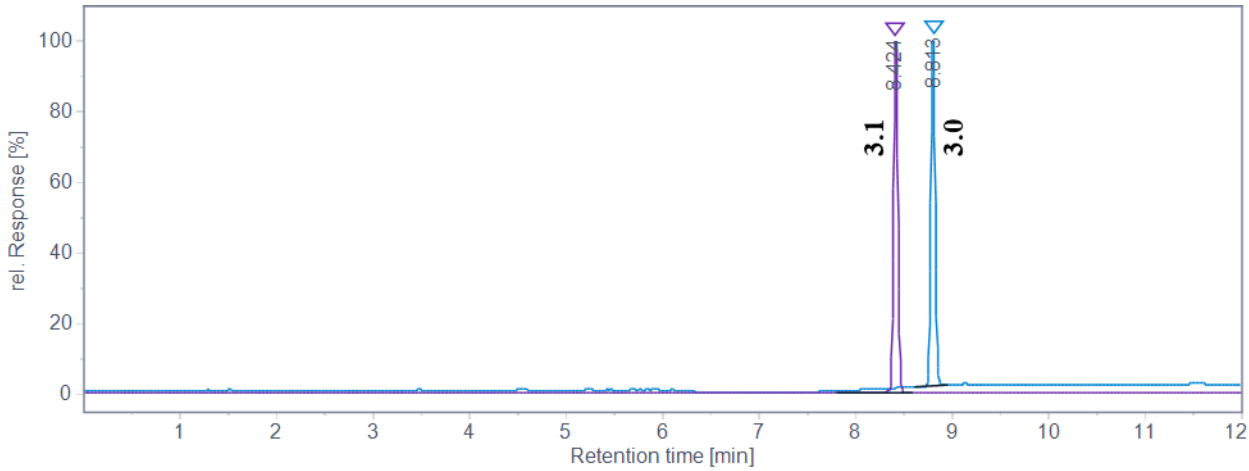


Figure 2.1.2.1.1. Representative chromatogram for **3.1** synthesis.

2.1.2.1.1 Relative Response Factors for 3.1

Standards of **3.0** and **3.1** were prepared at approximately 3 mg/mL and analyzed by the above method. The responses for these compounds were compared at a number of wavelengths from 210 nm to 290 nm in an effort to identify an isosbestic point – a point at which response factors are equal for the two compounds. As shown in Figure 2.1.2.1.1.1, the response for **3.1** is much greater at all wavelengths owing to addition of the acyl group which increased UV absorptivity.

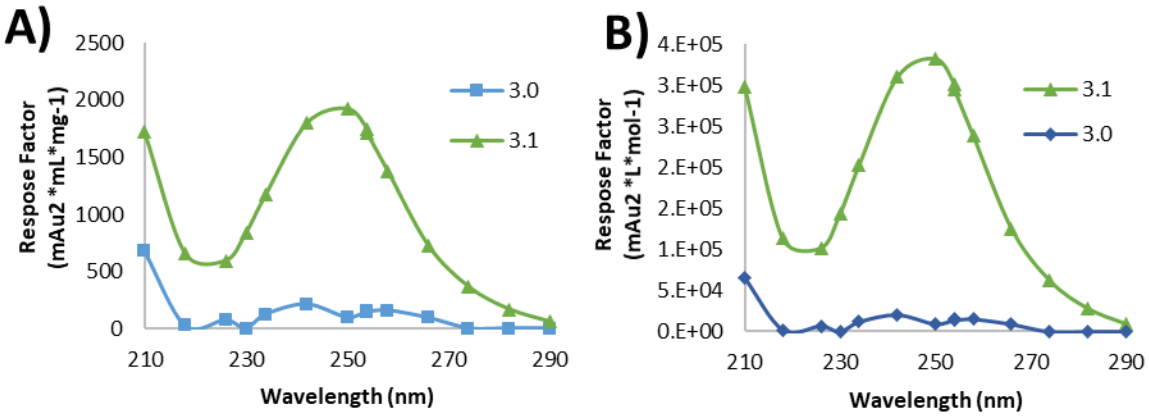


Figure 2.1.2.1.1.1. A) Mass-based and B) molarity-based response factors across the collected UV spectrum for **3.0** and **3.1**.

Because of this disparity, 254 nm was selected as the wavelength for detection of **3.1** as it is near to the **3.1** maximum. Because no wavelength offers an identical response for these two compounds, the use of reference standards is recommended when a quantitative determination of reaction conversion is desired. Relative response factors can be found in Table 2.1.2.1.1.

Table 2.1.2.1.1. Relative response factors for Milestone 1 are calculated based on concentration (mg/mL or M)

Compound	Retention Time (min)	RRF at 254 nm (mg/mL)	RRF at 254 nm (M)
3.0	8.81	0.089	0.049
3.1	8.42	1.00	1.00

2.1.2.1.2 Linearity for 3.1

The upper limit of linearity (UoL) and lower limit of linearity (LoL) were evaluated for **3.0** and **3.1** at 254 nm. The UoL was defined as the highest concentration which still produced a linear response, whereas LoL was defined by either the limit of quantitation (as defined Section 2.1.2.1.3) or the lowest tested concentration, whichever was higher. Calibration curves for **3.0** and **3.1** are shown in Figure 2.1.2.1.2.1. As expected, the relatively poor UV absorbance of **3.0** led to a much higher relative response for **3.1**. The UoL and LoL values for **3.0** and **3.1** are tabulated in Table 2.1.2.1.2.1.

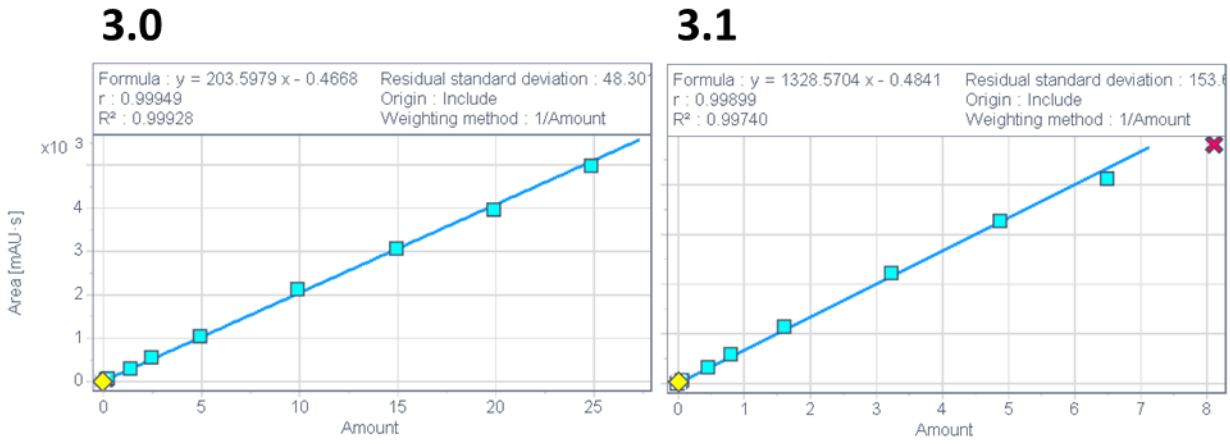


Figure 2.1.2.1.2.1. Linearity of **3.0** and **3.1** at 254 nm

Table 2.1.2.1.2.1. Quantitative metrics for **3.0** and **3.1** at 254 nm

Compound	Upper Limit of Linearity	Lower Limit of Linearity	Limit of Detection	Limit of Quantitation
3.0	24.9 mg/mL	0.030 mg/mL	0.0090 mg/mL	0.030 mg/mL
3.1	6.5 mg/mL	0.018 mg/mL	0.0175 mg/mL	0.0053 mg/mL

2.1.2.1.3 Limits of Detection (LOD) for **3.1**

Statistical limits of detection and quantitation were determined for **3.0** and **3.1** using a calibration curve limited to a lower concentration range. Limit of detection and quantitation were calculated using eqns. 2.1.2.1.3.1 and 2.1.2.1.3.2, below, where σ is the residual standard deviation and S is the slope of the calibration curve. The calculated values are listed in Table 2.1.2.1.2.1.

$$LoD = \frac{3 * \sigma}{S} \qquad \text{Eqn. 2.1.2.1.3.1}$$

$$LoQ = \frac{10 * \sigma}{S} \qquad \text{Eqn. 2.1.2.1.3.2}$$

*2.1.2.2 GC-MS Analysis for **3.1***

A GC-MS method was developed as a complimentary analysis to the LC-UV method outlined above. This method utilized an HP-5MS column (30 m x 0.250 mm; 0.25 µm) with a helium flow rate of 0.92 mL/min and a split ratio of 100:1. The initial temperature was set to 50 °C for 3 min, followed by a 25 °C/min ramp to 250 °C which was then held for 3 min. The temperature was then ramped again to 300 °C at 25°C/min followed by a final 3 min hold for a total run time of 19 min. A 1 µL injection was used. Additional method details are outlined in the Appendix 3.2 (GCMS_GAN_ComInt_v1). Figure 2.1.2.2.1 is a representative chromatogram for the starting material and product analyzed in the current method.

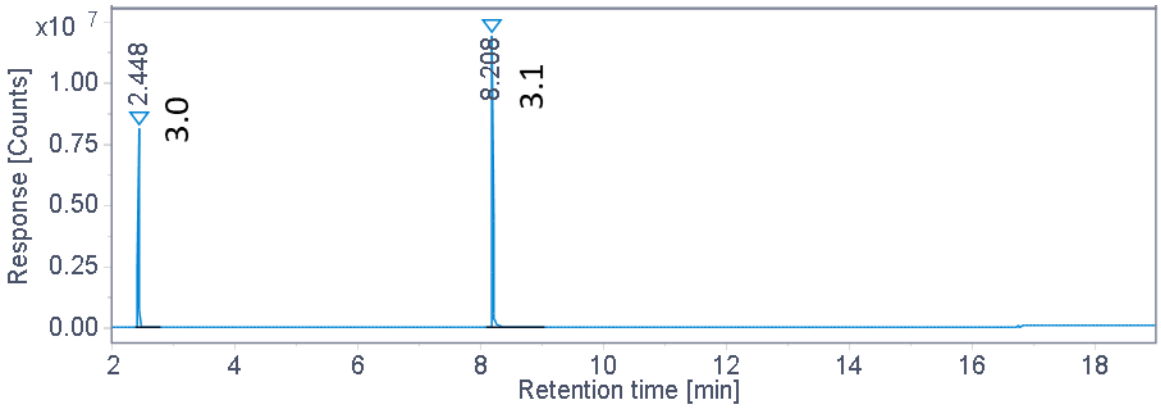


Figure 2.1.2.2.1. Representative GC-MS extracted ion chromatograms for **3.1** synthesis.

*2.1.2.3 LC-MS Analysis for **3.1***

A LC-MS method was developed for the confirmation of starting materials, products and impurity identification across all milestones; however, compounds **3.0** and **3.1** do not provide a LC-MS signal. Therefore, this method was not used for Milestone 1.

2.1.3 Impurities for 3.1

*2.1.3.1 Starting Material Impurities for **3.1***

Impurities were not specified nor determined for **3.1** starting materials.

*2.1.3.2 Synthesis Impurities for **3.1***

The Friedel Crafts acylation progressed was a high-yielding reaction with purity exceeding 95% purity by qNMR. No impurities were identified or tracked for this reaction.

2.1.4 Forced Degradation Studies for 3.1

Forced degradation studies were not performed for **3.1** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.1.5 Stability Testing for 3.1

Stability studies were not performed for **3.1** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.1.6 Methods for 3.1

All methods are described in detail in the Appendix.

2.1.6.1 Key Starting Materials for 3.1

Compound **3.0**, 4-fluorobenzene, was characterized by LC-UV (LCUV_GAN_ComInt_v1) as well as GC-MS (GCMS_GAN_ComInt_v1).

2.1.6.2 Reagents and Solvents for 3.1

All reagents and solvents for Milestone 1 were used based on the vendor-supplied certificates of analysis (CoA). No specific methods were developed in-house.

2.1.6.3 Intermediates for 3.1

No intermediates were analyzed for Milestone 1.

2.1.6.4 In-Process Controls (IPC) for 3.1

In-process controls were analyzed using method LCUV_GAN_ComInt_v1.

2.1.6.5 Final Product Analysis for 3.1

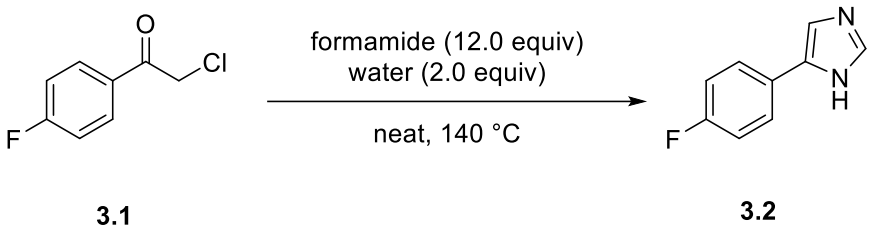
The final product was analyzed for weight percent and LC-UV area percent using LCUV_GAN_ComInt_v1. Weight percent assays were conducted using a calibration curve prepared with a reference standard characterized in-house for purity.

2.1.6.6 Method Appropriateness for 3.1

During development of the LCUV_GAN_ComInt_v1 method, certain performance characteristics were evaluated to select analytical conditions. These results are described above and include linearity. This method was not tested for specificity. Method validation was not performed. Specificity and validation were not included in the scope of this work.

2.2 3.1 to 3.2 (Milestone 2)

Milestone 2 comprised the preparation of **3.2** via a Bredereck imidazole synthesis from **3.1** as shown in Scheme 2.2.1.



3.1 **3.2**

Scheme 2.2.1. Preparation of **3.2** via a Bredereck imidazole synthesis

2.2.1 Pharmacopoeia Methods for 3.2

Neither compendial methods nor monographs from the United States Pharmacopoeia and/or the European Pharmacopeia are available for **3.2**.

2.2.2 Method Development for 3.2

2.2.2.1 In Process and Assay Analysis for 3.2

The same in-process method used in Milestone 1 was used for **3.2** analysis in Milestone 2 (Section 2.1.2.1). The full method is included in Appendix 3.1 (LCUV_GAN_ComInt_v1). Figure 2.2.2.1.1 is a representative chromatogram for the starting material, intermediates and product analyzed in the current method.

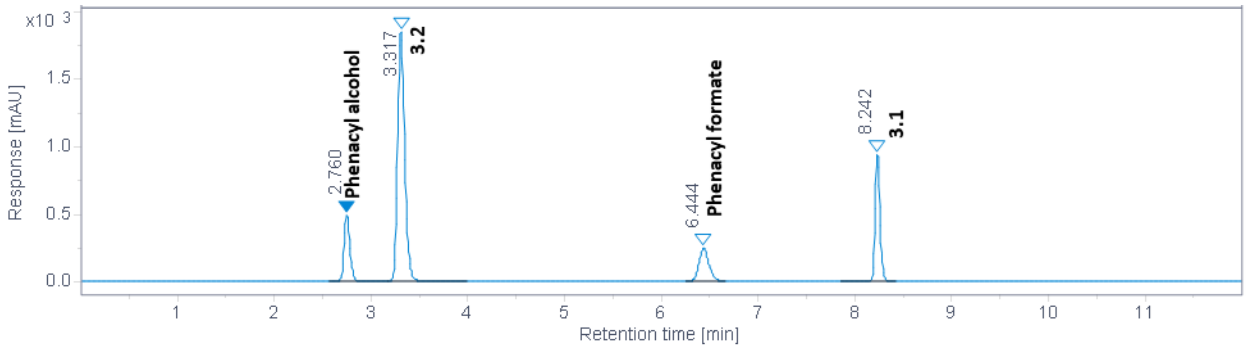


Figure 2.2.2.1.1. Reference chromatogram for **3.2** synthesis including two known intermediates.

2.2.2.1.1 Relative Response Factors for 3.2

Standards of **3.1** and **3.2** were prepared at 3 mg/mL and analyzed by the above method. The detector response was compared at several wavelengths between 210 nm and 290 nm in order to identify an isosbestic point – a point at which response factors are equal for the two compounds. As seen in Figure 2.2.2.1.1.1, no wavelength provided equal response factors between **3.1** and **3.2**

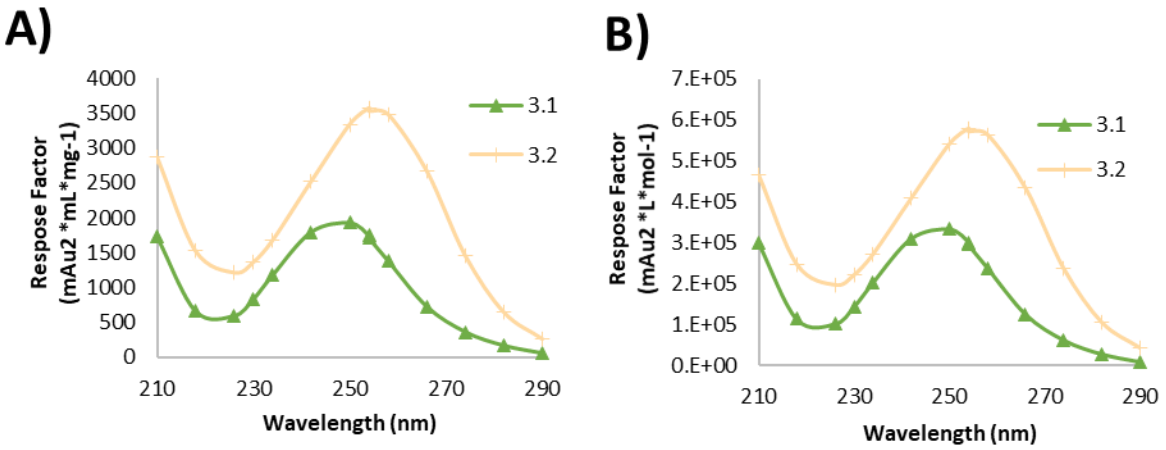


Figure 2.2.2.1.1.1. A) Mass-based and B) molarity based response factors across the collected UV spectrum for **3.1** and **3.2**.

To enable more accurate comparison of the ratio of **3.1** and **3.2** in the reaction, relative response factors (RRF) were calculated via the equation below (Eqn. 2.2.2.1.1) where area is the peak area at the specified wavelength and concentration is the measured in either mg/mL or mol/L. While no wavelength gave an RRF value of one, 234 nm was found to be the closest at 0.70. Prior to the isosbestic analysis, 254 nm was selected as the detection wavelength as it was close to the spectral maxima of many of the compounds across all milestones. As such, the RRF at 254 nm is also listed in Table 2.2.2.1.1.1; however, it is recommended that any future analysis utilize 234 nm.

$$\mathrm{RRF} = \frac{\left({}^{\mathrm{Area}}/_{\mathrm{Concentration}}\right)_{3.1}}{\left({}^{\mathrm{Area}}/_{\mathrm{Concentration}}\right)_{3.2}} \qquad \text{Eqn. 2.2.2.1.1}$$

Table 2.2.2.1.1.1. RRF Values for **3.1** and **3.2**

Compound	Retention Time (min)	RRF (mg/mL)		RRF (M)	
		234 nm	254 nm	234 nm	254 nm
3.1	8.24	0.70	0.49	0.74	0.61
3.2	3.29	1.00	1.00	1.00	1.00
Phenacyl formate	6.29	*Not determined*			
Phenacyl alcohol	2.76	*Not determined*			

2.2.2.1.2 Linearity for 3.2

The upper limit of linearity (UoL) and lower limit of linearity (LoL) were evaluated for **3.1** and **3.2** at 234 nm and 254 nm. The UoL was defined as the highest concentration which still produced a linear response, whereas LoL was defined by the limit of quantitation. The calibration curves are shown in Figure 2.2.2.1.2.1. The UoL and LoL values for **3.1** and **3.2** are tabulated in Table 2.2.2.1.2.1.

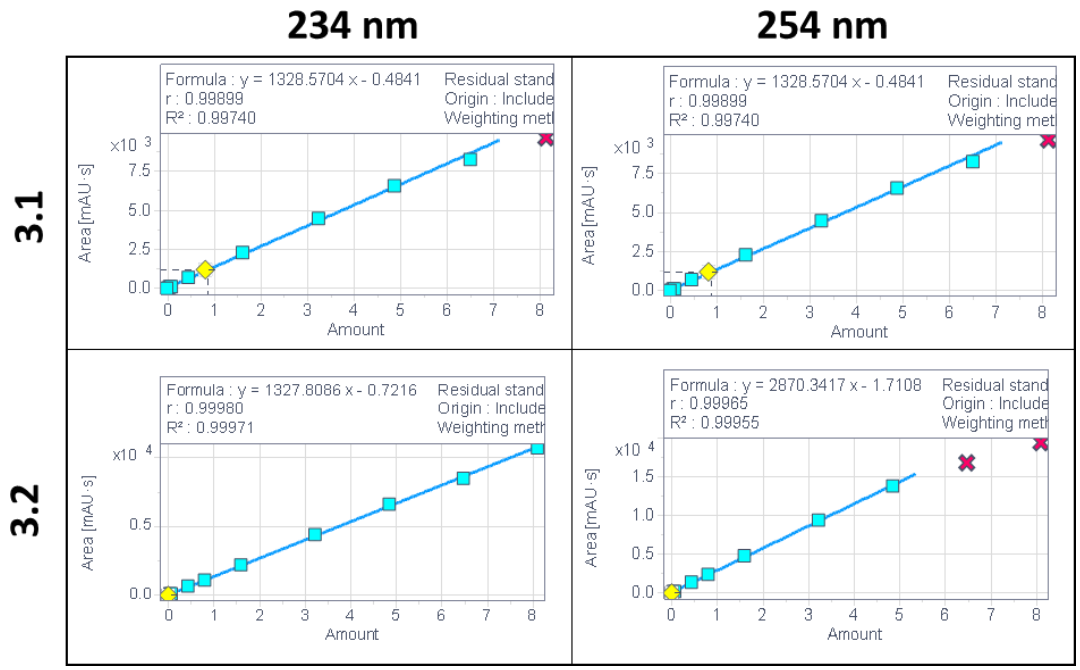


Figure 2.2.2.1.2.1. Linearity of **3.1** and **3.2** at 234 nm and 254 nm

Table 2.2.2.1.2.1. Quantitative metrics for **3.1** and **3.2**

Compound	Wavelength	Upper Limit of Linearity	Lower Limit of Linearity	Limit of Detection	Limit of Quantitation
3.1	234 nm	8.12 mg/mL	0.0049 mg/mL	0.0015 mg/mL	0.0049 mg/mL
	254 nm	6.50 mg/mL	0.0055 mg/mL	0.0016 mg/mL	0.0055 mg/mL
3.2	234 nm	8.09 mg/mL	0.0051 mg/mL	0.0015 mg/mL	0.0051 mg/mL
	254 nm	4.86 mg/mL	0.0028 mg/mL	0.00084 mg/mL	0.0028 mg/mL

2.2.2.1.3 Limits of Detection (LOD) for **3.2**

Statistical limits of detection and quantitation were determined for **3.1** and **3.1** using a calibration curve limited to a lower concentration range. Limit of detection and quantitation were calculated using equations 2.1.2.1.3.1 and 2.1.2.1.3.2. The calculated values are listed in Table 2.2.2.1.2.1.

*2.2.2.2 GC-MS Analysis for **3.2***

The same GC-MS method used in Milestone 1 was used for **3.2** analysis in Milestone 2 (Section 2.1.2.2). The full method is included in Appendix 3.2 (GCMS_GAN_ComInt_v1). Figure 2.2.2.2.1 is a representative chromatogram for the starting material, intermediates and product analyzed in the current method.

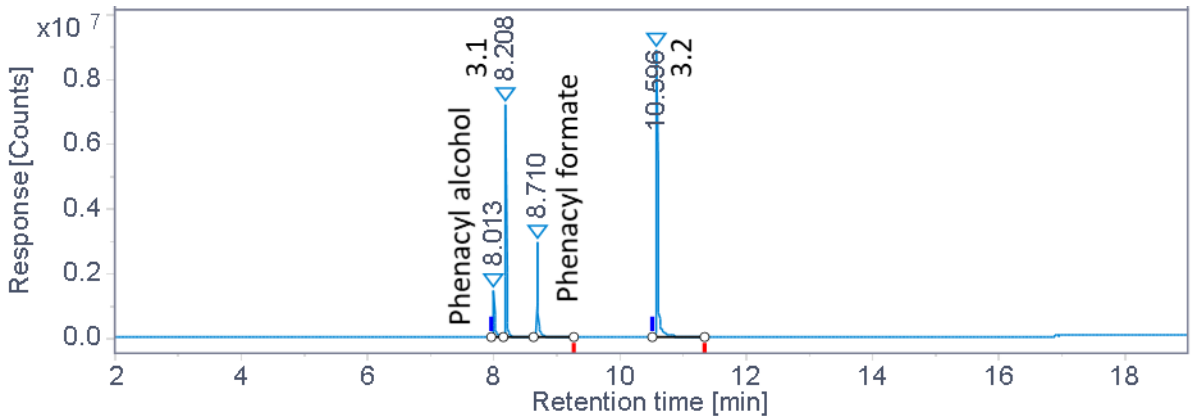


Figure 2.2.2.2.1. Representative GC-MS chromatograms for **3.2** synthesis.

2.2.2.3 LC-MS Analysis for 3.2

An LC-UV-MS method was developed for the confirmation of starting materials and products. The conditions were selected to mimic the primary in process LC-UV method while being compatible with mass spectrometric detection. An Agilent Eclipse XDB-C18 column (2.1 mm x 150 mm; 3.5 µm) was utilized and was held at a temperature of 30 °C with a mobile phase flow rate of 0.6 mL/min. The mobile phase consisted of 15 mM ammonium acetate at a pH 6.2 (A) and acetonitrile (B). Initially mobile phase B was held at 30% for 2.0 minutes, then ramped to 70% B over 2.0 minutes followed by a 1.0-minute hold. A 3.0-minute post-run equilibration was used. UV spectra were collected at a default wavelength of 260 nm and the MS was operated in both positive and negative electrospray ionization modes with a mass range of 40-1000 amu. Full method details are included in Appendix 3.3 (LCMS_GAN_v1).

Compound **3.1** does not ionize under these conditions. Compound **3.2** has precursor ions of *m/z* 163 and *m/z* 161 in positive and negative ESI modes, respectively. Chromatograms and mass spectra are included in Appendix 3.3.

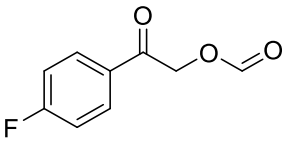
2.2.3 Impurities for 3.2

2.2.3.1 Starting Material Impurities for 3.2

No starting material impurities have been characterized for **3.1**.

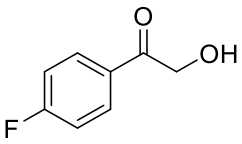
2.2.3.2 Synthesis Impurities for 3.2

No impurities have been characterized for Milestone 2. Reactive synthetic intermediates phenacyl formate and phenacyl alcohol shown in Figure 2.2.3.2.1 were identified and confirmed by independent synthesis. These intermediates did not carry forward into the isolated product.



Exact Mass: 182.04

Phenacyl formate



Exact Mass: 154.04

Phenacyl alcohol

Figure 2.2.3.2.1. Confirmed intermediate in the synthesis of **3.2**

2.2.4 Forced Degradation Studies for 3.2

Forced degradation studies were not performed for neither **3.2** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.2.5 Stability Testing for 3.2

Stability studies were not performed for neither **3.2** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.2.6 Methods for 3.2

All methods used are included in the Appendix 3.

2.2.6.1 Key Starting Materials for 3.2

Starting material **3.1** was analyzed by LCUV_GAN_ComInt_v1 for purity by LC area percent and assayed using the same method against a reference standard whose purity was determined in-house.

2.2.6.2 Reagents and Solvents for 3.2

All reagents and solvents for Milestone 1 were used based on the vendor-supplied certificates of analysis (CoA). No specific methods were developed in-house.

2.2.6.3 Intermediates for 3.2

There were no stable, isolated intermediates in the reaction of **3.1** to **3.2**. The reactive intermediates are tracked by LCUV_GAN_ComInt_v1 during the in-process controls.

2.2.6.4 *In-Process Controls (IPC) for **3.2***

In-process controls were analyzed via LCUV_GAN_ComInt_v1. The samples are pulled from the reaction, diluted in an appropriate solvent prior to analysis.

2.2.6.5 *Final Product Analysis for **3.2***

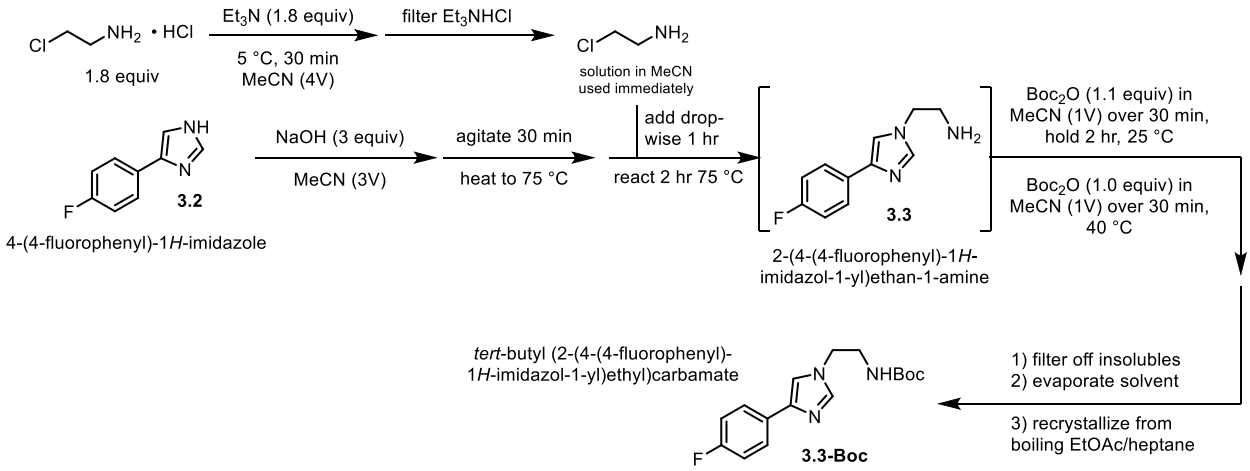
The product **3.2** was assayed using a calibration curve prepared from reference standards whose purity was determined in-house. LCUV_GAN_ComInt_v1 was used for analysis.

2.2.6.6 *Method Appropriateness for **3.2***

During development of the LCUV_GAN_ComInt_v1 method, certain performance characteristics were evaluated to select analytical conditions. These results are described above and include linearity. This method was not tested for specificity. Method validation was not performed. Specificity and validation were not included in the scope of this work.

2.3 3.2 to 3.3 and 3.3 to 3.3-Boc (Milestone 3)

Milestone 3 consisted of the two telescoped chemical transformations. First, **3.2** was N-alkylated to product **3.3** which was then Boc protected to yield **3.3-Boc** as a solid. **3.3-Boc** was then recrystallized to purge process impurities and achieve a high **3.3-Boc** purity (Scheme 2.3.1).



Scheme 2.3.1 M4All telescoped process for **3.3-Boc** synthesis.

2.3.1 Pharmacopoeia Methods for 3.3-Boc

Neither compendial methods nor monographs from the United States Pharmacopoeia and/or the European Pharmacopeia are available for **3.3-Boc**.

2.3.2 Method Development for 3.3-Boc

2.3.2.1 In Process and Assay Analysis for 3.3-Boc

The same in process method used in Milestone 1 was used for **3.2** analysis in Milestone 3 (Section 2.1.2.1) The full method is included in Appendix 3.1 (LCUV_GAN_ComInt_v1). Figure 2.3.2.1.1 is a representative chromatogram for the starting material, intermediate, and product analyzed in the current method.

A limitation of this method for Milestone 3 is the inability to separate the regioisomers of **3.3** and **3.3-Boc**. The *N*-alkylation of **3.2** occurs primarily on the desired nitrogen; however, a portion of the **3.2** undergoes alkylation on the undesired nitrogen giving the regioisomer ***iso*-3.3**. Subsequent Boc protection give ***iso*-3.3Boc**. These regioisomers can be detected by a secondary, GC-MS method described in Section 2.3.2.2., below.

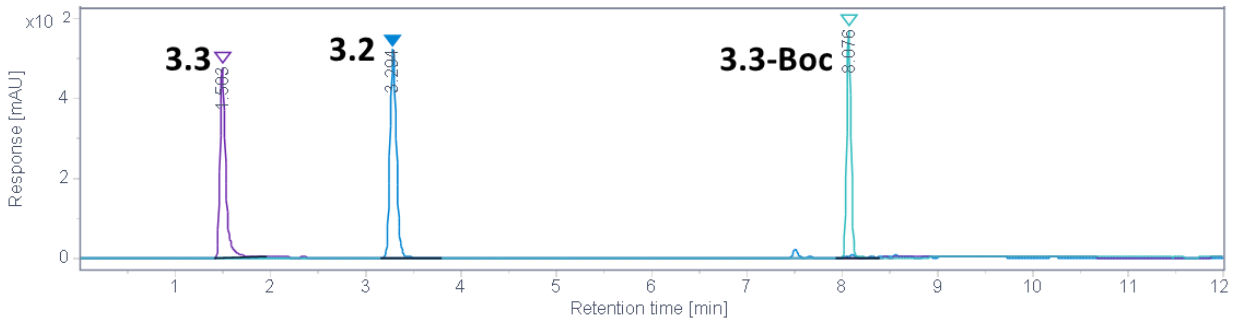


Figure 2.3.2.1.1. Representative chromatogram for **3.3-Boc** synthesis.

2.3.2.1.1 Relative Response Factors for 3.3-Boc

Standards of **3.2, 3.3**, and **3.3-Boc** were prepared at 3 mg/mL and analyzed by the above method. The detector response was compared at several wavelengths between 210 nm and 290 nm in order to identify an isosbestic point – a point at which response factors are equal for the two compounds.

As seen in Figure 2.3.2.1.1.1. No wavelength provided equal mass-based response factors between all three compounds, with **3.2** showing higher UV absorptivity across the entire spectrum. When compared on a molar basis (Figure 2.3.2.1.1.1), 234 nm provides a uniform response across all 3 compounds. For all of the process development work performed to date, 254 nm was used for detection as it was a shared maximum across the three compounds; however, it is recommended that 234 nm be used for reaction monitoring. RRF values are calculated in Table 2.3.2.1.1.1 for both 234 nm and 254 nm.

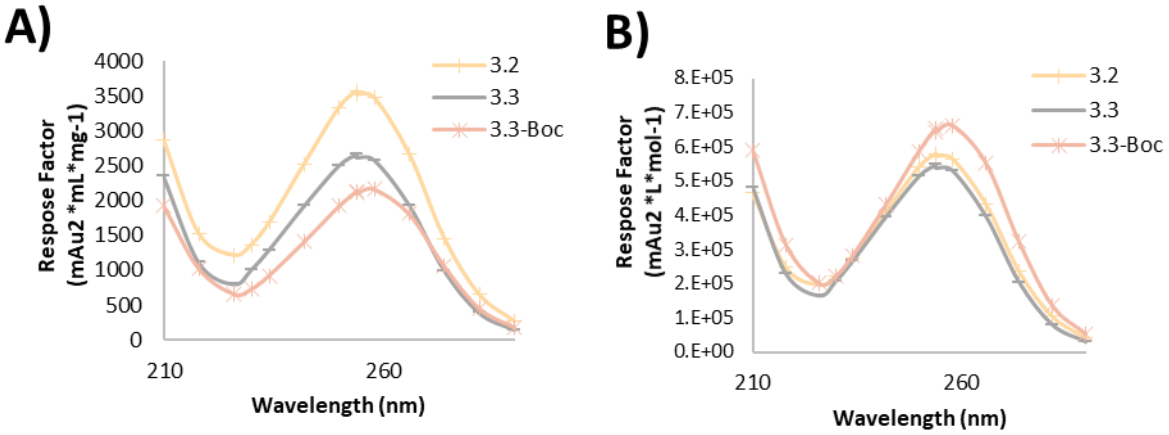


Figure 2.3.2.1.1.1. A) Mass-based and B) molarity based response factors across the collected UV spectrum for **3.2**, **3.3** and **3.3-Boc**

Taking the individual response factors for each compound shown graphically in Figure 2.3.2.1.1.1, relative response factors (RRF) were calculated relative to **3.3-Boc** (Eqn. 2.3.2.1.1.1). These RRF values are tabulated in Table 2.3.2.1.1.1.

$$\mathrm{RRF} = \frac{\left(\mathrm{Area}/\mathrm{Concentration}\right)_{\mathrm{Analyte}}}{\left(\mathrm{Area}/\mathrm{Concentration}\right)_{3.3-Boc}} \qquad \text{Eqn. 2.3.1.2.1.1}$$

Table 2.3.2.1.1.1. RRF Values for **3.2**, **3.3**, and **3.3-Boc**

Compound	Retention Time (min)	RRF (mg/mL)		RRF (M)	
		234 nm	254 nm	234 nm	254 nm
3.2	3.29	1.82	1.67	0.97	0.89
3.3	1.49	1.40	1.24	0.94	0.84
3.3-Boc	8.10	1.00	1.00	1.00	1.00

2.3.2.1.2 Linearity for 3.3-Boc

For each compound, the upper and lower limits of linearity (UoL and LoL) were determined. UoL is defined as the highest concentration at which the peak response gave a linear response with respect to concentration (Figure 2.3.2.1.2.1). In this work, the LoL is defined as the limit of quantitation (LoQ) or the lowest calibration point analyzed, whichever is higher (Table 2.3.2.1.2.1).

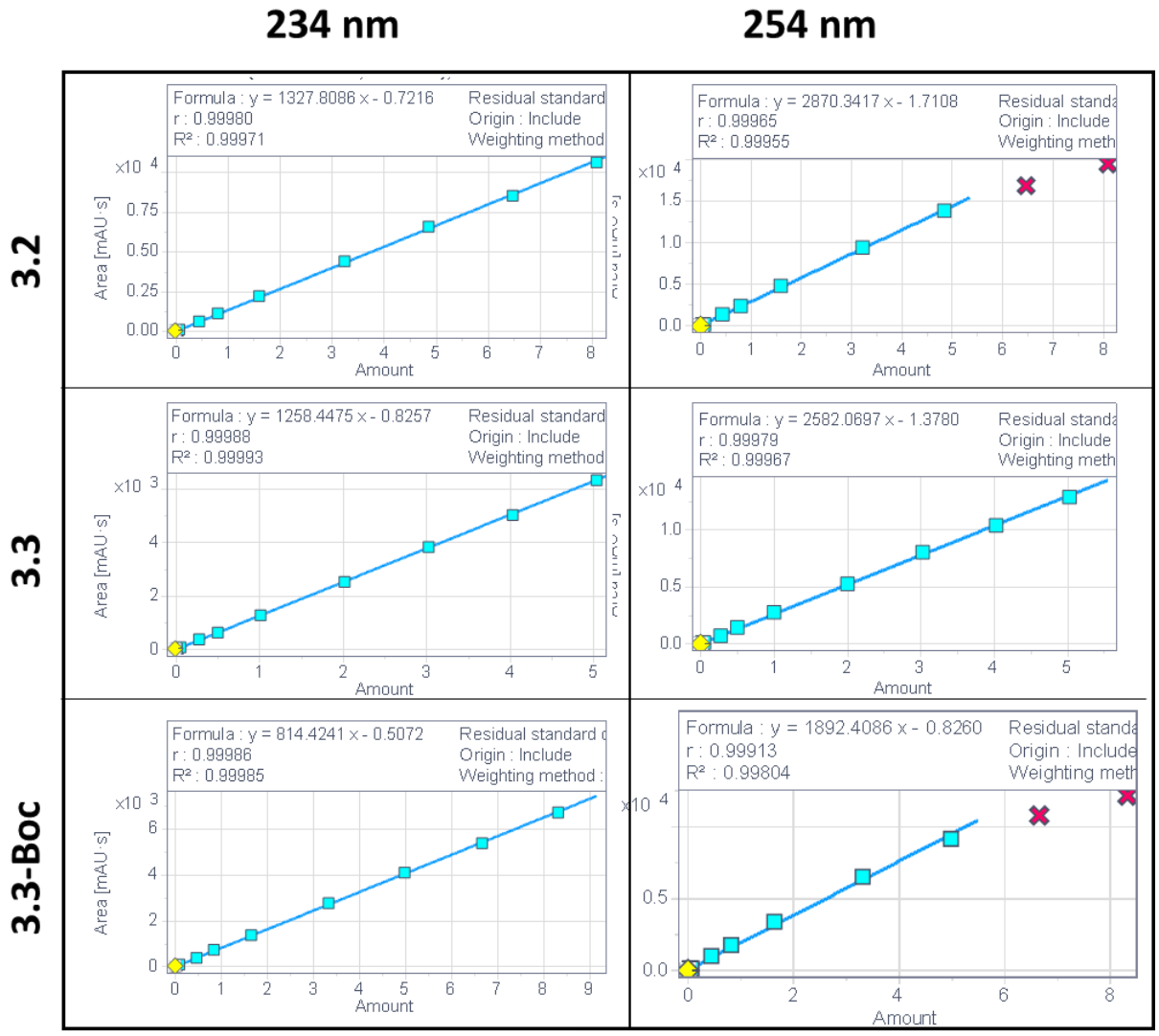


Figure 2.3.2.1.2.1. Calibration curves for each of the compounds in Milestone 3 at 234 nm and 254 nm

Table 2.3.2.1.2.1. Quantitative metrics for **3.2**, **3.3**, and **3.3-Boc** at 234 nm and 254 nm

Compound	Wavelength	Upper Limit of Linearity	Lower Limit of Linearity	Limit of Detection	Limit of Quantitation
3.2	234 nm	8.09 mg/mL	0.0051 mg/mL	0.0015 mg/mL	0.0051 mg/mL
	254 nm	4.86 mg/mL	0.0028 mg/mL	0.00084 mg/mL	0.0028 mg/mL
3.3	234 nm	5.05 mg/mL	0.00052 mg/mL	0.00015 mg/mL	0.00052 mg/mL
	254 nm	5.05 mg/mL	0.00082 mg/mL	0.00025 mg/mL	0.0016 mg/mL
3.3-Boc	234 nm	8.32 mg/mL	0.0060 mg/mL	0.0018 mg/mL	0.0060 mg/mL
	254 nm	4.99 mg/mL	0.0056 mg/mL	0.0017 mg/mL	0.0056 mg/mL

2.3.2.1.3 Limits of Detection (LOD) for 3.3-Boc

Statistical limits of detection and quantitation were determined for **3.2**, **3.3**, and **3.3-Boc** using a calibration curve limited to a lower concentration range. Limit of detection and quantitation were calculated using eqns. 2.1.2.1.3.1 and 2.1.2.1.3.2. The calculated values are listed in Table 2.3.2.1.2.1.

2.3.2.2 GC-MS Analysis for 3.3-Boc

The same GC-MS method used in Milestone 1 was used for **3.3-Boc** analysis in Milestone 3 (Section 2.1.2.2). The full method is included in Appendix 3.2 (GCMS_GAN_ComInt_v1). Figure 2.3.2.2.1 is a representative chromatogram for the starting material, intermediate, impurities and product analyzed in the current method.

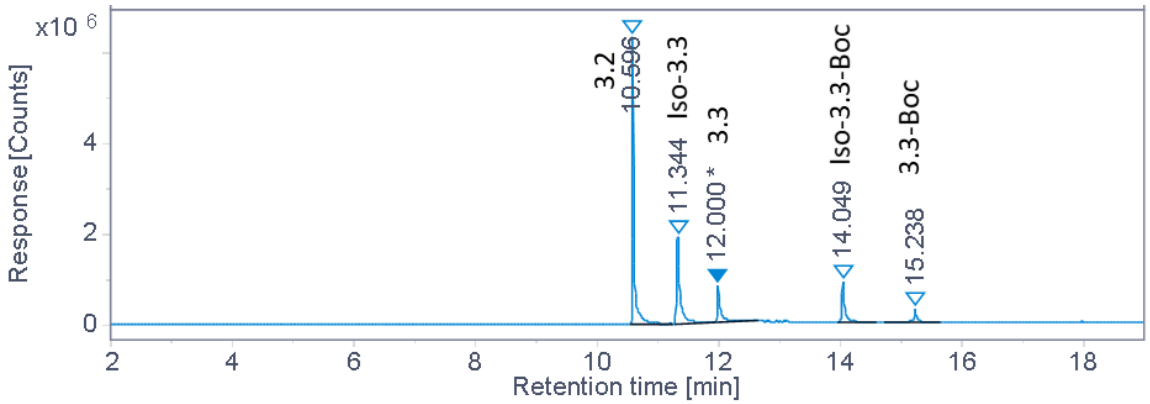


Figure 2.3.2.2.1. Representative GC-MS chromatogram for **3.3-Boc** synthesis.

2.3.2.3 LC-MS Analysis for 3.3-Boc

The same LC-MS method used in Milestone 2 was used for **3.3-Boc** analysis in Milestone 3 (Section 2.1.2.2). The full method is included in Appendix 3.2 (LCMS_GAN_ComInt_v1).

2.3.3 Impurities for 3.3-Boc

2.3.3.1 Starting Material Impurities for 3.3-Boc

No identified impurities were carried forth from the synthesis of the starting material, **3.2**, described in Milestone 2.

2.3.3.2 Synthesis Impurities for 3.3-Boc

During the alkylation of **3.2**, the addition of the chloroethylamine can alkylate at the other nitrogen of the imidazole. This is found in an approximately 10:1 ratio of **3.3**:**iso-3.3** as observed by NMR and GC-MS. This material is carried forward into the Boc protection where the corresponding Boc-protected impurity, **iso-3.3-Boc**, is formed – again in a 10:1 ratio with **3.3-Boc**. These regioisomers are purged during recrystallization.

The primary LC-UV method (LCUV_GAN_ComInt_v1) does not show resolution of the isomeric pairs. For detection of **iso-3.3** and **iso-3.3-Boc**, GC-MS can be used (GCMS_GAN_ComInt_v1) as shown in Figure 2.3.2.2.1

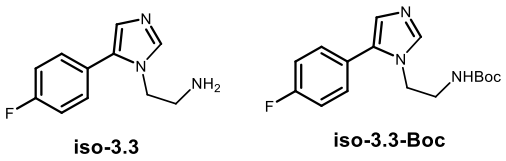


Figure 2.3.3.2.1. Regioisomers formed in Milestone 3.

2.3.4 Forced Degradation Studies for 3.3-Boc

Forced degradation studies were not performed for neither **3.3-Boc** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.3.5 Stability Testing for 3.3-Boc

Stability studies were not performed for neither **3.3-Boc** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.3.6 Methods for 3.3-Boc

All methods used are described in detail in the Appendix.

2.3.6.1 Key Starting Materials for 3.3-Boc

Compound **3.2** was assayed for weight percent via LC-UV using method LCUV_GAN_ComInt_v1. Chromatographic purity was also determined via the same method.

2.3.6.2 Reagents and Solvents for 3.3-Boc

All reagents and solvents for Milestone 3 were used based on the vendor-supplied certificates of analysis (CoA). No specific methods were developed in-house.

2.3.6.3 Intermediates for 3.3-Boc

Compound **3.3** was analyzed using method LCUV_GAN_ComInt_v1 for chromatographic purity and weight percent assay. The ratio of **3.3** to the corresponding regioisomer **iso-3.3** was determined via GC-MS (GCMS_GAN_ComInt_v1).

2.3.6.4 *In-Process Controls (IPC) for **3.3-Boc***

LC-UV was used for in process controls. LC peak area percent at 254 nm was used for monitoring the progress of the reaction.

2.3.6.5 *Final Product Analysis for **3.3-Boc***

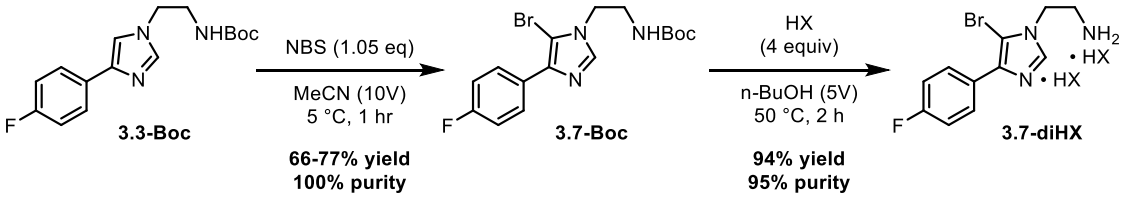
The product, **3.3-Boc**, was analyzed via LC-UV using LCUV_GAN_ComInt_v1 for chromatographic purity and weight percent assay. The absence of **3.3-Boc** was confirmed via GC-MS (GCMS_GAN_ComInt_v1).

2.3.6.6 *Method Appropriateness for **3.3-Boc***

During development of the LCUV_GAN_ComInt_v1 method, certain performance characteristics were evaluated to select analytical conditions. These results are described above and include linearity. This method was not tested for specificity. Method validation was not performed. Specificity and validation were not included in the scope of this work.

2.4 3.3-Boc to 3.7-diHX (Milestone 4)

The synthesis of the penultimate compound, **3.7**, is accomplished in two discrete steps. First, **3.3-Boc** is brominated to yield **3.7-Boc**. Subsequently, an acid mediated deprotection yield **3.7** which precipitates as salt, **3.7-diHX**. The compound has been isolated as both the dihydrochloride and dihydrobromide salts (Scheme 2.4.1).



Scheme 2.4.1 NBS-promoted bromination and the subsequent Boc deprotection.

2.4.1 Pharmacopoeia Methods for 3.7-diHX

Neither compendial methods nor monographs from the United States Pharmacopoeia and/or the European Pharmacopeia are available for **3.7-diHX**.

2.4.2 Method Development for 3.7-diHX

2.4.2.1 In Process and Assay Analysis for 3.7-diHX

The same in-process method used in Milestone 1 was used for **3.7-diHX** analysis in Milestone 4 (Section 2.1.2.1) The full method is included in Appendix 3.1 (LCUV_GAN_ComInt_v1). Figure 2.3.2.1.1 is a representative chromatogram for the starting material, intermediate, and product analyzed in the current method.

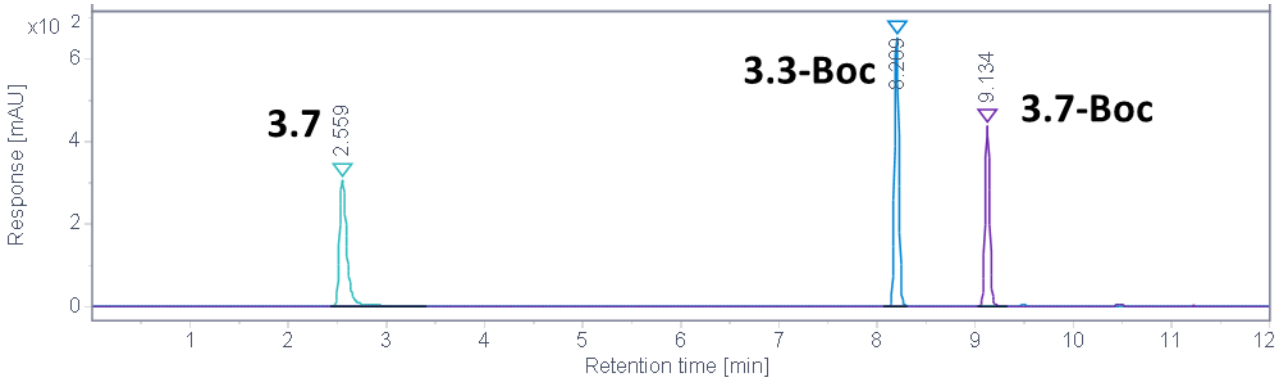


Figure 2.4.2.1.1. Representative LC-UV chromatogram used for monitoring **3.3-Boc** to **3.7-Boc** to **3.7-diHX**.

2.4.2.1.1 Relative Response Factors for 3.7-diHX

The UV responses of **3.3-Boc, 3.7-Boc**, and **3.7-diHx** at various wavelengths were analyzed in order to select the ideal wavelength for detection in the LC-UV method (LCUV_GAN_ComInt_v1). Standards of each compound were prepared and analyzed by the above method at wavelengths between 210 nm and 290 nm in order identify an isosbestic point – a point at which response factors are equal for the two compounds. A detection wavelength of 226 nm provides the most uniform response across these compounds. Because early process

development was performed using a detection wavelength at 254 nm – near the maxima for these compounds – this is included in the RRF table below.

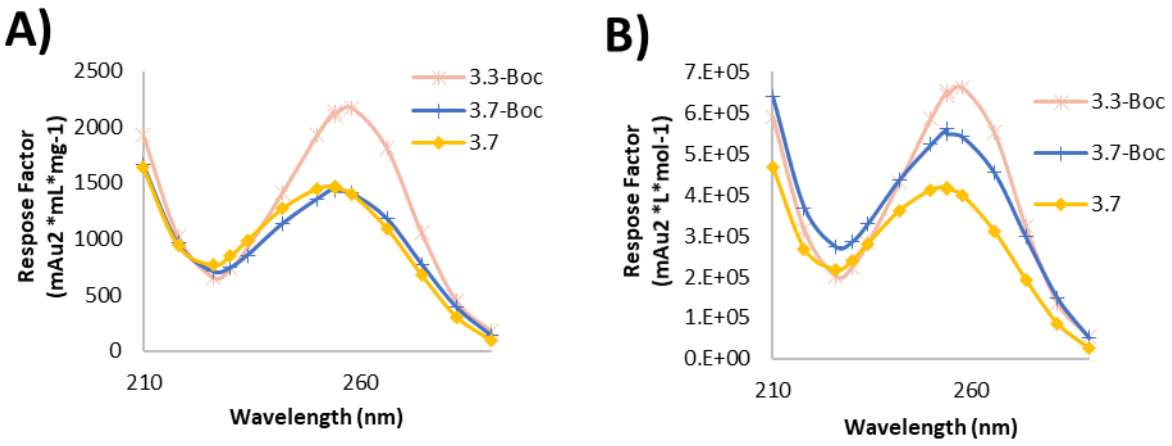


Figure 2.4.2.1.1.1. A) Mass-based and B) molarity based (**B**) response factors across the collected UV spectrum for **3.3-Boc, 3.7-Boc**, and **3.7** (as a free base).

Taking the individual response factors for each compound shown graphically in Figure 2.4.2.1.1.1, relative response factors (RRF) were calculated relative to **3.7** as the free base. These RRF values are tabulated in Table 2.4.2.1.1.1.

Table 2.4.2.1.1.1. RRF Values for **3.3-Boc**, **3.7-Boc**, and **3.7**

Compound	Retention Time (min)	RRF (mg/mL)		RRF (M)	
		226 nm	254 nm	226 nm	254 nm
3.3-Boc	8.10	0.86	1.46	0.92	1.56
3.7-Boc	9.02	0.93	0.99	1.25	1.34
3.7 (free base)	2.50	1.00	1.00	1.00	1.00

2.4.2.1.2 Linearity for 3.7-diHX

For each compound, the upper and lower limits of linearity (UoL and LoL) were determined. UoL is defined as the highest concentration at which the peak response gave a linear response with respect to concentration (Figure 2.4.2.1.2.1). In this work, the LoL is defined as the limit of quantitation (LoQ) or the lowest calibration point analyzed, whichever is higher (Table 2.4.2.1.2.1).

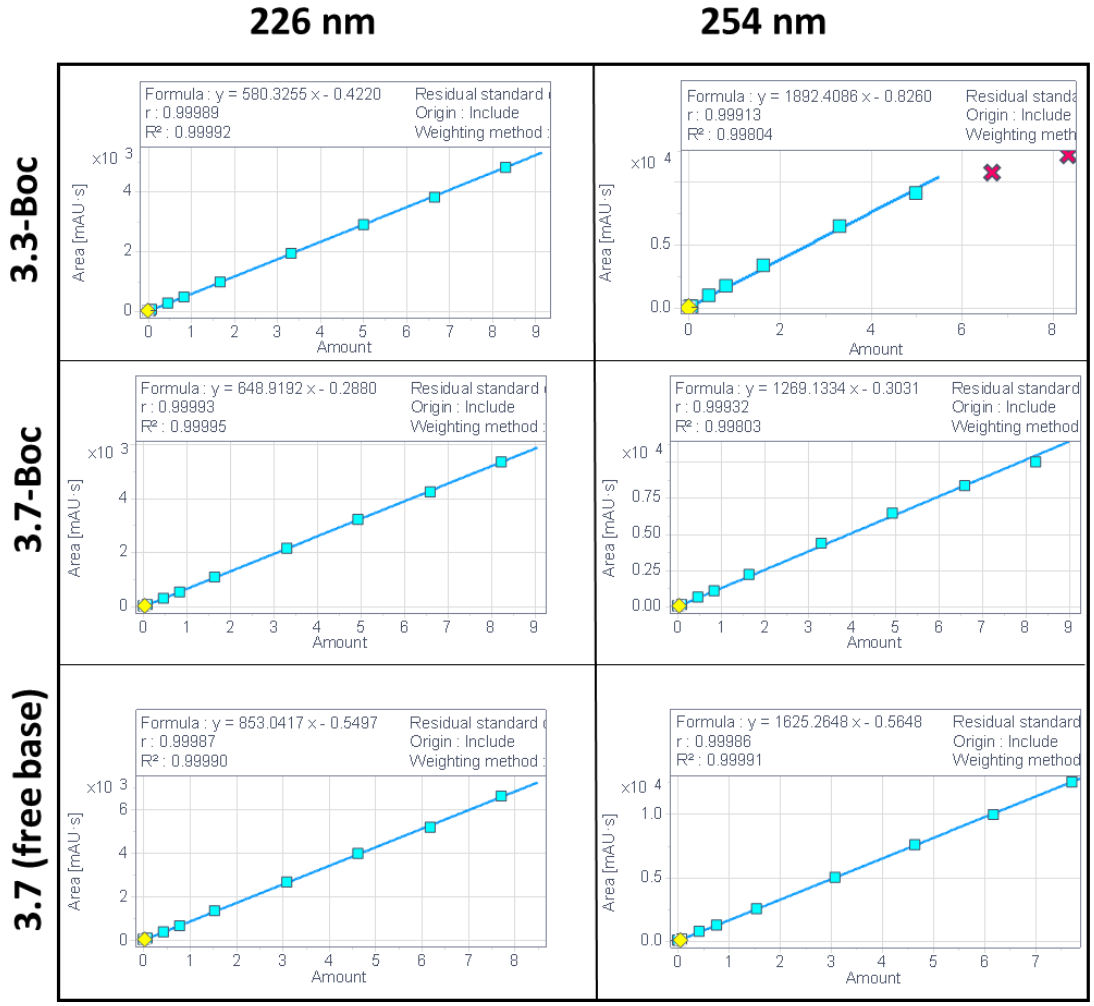


Figure 2.4.2.1.2.1. Calibration curves for each of the compounds in Milestone 4 at 226 nm and 254 nm

Table 2.3.2.1.2.1. Quantitative metrics for **3.3-Boc**, **3.7-Boc**, and **3.7** at 226 nm and 254 nm

Compound	Wavelength	Upper Limit of Linearity	Lower Limit of Linearity	Limit of Detection	Limit of Quantitation
3.3-Boc	226 nm	8.32 mg/mL	0.0057 mg/mL	0.0017 mg/mL	0.0057 mg/mL
	254 nm	4.99 mg/mL	0.0056 mg/mL	0.0017 mg/mL	0.0056 mg/mL
3.7-Boc	226 nm	8.24 mg/mL	0.0059 mg/mL	0.0018 mg/mL	0.0059 mg/mL
	254 nm	8.24 mg/mL	0.0085 mg/mL	0.0025 mg/mL	0.0085 mg/mL
3.7 (free base)	226 nm	7.73 mg/mL	0.0088 mg/mL	0.0026 mg/mL	0.0088 mg/mL
	254 nm	7.73 mg/mL	0.011 mg/mL	0.003 mg/mL	0.0011 mg/mL

2.4.2.1.3 Limits of Detection (LOD) for 3.7-diHX

Statistical limits of detection and quantitation were determined for **3.3-Boc**, **3.7-Boc**, and **3.7** (as the free base) using a calibration curve limited to a lower concentration range. Limit of detection and quantitation were calculated using eqns. 2.1.2.1.3.1 and 2.1.2.1.3.2. The calculated values are listed in Table 2.4.2.1.2.1.

2.4.2.2 GC-MS Analysis for 3.7-diHX

The same GC-MS method used in Milestone 1 was used for **3.7-diHx** analysis in Milestone 4 (Section 2.1.2.2). **3.7-Boc** is not detected in this method, likely due to its high molecular weight and/or potential thermal instability. The full method is included in Appendix 3.2 (GCMS_GAN_ComInt_v1). Figure 2.3.2.2.1 is a representative chromatogram for the starting material, intermediate, impurities and product analyzed in the current method.

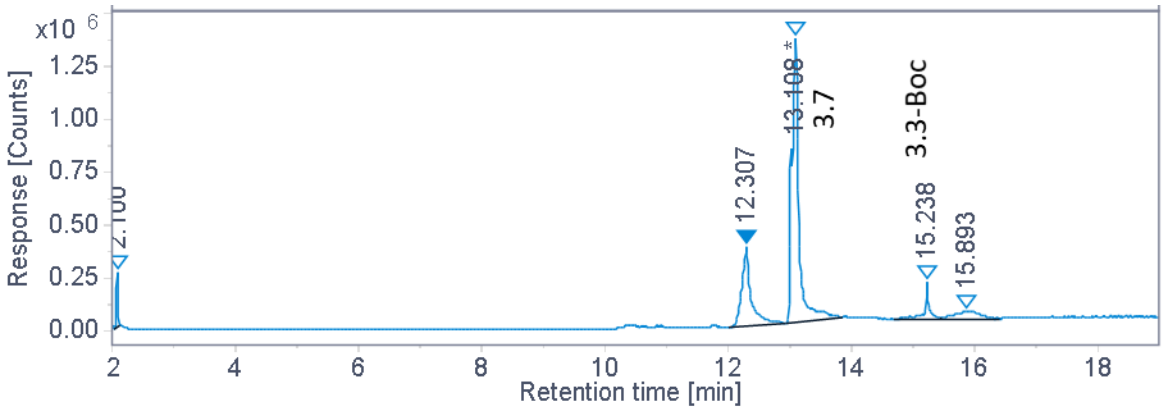


Figure 2.4.2.2.1. Representative GC-MS chromatogram for the synthesis of **3.7-diHx**. Unlabeled peak at 12.31 peak is potentially a degradation product of 3.7-Boc in the inlet.

2.4.2.3 LC-MS Analysis for 3.7-diHX

The same LC-MS method used in Milestone 2 was used for **3.7-diHX** analysis in Milestone 4 (Section 2.1.2.2). The full method is included in Appendix 3.2 (LCMS_GAN_ComInt_v1).

2.4.2.4 LC-ELSD Analysis for Counterions of 3.7-diHx

The counterion – chloride or bromide – was assayed via LC with an evaporative light scattering detector (ELSD). This method (Salts_HILIC-ELSD_v5) utilizes an Agilent Poroshell 120 HILIC-Z (3.0 x 150 mm, 2.7 µm) column held at 30 °C with a mobile phase flow rate of 0.8 mL/min. A binary gradient is used for elution using aqueous 10 mM ammonium acetate buffer at pH 4.0 (A) and 90% acetonitrile:10% aqueous 100 mM ammonium acetate buffer at pH 4.0 (B). Initial conditions were set to 90% B held for 1.0 min, followed by a 5 min ramp to 80% B. Immediately, the mobile phase was again ramped over 5 min to reach 20% B, which was then held for 4 min. A 3.0 min post run equilibration time was used. It should be noted that a quadratic fit is used for all LC-ELSD calibration curves.

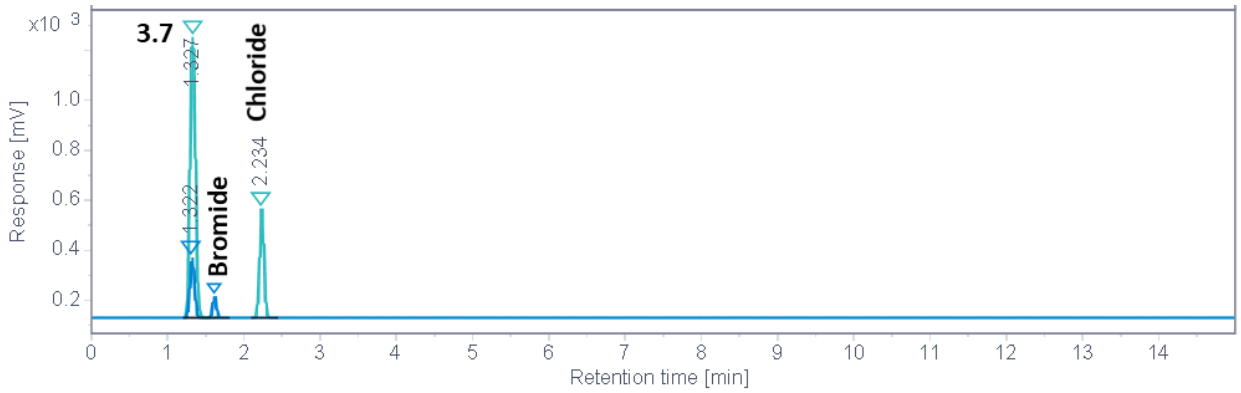


Figure 2.4.2.4.1. Representative ELSD chromatogram from Salts_HILIC-ELSD_v5 method for measuring counterions

When **3.7** precipitates from the reaction as a salt, it does so at a 1:2 stoichiometric ratio of **3.7**: counter ion. This corresponds to 19.9% or 35.8% by weight of chloride or bromide, respectively, in the corresponding **3.7-diHX** molecule. As shown in Figure 2.4.2.4.2, a range of 0.2 – 1.0 mg/mL gives a suitable calibration curve for both chloride and bromide. For analysis, samples are prepared at approximately 2.0 mg/mL in a 3:1 acetonitrile: water diluent which gives theoretical values of 0.4 mg/mL and 0.7 mg/mL for chloride and bromide, respectively.

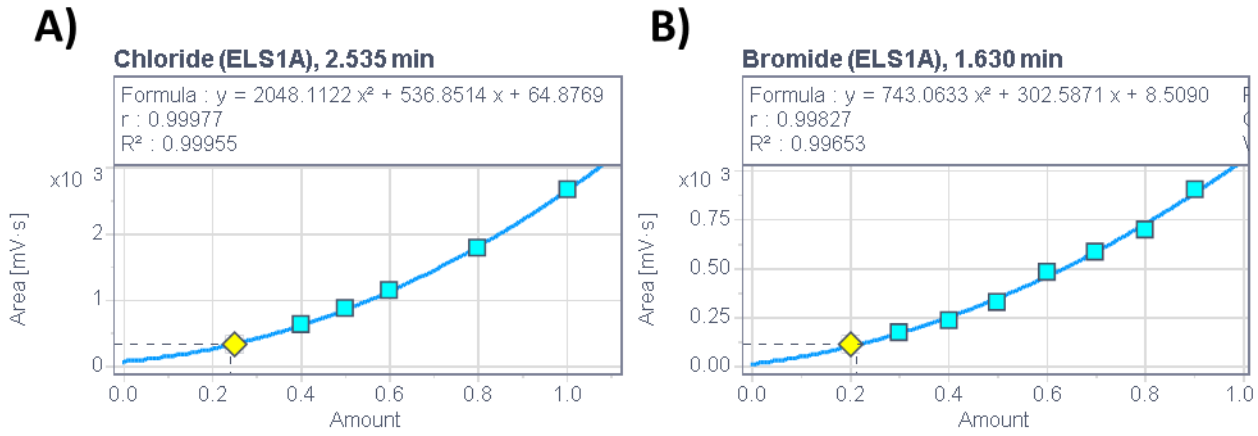


Figure 2.4.2.4.2. A) Chloride and B) bromide calibration curves in the relevant operating range

2.4.3 Impurities for 3.7-diHX

2.4.3.1 Starting Material Impurities for 3.7-diHX

As described in 2.3.3.2, ***iso*-3.3-Boc,** a regioisomer of the **3.3-Boc** starting material, is generated during its synthesis. This is efficiently purged in the workup of that synthesis. GC-MS was used to confirm its absence in the **3.3-Boc** starting material

2.4.3.2 Synthesis Impurities for 3.7-diHX

The first step of Milestone 4 to form **3.7-Boc** produces several minor impurities. LC-MS reveals multiple high molecular weight products suggestive of dimerization-like side reactions; however, no impurities were definitively identified or isolated. These impurities were effectively purged through an optimized workup procedure to give LC weight percent assay values of >98%. The subsequent deprotection of **3.7-Boc** and salt formation to generate **3.7-diHX** proceeded cleanly. No impurities were identified that required characterization.

2.4.4 Forced Degradation Studies for 3.7-diHX

Forced degradation studies were not performed for **3.7-diHX** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.4.5 Stability Testing for 3.7-diHX

Stability studies were not performed for **3.7-diHX** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.4.6 Methods for 3.7-diHX

All methods used for Milestone 4 are described in detail in the Appendix.

2.4.6.1 Key Starting Materials for 3.7-diHX

The key starting material, **3.3-Boc**, was analyzed via LC-UV using LCUV_GAN_ComInt_v1 for chromatographic purity and weight percent assay. The absence of ***iso*-3.3-Boc** was confirmed via GC-MS (GCMS_GAN_ComInt_v1).

2.4.6.2 Reagents and Solvents for 3.7-diHX

All reagents and solvents for Milestone 4 were used based on the vendor-supplied certificates of analysis (CoA). No specific methods were developed in-house.

2.4.6.3 Intermediates for 3.7-diHX

The intermediate, **3.7-Boc**, was characterized by LC-UV for chromatographic purity and for weight percent assay against a reference standard that was characterized in-house for purity.

2.4.6.4 In-Process Controls (IPC) for 3.7-diHX

In-process reaction progress was analyzed by LC-UV (LCUV_GAN_ComInt_v1). Reaction mixtures were diluted in methanol and analyzed.

2.4.6.5 Final Product Analysis for 3.7-diHX

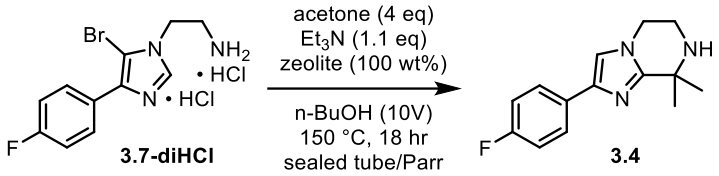
The final product, **3.7-diHX**, was characterized via LC-UV for chromatographic purity as well as weight percent assay of **3.7** using a reference standard whose purity was characterized in-house. The counterion – chloride or bromide – was quantified via LC-ELSD (Salts_HILIC-ELSD_v5) against a commercially sourced standard. Moisture content was quantified via Karl Fischer titration.

2.4.6.6 Method Appropriateness for 3.7-diHX

During development of the LCUV_GAN_ComInt_v1 method, certain performance characteristics were evaluated to select analytical conditions. These results are described above and include linearity. This method was not tested for specificity. Method validation was not performed. Specificity and validation were not included in the scope of this work.

2.5 3.7-diHCl to 3.4 (Milestone 5)

The preparation of **3.4**, the final molecule in the report, is accomplished via a Pictet-Spengler reaction as shown in Scheme 2.5.1 where **3.7-diHX** is reacted with acetone and an acid catalyst.



Scheme 2.5.1. Conversion of **3.7-diHCl** to **3.4** via Pictet-Spengler reaction.

2.5.1 Pharmacopoeia Methods for **3.4**

Neither compendial methods nor monographs from the United States Pharmacopoeia and/or the European Pharmacopeia are available for **3.4**.

2.5.2 Method Development for **3.4**

*2.5.2.1 In Process and Assay Analysis for **3.4***

The same in process method used in Milestone 1 was used for **3.4** analysis in Milestone 5 (Section 2.1.2.1) The full method is included in Appendix 3.1 (LCUV_GAN_ComInt_v1). Figure 2.5.2.1.1 is a representative chromatogram for the starting material, intermediate and product analyzed in the current method.

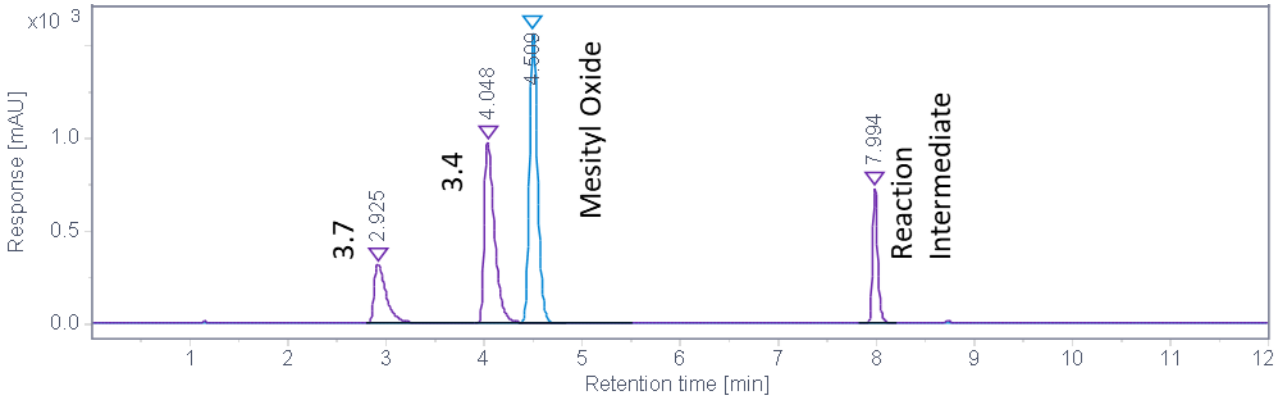


Figure 2.5.1.1. Representative chromatogram at 234 nm (top) and 254 nm (bottom) for Milestone 5 showing **3.7-diHX** and **3.4** as well as a reaction intermediate.

2.5.2.1.1 Relative Response Factors for 3.4

Relative response factors (RRFs) were subsequently determined for starting materials and each available known impurity using the method put forth in Section 2.1.2.1 (Figure 2.5.2.1.1.1, Table 2.1.2.1.1). Impurities were synthesized and purified in-house (refer to GFN-002-PDR). Samples were prepared at 1 mg/mL in acetonitrile and relative response factors were calculated using Equation 2.1.2.1.1. Table 2.5.2.1.1.1 lists the approximate retention times and RRFs (based on mg/mL and M basis) for each analyte.

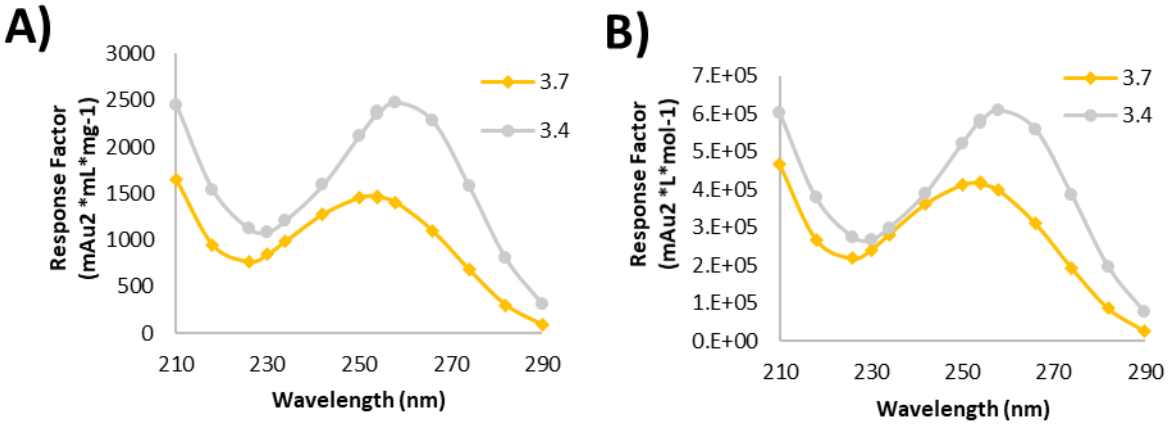


Figure 2.5.2.1.1.1. A) Mass-based and B) molarity based response factors across the collected UV spectrum for **3.7** (free base) and **3.4**.

Table 2.5.2.1.1.1. Relative response factors for starting material and product, **3.7** and **3.4**, in milestone 5

Compound	Retention Time (min)	RRF (mg/mL)		RRF (M)	
		234 nm	254 nm	234 nm	254 nm
3.7 (free base)	2.74	0.81	0.62	0.94	0.72
3.4	3.97	1.00	1.00	1.00	1.00
Reaction Intermediate	8.00	*Not determined*			
Mesityl Oxide	4.51	*Not determined*			

2.5.2.1.2 Linearity for **3.4**

For the starting material and product, the upper and lower limits of linearity (UoL and LoL) were determined. UoL is defined as the highest concentration at which the peak response gave a linear response with respect to concentration (Figure 2.5.2.1.2.1). In this work, the LoL is defined as the limit of quantitation (LoQ) or the lowest calibration point analyzed, whichever is higher (Table 2.5.2.1.2.1).

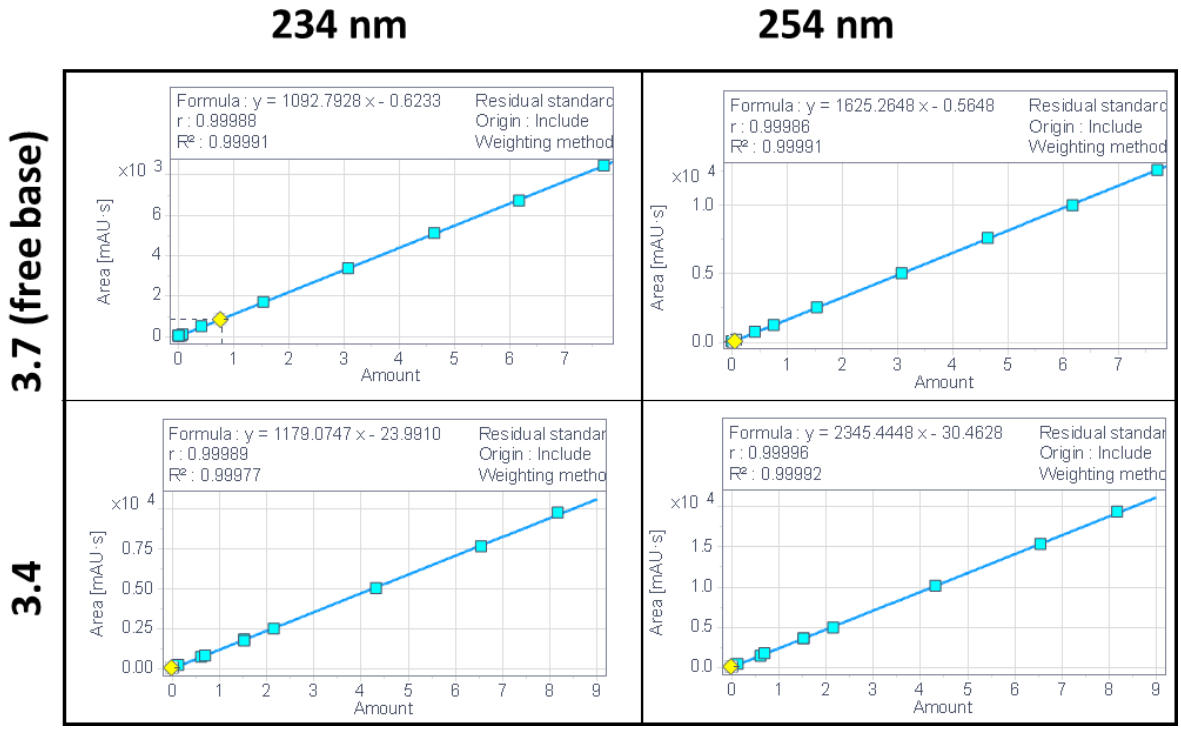


Figure 2.5.2.1.2.1. Calibration curves for starting material (**3.7**) and product (**3.4**) in Milestone 5 at 226 nm and 254 nm

Table 2.5.2.1.2.1. Quantitative metrics for **3.7**, **3.4** and the reaction intermediate at 234 nm and 254 nm

Compound	Wavelength	Upper Limit of Linearity	Lower Limit of Linearity	Limit of Detection	Limit of Quantitation
3.7 (free base)	234 nm	7.73 mg/mL	0.0073 mg/mL	0.0022 mg/mL	0.0073 mg/mL
	254 nm	7.73 mg/mL	0.0011 mg/mL	0.0032 mg/mL	0.0011 mg/mL
3.4	234 nm	8.20 mg/mL	0.0024 mg/mL	0.00073 mg/mL	0.0024 mg/mL
	254 nm	8.20 mg/mL	0.0054 mg/mL	0.0016 mg/mL	0.0054 mg/mL
Reaction intermediate	234 nm	Not determined	Not determined	0.0018 mg/mL	0.00054 mg/mL
	254 nm	Not determined	Not determined	0.00038 mg/mL	0.0013 mg/mL

2.5.2.1.3 Limits of Detection (LOD) for **3.4**

Statistical limits of detection and quantitation were determined for starting material **3.**7 and product **3.4** as well as a reaction intermediate using a calibration curve limited to a lower concentration range. Limit of detection and quantitation were calculated using eqns. 2.1.2.1.3.1 and 2.1.2.1.3.2. The calculated values are listed in Table 2.5.2.1.2.1.

*2.5.2.2 GC-MS Analysis for **3.4***

The same GC-MS method used in Milestone 1 was used for **3.4** analysis in Milestone 5 (Section 2.1.2.2). The full method is included in Appendix 3.2 (GCMS_GAN_ComInt_v1). Figure 2.5.2.2.1 is a representative chromatogram for the starting material, intermediate, impurities and product analyzed in the current method.

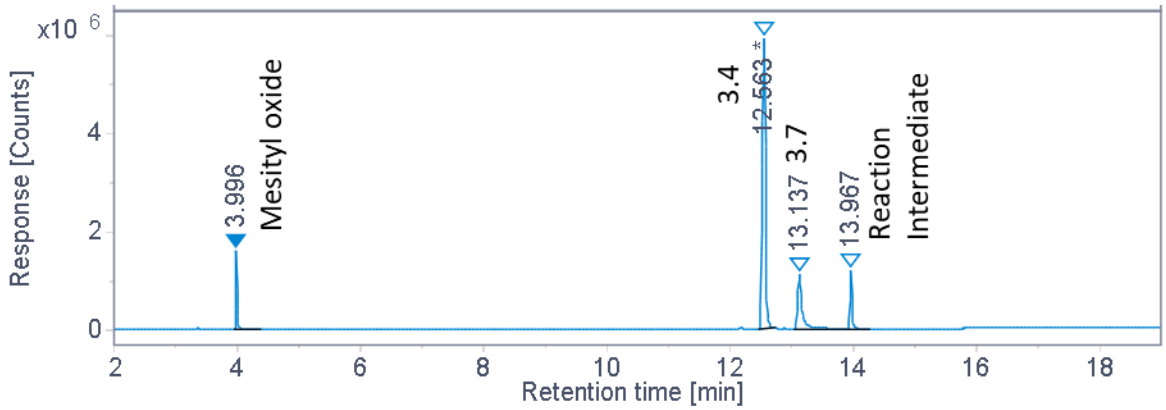


Figure 2.5.2.2.1. Representative chromatogram for the synthesis of **3.4**

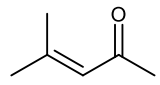
*2.5.2.3 LC-MS Analysis for **3.4***

The same LC-MS method used in Milestone 2 was used for **3.4** analysis in Milestone 5 (Section 2.1.2.2). The full method is included in Appendix 3.2 (LCMS_GAN_ComInt_v1).

2.5.3 Impurities for 3.4

*2.5.3.1 Synthesis Impurities for **3.4***

Two impurities have been identified in the synthesis of **3.4** from **3.7-diHX** as shown in Figure 2.5.3.2.1. Mesityl oxide is formed by an aldol self-condensation of the acetone reagent. This gives a significant UV signal in the LC-UV, but does not consume or otherwise interact with **3.7-diHX** or **3.4**.



Mesityl oxide
Exact Mass: 98.07

Figure 2.5.3.2.1. Impurities identified in milestone 5

*2.5.3.2 Starting Material Impurities for **3.4***

Starting material **3.7-diHX** is obtained in high purity from Milestone 4. No impurities have been identified or characterized.

2.5.4 Forced Degradation Studies for 3.4

Forced degradation studies were not performed for **3.4** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.5.5 Stability Testing for 3.4

Stability studies were not performed for **3.4** nor its starting materials, intermediates and impurities. (Not in the scope of this work.)

2.5.6 Methods for 3.4

All methods used are described in detail in the Appendix.

2.5.6.1 Key Starting Materials for 3.4

The characterization of starting material **3.7-diHX** is described in Milestone 4 (2.4.4.5), above.

2.5.6.2 Reagents and Solvents for 3.4

All reagents and solvents for Milestone 5 were used based on the vendor-supplied certificates of analysis (CoA). No specific methods were developed in-house.

2.5.6.3 Intermediates for 3.4

No intermediates were isolated in Milestone 5 for characterization.

2.5.6.4 In-Process Controls (IPC) for 3.4

In-process reaction progress was analyzed by LC-UV (LCUV_GAN_ComInt_v1). Reaction mixtures were diluted in methanol and analyzed.

2.5.6.5 Final Product Analysis for 3.4

The final product **3.4** was analyzed by LC-UV (LCUV_GAN_ComInt_v1) for both chromatographic purity and weight percent assay. For the latter, the sample was quantified using

a reference standard whose purity was characterized in-house. Water content was determined via Karl Fisher Titration.

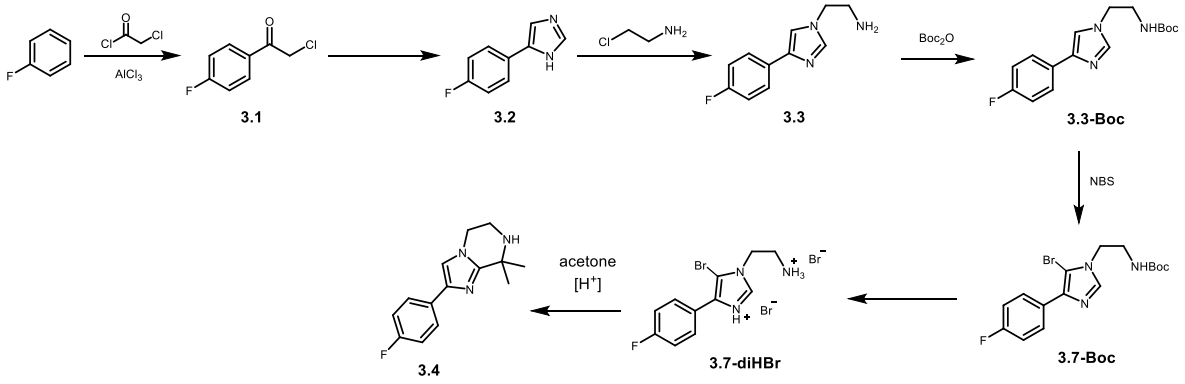
*2.5.6.6 Method Appropriateness for **3.4***

During development of the LCUV_GAN_ComInt_v1 method, certain performance characteristics were evaluated to select analytical conditions. These results are described above and include linearity. This method was not tested for specificity. Method validation was not performed. Specificity and validation were not included in the scope of this work.

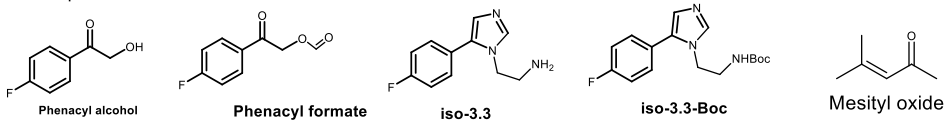
3 Appendix

3.1 LC-UV Method For Assay and Impurities (LCUV_GAN_ComInt_v1)

Structures & IDs:



Known Impurities



Instrument Type: Agilent 1100 liquid chromatograph (LC) with diode array detector (DAD)

Conditions:

Column: Agilent ZORBAX Eclipse XDB-C18, 4.6 x 250 mm, 5 µm

Mobile Phase A: 25 mM potassium phosphate – pH 6.2 ± 0.1

(Add 4.36 g of K_2HPO_4 to 1 L of water. Adjust to pH 6.2 ± 0.1 with phosphoric acid)

Mobile Phase B: Acetonitrile

Injection volume: 1 µL Column temp: 30 °C Flow rate: 2.0 mL/min

Detector wavelength(s): 254 nm (Milestone 1, monitoring and comparison), 234 nm (Milestones 2, 3, 5), 226 nm (Milestone 4)

LC Gradient Table:

Time (min)	%A	%B
0	70	30
4.5	70	30
7.5	30	70
12	30	70

Post-run equilibration: 4 min

Sample preparation:

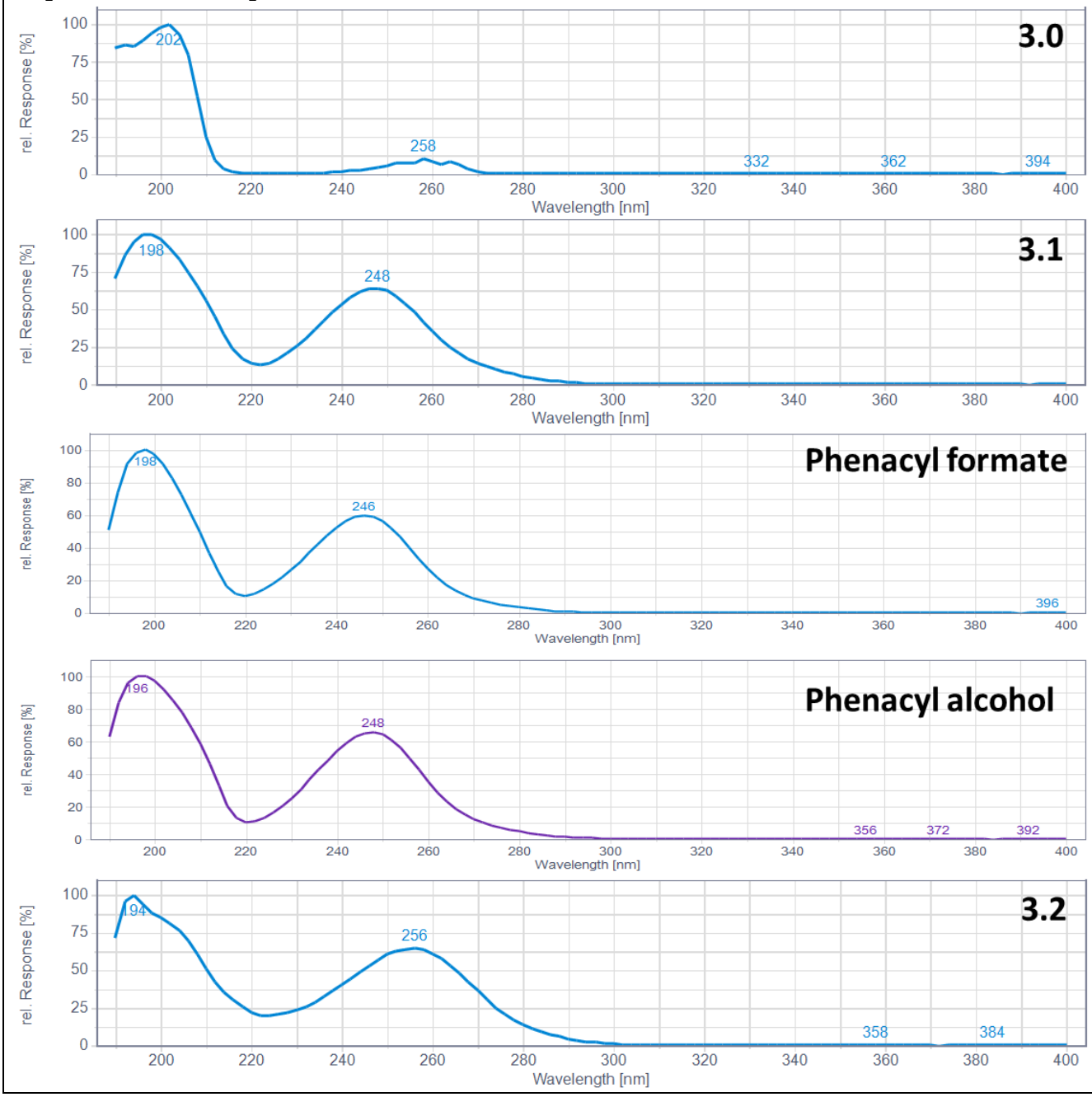
Prepare samples at approximately 5 mg/mL in a suitable diluent. Recommended acetonitrile:water in a 3:1 ratio.

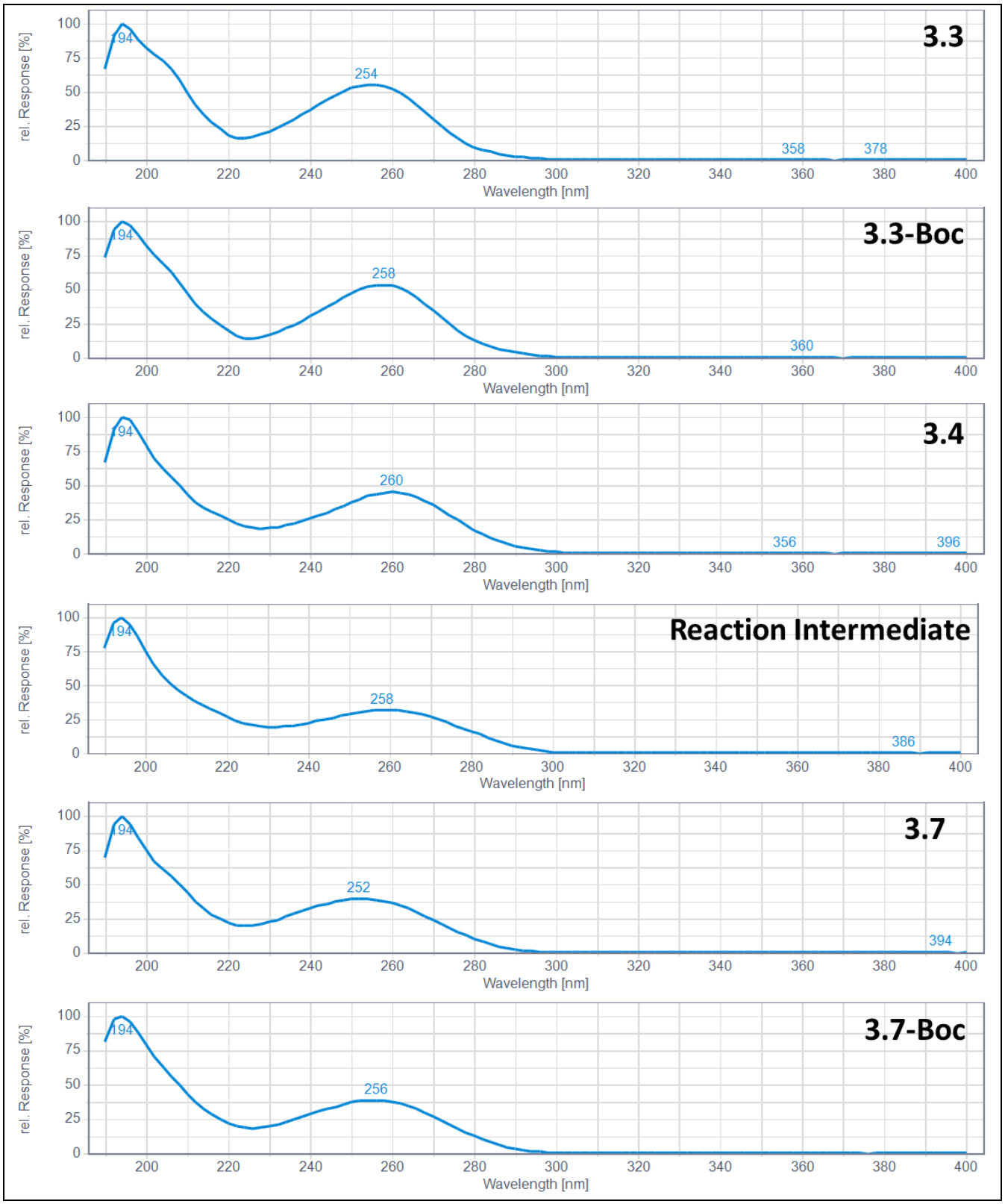
Retention Times								
Compound	Time (min)	RRT	RRF (mg/mL)			RRF (mg/mL)		
			226 nm	234 nm	254 nm	226 nm	234 nm	254 nm
MILESTONE 1								
3.0	8.64	1.05	-	-	0.089	-	-	0.049
3.1	8.24	1.00	-	-	1.00	-	-	1.00
MILESTONE 2								
3.1	8.24	2.50	-	0.70	0.49	-	0.74	0.61
Phenacyl alcohol	2.76	0.84	-	ND	ND	-	ND	ND
Phenacyl formate	6.44	1.96	-	ND	ND	-	ND	ND
3.2	3.29	1.00	-	1.00	1.00	1.00	1.00	1.00
MILESTONE 3								
3.2	3.29	0.41	-	1.82	1.67	-	0.97	0.89
3.3	1.49	0.18	-	1.40	1.24	-	0.94	0.84
3.3-Boc	8.10	1.00	-	1.00	1.00	-	1.00	1.00
MILESTONE 4								
3.3-Boc	8.10	3.24	0.86	-	1.46	0.92	-	1.56
3.7-Boc	9.02	3.61	0.93	-	0.99	1.25	-	1.34
3.7 (free base)	2.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MILESTONE 5								
3.7 (free base)	2.50	0.64	-	0.81	0.62	-	0.94	0.72
Mesityl oxide	4.00	1.03	-	ND	ND	-	ND	ND
3.5	7.94	2.04	-	ND	ND	-	ND	ND
3.4	3.9	1.00	-	1.00	1.222	-	1.00	1.00

Notes:
ND = Not determined

$$*Relative\ RF\ (RRF) = \frac{\left(Analyte\ 1\ Peak\ Area / Analyte\ 1\ Conc\right)}{\left(Product\ Peak\ Area / Product\ Conc\right)}$$

Representative UV Spectra




3.3
rel. Response [%]
100
75
50
25
0
194
254
358
378
200
220
240
260
280
300
320
340
360
380
400
Wavelength [nm]
3.3-Boc
194
258
360
3.4
194
260
356
396
Reaction Intermediate
194
258
386
3.7
194
252
394
3.7-Boc
194
256

3.2 GC-MS Method for Impurities (GCMS_GAN_ComInt_v1)

Conditions:

Column: HP-5MS; 30M X 0.250 mm; 0.25 µM film

Inlet Pressure: 6.7 psi | Split Ratio: 100:1 | Split Flow: 91.8 mL/min

Column flow: 0.92 mL/min | Injection Temp: 250 °C | Injection volume: 1 µL

Solvent Delay: 3 min | Runtime: 19 min

Temperature Program:

Temp (°C)	Ramp (°C/min)	Hold (min)
50	0	3
250	25	3
300	25	3

MS Parameters:

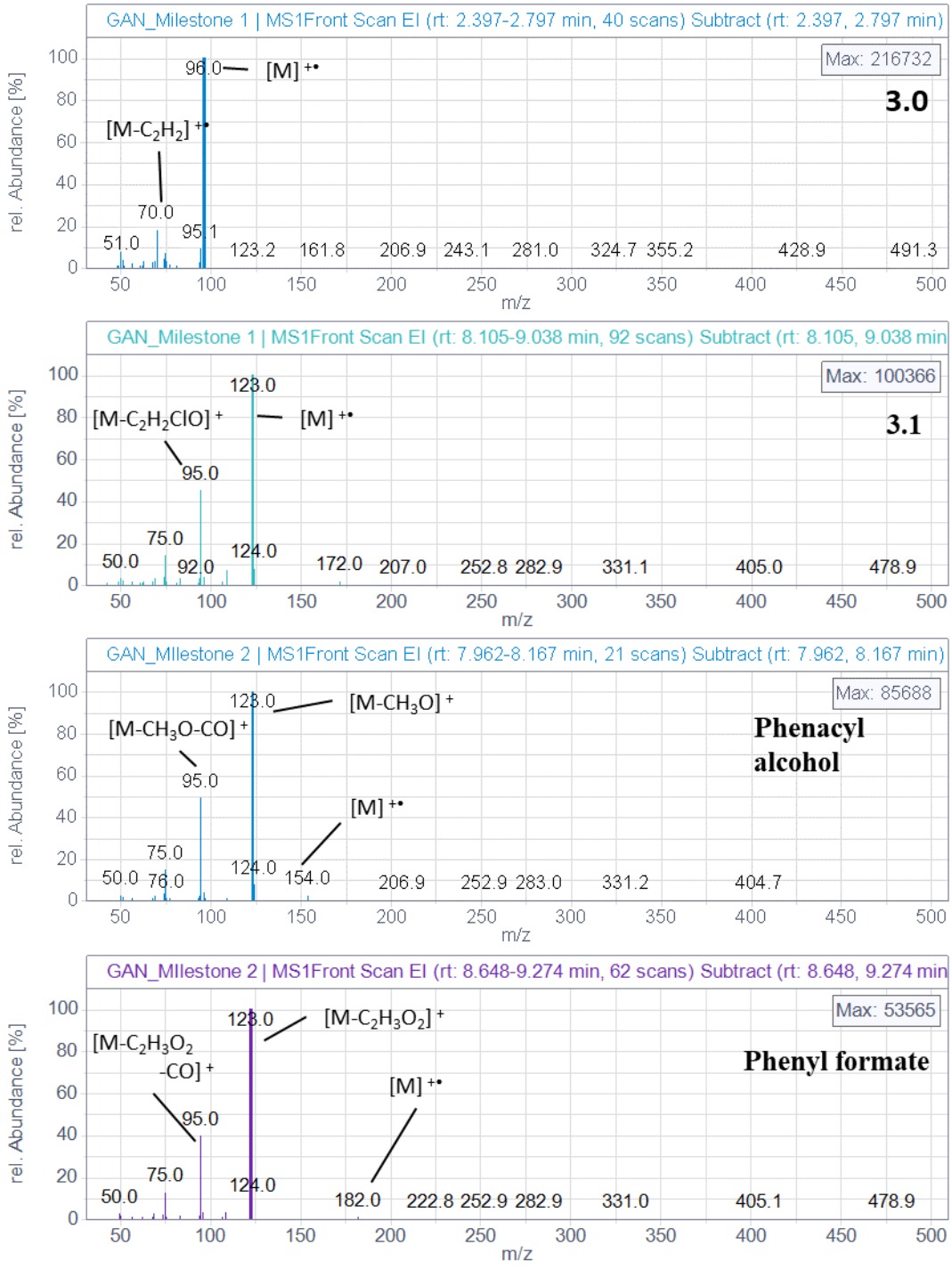
Transfer Line Temp (°C)	250
Source Temp (°C)	230
Quad Temp (°C)	150
Electron Energy (eV)	70

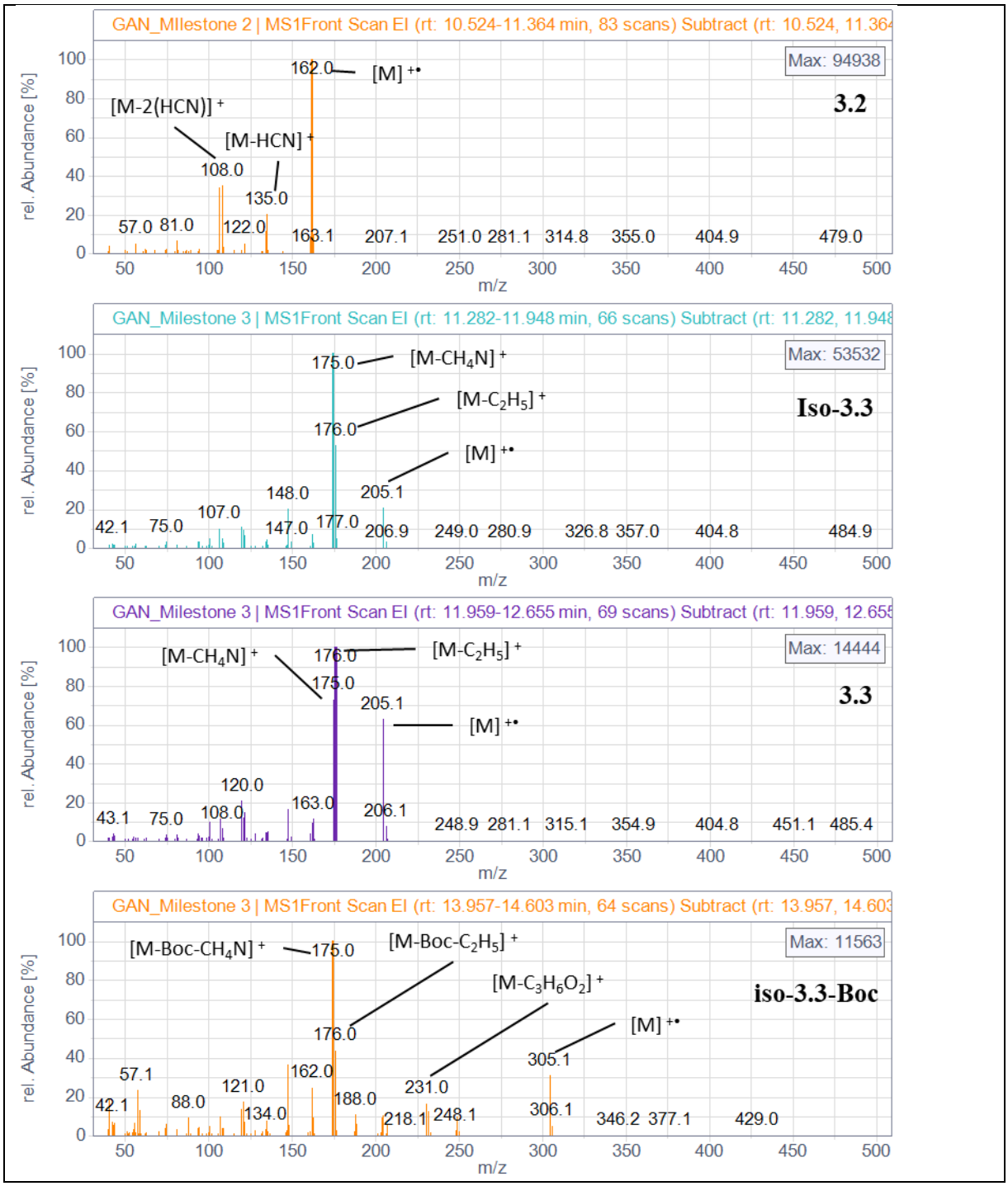
Sample preparation: Diluted in MeCN:H_2O (75:25)

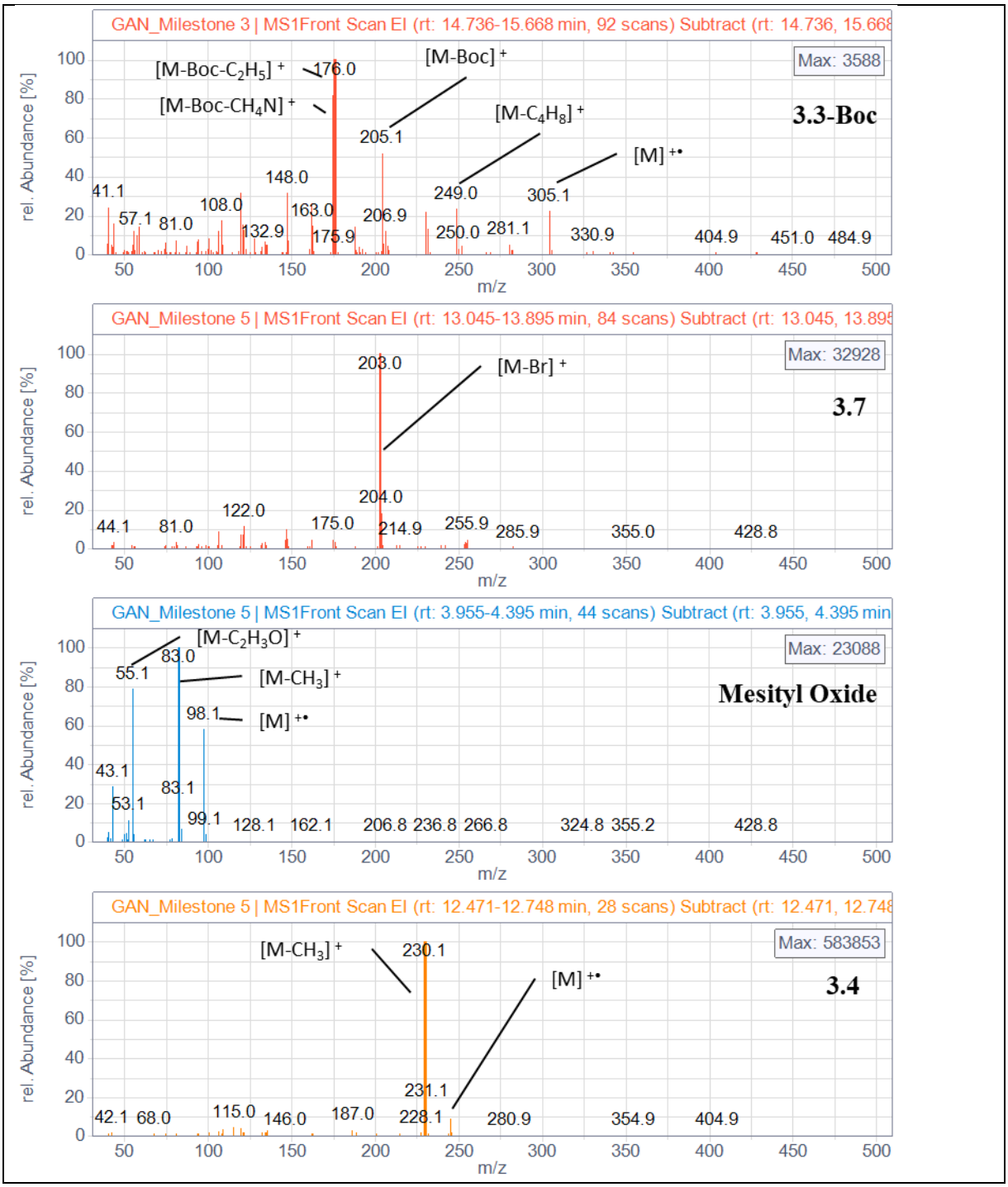
Retention Times			
Compound	***m/z***	**Time (min)**	**RRT**
MILESTONE 1			
3.0	96	2.45	0.30
3.1	172, 123, 95	8.21	1.00
MILESTONE 2			
3.1	172, 123, 95	8.21	0.77
Phenacyl alcohol	154, 123	8.01	0.76
Phenacyl formate	182, 123	8.71	0.82
3.2	162, 135, 107	10.60	1.00
MILESTONE 3			
3.2	162, 135, 107	10.60	0.70
iso-3.3	205, 176	11.34	0.74
3.3	205, 176	12.00	0.79
iso-3.3-Boc	305, 175	14.05	0.92
3.3-Boc	305, 249, 232, 176	15.24	1.00
MILESTONE 4			
3.3-Boc	305, 249, 232, 176	15.24	0.86
3.7-Boc*	-	-	-
3.7	283, 254, 203, 122	13.14	1.00
MILESTONE 5			
3.7	283, 254, 203, 122	13.14	1.05
Mesityl oxide	98, 83	4.00	0.32
3.4	245, 230	12.56	1.00

*3.7-Boc is not observed in this method, likely due to its high molecular weight and/or potential thermal degradation

Representative GC-MS Spectra




GAN_MIlestone 2 | MS1Front Scan EI (rt: 10.524-11.364 min, 83 scans) Subtract (rt: 10.524, 11.364
Max: 94938
3.2
[M-2(HCN)]+
[M-HCN]+
[M]+•
rel. Abundance [%]
m/z
GAN_Milestone 3 | MS1Front Scan EI (rt: 11.282-11.948 min, 66 scans) Subtract (rt: 11.282, 11.948
Max: 53532
Iso-3.3
[M-CH4N]+
[M-C2H5]+
[M]+•
rel. Abundance [%]
m/z
GAN_Milestone 3 | MS1Front Scan EI (rt: 11.959-12.655 min, 69 scans) Subtract (rt: 11.959, 12.655
Max: 14444
3.3
[M-CH4N]+
[M-C2H5]+
[M]+•
rel. Abundance [%]
m/z
GAN_Milestone 3 | MS1Front Scan EI (rt: 13.957-14.603 min, 64 scans) Subtract (rt: 13.957, 14.603
Max: 11563
iso-3.3-Boc
[M-Boc-CH4N]+
[M-Boc-C2H5]+
[M-C3H6O2]+
[M]+•
rel. Abundance [%]
m/z


GAN_Milestone 3 | MS1Front Scan EI (rt: 14.736-15.668 min, 92 scans) Subtract (rt: 14.736, 15.668
Max: 3588
3.3-Boc
[M-Boc-C2H5] +
[M-Boc-CH4N] +
[M-Boc] +
[M-C4H8] +
[M] +•
176.0
205.1
249.0
305.1
148.0
108.0
163.0
206.9
41.1
57.1
81.0
132.9
175.9
250.0
281.1
330.9
404.9
451.0
484.9
GAN_Milestone 5 | MS1Front Scan EI (rt: 13.045-13.895 min, 84 scans) Subtract (rt: 13.045, 13.895
Max: 32928
3.7
208.0
[M-Br] +
204.0
122.0
44.1
81.0
175.0
214.9
255.9
285.9
355.0
428.8
GAN_Milestone 5 | MS1Front Scan EI (rt: 3.955-4.395 min, 44 scans) Subtract (rt: 3.955, 4.395 min
Max: 23088
Mesityl Oxide
[M-C2H3O] +
[M-CH3] +
[M] +•
83.0
55.1
98.1
43.1
83.1
53.1
99.1
128.1
162.1
206.8
236.8
266.8
324.8
355.2
428.8
GAN_Milestone 5 | MS1Front Scan EI (rt: 12.471-12.748 min, 28 scans) Subtract (rt: 12.471, 12.748
Max: 583853
3.4
[M-CH3] +
[M] +•
230.1
231.1
42.1
68.0
115.0
146.0
187.0
228.1
280.9
354.9
404.9
rel. Abundance [%]
m/z

3.3 LC-MS Method (LCMS_GAN_ComInt_v1)

Instrument Type: Agilent 1260 liquid chromatograph (LC) with diode array detector (DAD) and 6125 mass spectrometer detector (MSD)

Conditions:

Column: Agilent Eclipse XDB C18, 2.1 x 150 mm, 3.5 µm

Mobile Phase A: 15 mM Ammonium acetate (pH = 6.2 ± 0.05)

Mobile Phase B: Acetonitrile

Injection volume: 1 µL Column temp: 30 ºC Flow rate: 0.60 mL/min

Detector wavelength(s): 260 nm (primary), 210 nm (secondary)

LC Gradient Table:

Time (min)	%A	%B
0.0	70%	30%
2.0	70%	30%
4.0	30%	70%
5.0	30%	70%

Post-run equilibration: 3.0 min

MSD Parameters:

Source Parameters			Signal 1 (+)	Signal 2 (-)
Ion Source	ESI	Time Filter	0.02 min	
Gas Temp.	350˚C	Polarity	Positive	Negative
Gas Flow	11.0 L/min	Mass Range (amu)	40 – 1000	40 – 1000
Nebulizer	35.0 psig	Gain Factor	1	1
VCap (+)	4000 V	Fragmentor	15	15
VCap (-)	4000 V	Threshold	0	0

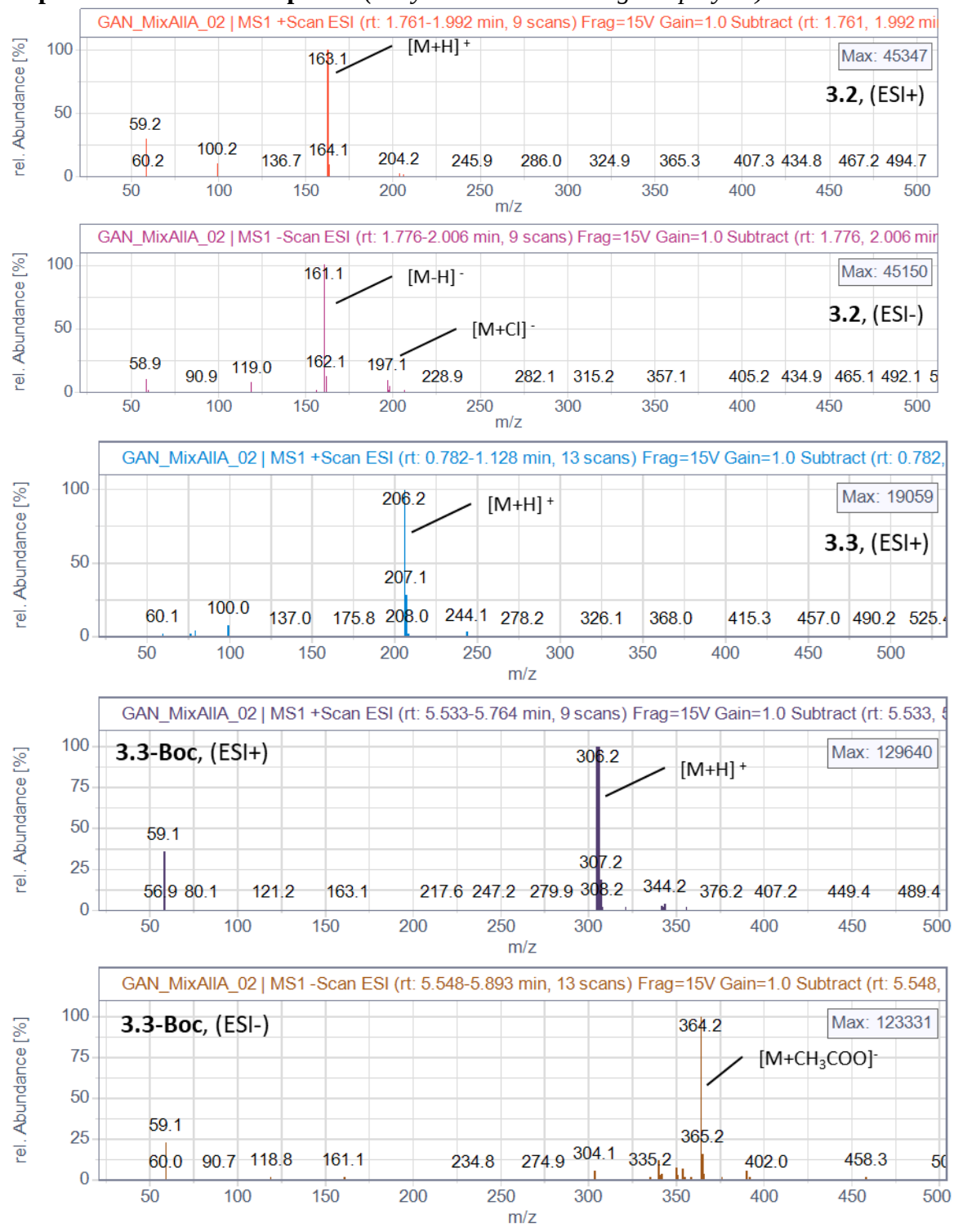
Sample preparation: Prepare sample at a target concentration of 2 – 5 mg/mL in a suitable solvent (i.e., methanol, acetonitrile, water).

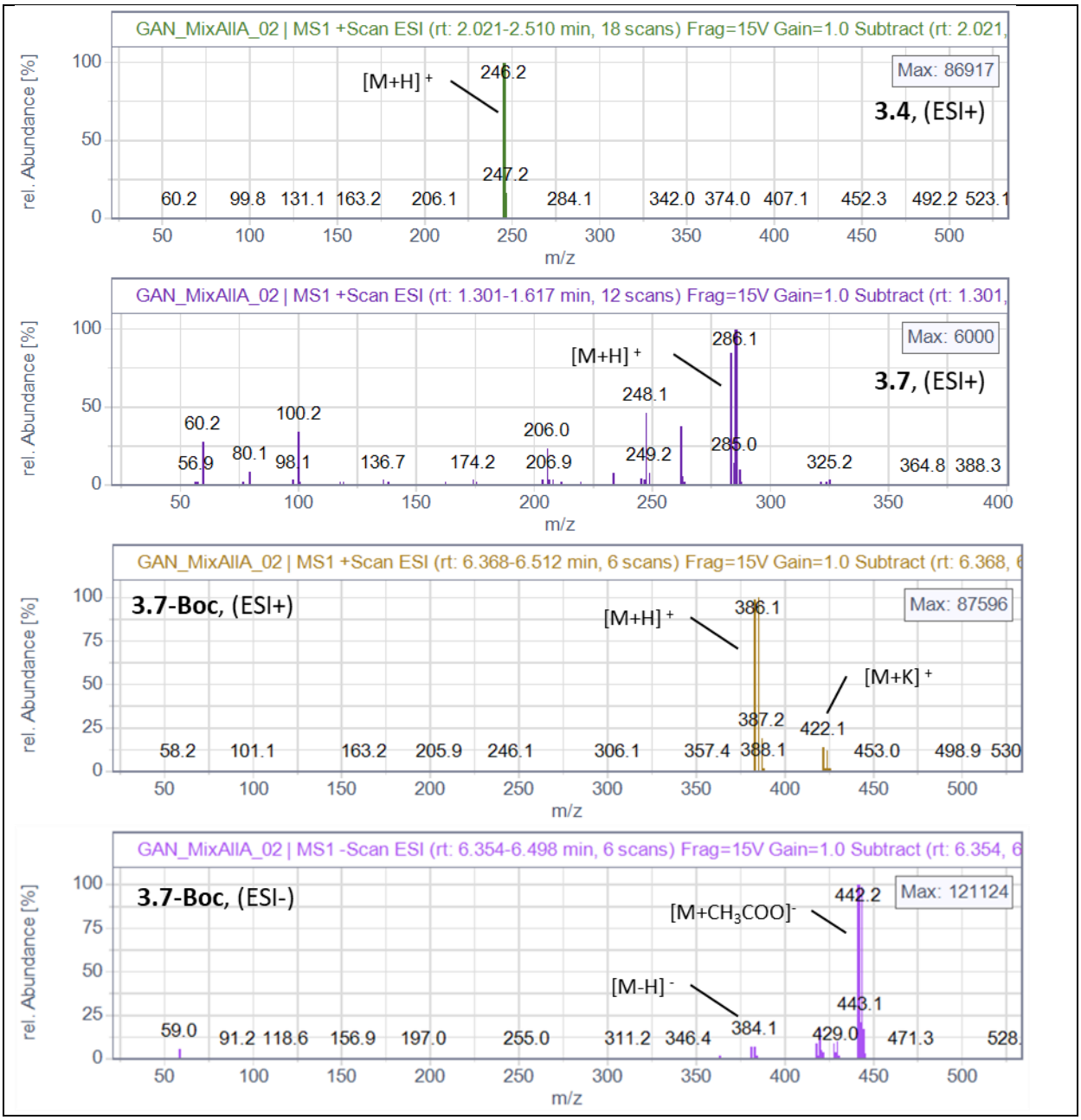
Retention Times

Compound	Time (min)	*m/z* (ionization mode)
3.0	8.05	No ionization
3.1	5.91	No ionization
3.2	1.83	163 (+) 161 (-)
3.3	0.87	206 (+)
3.3-Boc	5.62	306 (+) 364 (-) as an acetic acid adduct
3.4	2.08	246 (+)
3.7	1.35	284 (+)
3.7-Boc	6.40	384 (+) 442 (-) as an acetic acid adduct

Notes: Impurities phenacyl alcohol, phenacyl formate do not ionize in ESI and are therefore excluded from analysis in this method. *Iso-3.3* and *iso*-3.3Boc coelute with 3.3 and 3.3-Boc, respectively, and give identical ionization therefore are indistinguishable in this method.

Representative LC-MS Spectra *(Only relevant mass range displayed.)*




GAN_MixAllA_02 | MS1 +Scan ESI (rt: 2.021-2.510 min, 18 scans) Frag=15V Gain=1.0 Subtract (rt: 2.021,
Max: 86917
3.4, (ESI+)
[M+H]+
246.2
247.2
rel. Abundance [%]
m/z
GAN_MixAllA_02 | MS1 +Scan ESI (rt: 1.301-1.617 min, 12 scans) Frag=15V Gain=1.0 Subtract (rt: 1.301,
Max: 6000
3.7, (ESI+)
[M+H]+
286.1
248.1
285.0
100.2
60.2
206.0
249.2
rel. Abundance [%]
m/z
GAN_MixAllA_02 | MS1 +Scan ESI (rt: 6.368-6.512 min, 6 scans) Frag=15V Gain=1.0 Subtract (rt: 6.368,
3.7-Boc, (ESI+)
Max: 87596
[M+H]+
386.1
[M+K]+
387.2
422.1
rel. Abundance [%]
m/z
GAN_MixAllA_02 | MS1 -Scan ESI (rt: 6.354-6.498 min, 6 scans) Frag=15V Gain=1.0 Subtract (rt: 6.354, 6
3.7-Boc, (ESI-)
Max: 121124
442.2
[M+CH3COO]-
[M-H]-
384.1
443.1
429.0
rel. Abundance [%]
m/z

3.4 LC-ELSD Method For Salt Analysis (Salts-HILIC-ELSD_v5)

Conditions:

Column: Agilent InfinityLab Poroshell 120 HILIC-Z, 3.0 x 150 mm, 2.7 µm
Buffer Solution: 100 mM Ammonium acetate, pH 4.00 ± 0.02
Mobile Phase A: 90% Water + 10% *Buffer Solution*
Mobile Phase B: 90% Acetonitrile + 10% *Buffer Solution*
Injection volume: 1.5 µL Column temp: 30 °C Flow rate: 0.8 mL/min
Detector: ELSD

LC Gradient Table:

Time (min)	%A	%B
0	10%	90%
1	10%	90%
6	20%	80%
11	80%	20%
15	80%	20%

ELSD Parameters:

Evaporator Temp.	30 °C
Nebulizer Temp.	30 °C
Gas Flow Rate (SLM)	1.60
Data Rate (Hz)	80
LED Intensity	100%
Smoothing	30
PMT Gain	1.0

Post-run equilibration: 3 min
Sample preparation: 2mg/mL in MeCN:H_2O in a 3:1 ratio.

Retention Times		
Compound	**Time (min)**	**Operating Range***
3.7	1.3	ND
Bromide	1.6	0.05 – 1.00 mg/mL
Chloride	2.4	0.05 – 1.00 mg/mL
Sodium	4.0	ND

Notes: *The ELSD response is non-linear. A quadratic fit is used for calibration curves. Operating range indicates the concentration range over which the accuracy of a quadratic calibration curve has been established

ND = Not determined. These compounds are not quantified with this method

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5 References

(1) *Phase 3 clinical trial started for the first malaria treatment combining three drugs*. Medicines for Malaria Venture. https://www.mmv.org/newsroom/news-resources-search/phase-3-clinical-trial-started-first-malaria-treatment-combining (accessed 2025-10-03).

(2) *UNAIDS*. https://www.unaids.org/en/Homepage (accessed 2025-10-02).

(3) *The United States President's Emergency Plan for AIDS Relief - United States Department of State %*. United States Department of State. https://www.state.gov/pepfar/ (accessed 2025-10-02).

(4) Anderer, S. WHO Recommends Lenacapavir for HIV Prevention in New Guidelines. *JAMA* **2025**, *334* (9), 757. https://doi.org/10.1001/jama.2025.10988.

(5) Segal-Maurer, S.; DeJesus, E.; Stellbrink, H.-J.; Castagna, A.; Richmond, G. J.; Sinclair, G. I.; Siripassorn, K.; Ruane, P. J.; Berhe, M.; Wang, H.; Margot, N. A.; Dvory-Sobol, H.; Hyland, R. H.; Brainard, D. M.; Rhee, M. S.; Baeten, J. M.; Molina, J.-M. Capsid Inhibition with Lenacapavir in Multidrug-Resistant HIV-1 Infection. *N. Engl. J. Med.* **2022**, *386* (19), 1793–1803. https://doi.org/10.1056/NEJMoa2115542.

(6) Nagle, A.; Wu, T.; Kuhen, K.; Gagaring, K.; Borboa, R.; Francek, C.; Chen, Z.; Plouffe, D.; Lin, X.; Caldwell, C.; Ek, J.; Skolnik, S.; Liu, F.; Wang, J.; Chang, J.; Li, C.; Liu, B.; Hollenbeck, T.; Tuntland, T.; Isbell, J.; Chuan, T.; Alper, P. B.; Fischli, C.; Brun, R.; Lakshminarayana, S. B.; Rottmann, M.; Diagana, T. T.; Winzeler, E. A.; Glynne, R.; Tully, D. C.; Chatterjee, A. K. Imidazolopiperazines: Lead Optimization of the Second-Generation Antimalarial Agents. *J. Med. Chem.* **2012**, *55* (9), 4244–4273. https://doi.org/10.1021/jm300041e.

(7) Chatterjee, A. K.; Nagle, A.; Wu, T.; Tully, D.; Kuhen, K. L. Compounds and Compositions for the Treatment of Parasitic Diseases. WO2011006143A2, January 13, 2011. https://patents.google.com/patent/WO2011006143A2/en (accessed 2025-10-02).

(8) Von Keutz, T.; Cantillo, D.; Kappe, C. O. Continuous Flow Synthesis of Terminal Epoxides from Ketones Using in Situ Generated Bromomethyl Lithium. *Org. Lett.* **2019**, *21* (24), 10094–10098. https://doi.org/10.1021/acs.orglett.9b04072.

(9) US EPA, O. *Biden-Harris Administration Finalizes Ban on Most Uses of Methylene Chloride, Protecting Workers and Communities from Fatal Exposure*. https://www.epa.gov/newsreleases/biden-harris-administration-finalizes-ban-most-uses-methylene-chloride-protecting (accessed 2025-10-02).