The Degradation of 4-Morpholinoaniline in Aqueous Solution

By

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Abstract

The reactivity of 4-morpholinoaniline with water was examined in aqueous solutions at pH 1 – 4. Two dissociation constant values were identified for 4-morpholinoaniline using spectrophotometric titration: pK_{a1}, which was assigned to the aniline ammonia nitrogen, was 1.8 and pK_{a2}, assigned to the morpholine nitrogen, was 4.9. At pH < pK_{a1}, 4-morpholinoaniline reacted with water to form 4-morpholinophenol, which further degraded to hydroquinone. The hydrolytic degradation reactions of 4-morpholinoaniline to 4-morpholinophenol and 4-morpholinophenol to hydroquinone both exhibited pseudo-first order kinetics under these reaction conditions. 4-Morpholinophenol is proposed to form by nucleophilic aromatic substitution of the aniline ammonia by water via an intermediate whose electron-rich aromatic ring is electrostatically stabilized by the positive charge on the morpholino nitrogen and the positive charge on the incoming nucleophilic water molecule. As pH exceeded pK_{a1} and the aniline ammonia nitrogen was deprotonated, the nucleophilic aromatic substitution reaction was disfavored and accounted for less than 10% of 4-morpholinoaniline degradation at pH ≥ 2.5. Instead, 4-Morpholinoaniline degradation in these higher pH aqueous solutions yielded multiple degradation products, and the kinetics of 4-morpholinoaniline degradation were more complicated than zero-, first-, or second-order. Characterization and structural identification of the major degradation product observed in these higher pH solutions yielded a highly conjugated molecule that appears to be a dimerization product. Without identification of intermediates or additional degradation products observed by chromatography, the mechanism by which this product forms could not be resolved; however, metal catalysis and direct reaction of 4-morpholinoaniline with 4-morpholinophenol were disproven.

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I would like to thank my many colleagues at Gilead Sciences who supported me and contributed their expertise to this project. Specifically, I offer my sincerest gratitude to Eugene Pan for analytical expertise and for performing NMR experiments, and to Brian Wiest for training me on the QTOF and MassHunter software. I am also grateful to Sandy Koppenol, Sarina Ma, Roshy Pakdaman, and Reza Oliyai for being amazing mentors and managers, for helping me develop professionally, and for believing in me.

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Introduction

Background

In recent years, deregulation of kinase function has been implicated in cancer and in disorders of the immunologic, neurologic, and metabolic systems. In healthy cells, these kinases initiate or perpetuate a cellular signal through the binding of ATP and subsequent catalysis of the transfer of the terminal phosphate of ATP to a serine, threonine, or tyrosine residue on a substrate protein in a signaling cascade. When these kinases undergo a genetic mutation or translocation, they become impervious to the cell's normal regulatory mechanisms, leading to constitutive phosphorylation activity and up-regulated cell signaling. The identification of this faulty kinase activity in the development and perpetuation of disease has generated considerable interest in protein kinases as drug targets, leading to the development of small molecule kinase inhibitors as therapeutic agents.[1] The majority of these inhibitors competitively inhibit ATP binding by inhabiting the kinase's highly conserved ATP binding cleft and forming key hydrogen bonds with a portion of the kinase known as the hinge, which connects the amino- and carboxy-terminal kinase domains. Due to the high-profile success of small molecule kinase inhibitors such as imitanib in the treatment of previously fatal cancers, an estimated 20% of efforts in the pharmaceutical industry are dedicated to the discovery and development of this class of small molecule therapeutics, targeting approximately 30 distinct kinase targets.[2] In fact, the FDA approved 15 new small molecule kinase inhibitors between January 2012 and February 2015.[1]

The Gilead Sciences, Inc. pipeline includes small molecule kinase inhibitors for the treatment of a variety of diseases. This discussion will focus on two SMKI's in Gilead's

oncology pipeline. Momelotinib (Figure 1) is a JAK-1 inhibitor in clinical trials for the treatment of myelofibrosis and Entospletinib (Figure 2) is a SYK inhibitor under evaluation for treatment of hematological malignancies such as acute myeloid leukemia. Common to both of these compounds is a 4-morpholinoaniline group (Figure 3), which is included for its ability to form hydrogen bonds between the aniline nitrogen and the hinge in the kinase ATP binding cleft and for the improved pharmacokinetics imparted by the morpholine component.[3, 4] These favorable characteristics have led to the inclusion of 4-morpholinoaniline in the structures of other JAK2 inhibitors in preclinical and clinical studies.[5, 6]

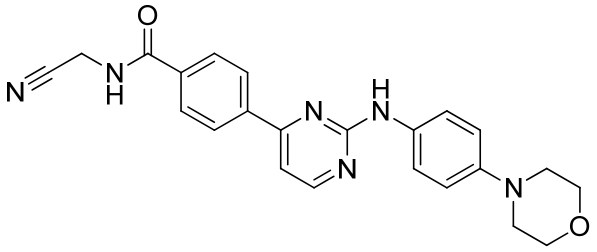


Figure 1. Momelotinib, a Gilead Sciences, Inc. small molecule JAK-1 inhibitor developed for the treatment of myelofibrosis

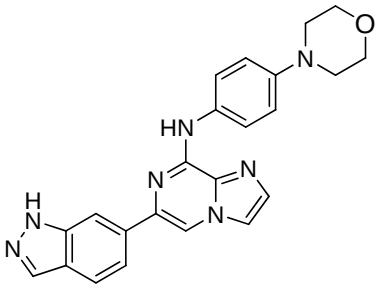


Figure 2. Entospletinib, a Gilead Sciences, inc. SYK inhibitor in evaluation for treatment of hematological malignancies

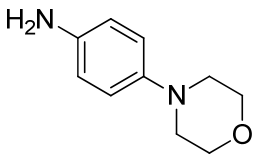


Figure 3. Chemical structure of 4-morpholinoaniline

Although the 4-morpholinoaniline structural motif was found to be important in improving pharmacokinetics and in promoting specificity for the ATP binding site of the target kinases, this chemical moiety has been implicated in complicated chemical degradation pathways observed for both molecules. Because both molecules were developed as acidic salts, the microenvironment experienced by these molecules is expected to have an acidic pH, which may contribute to the observed chemical degradation. Elucidation of these low pH degradation pathways is critical to understanding the drug substance and drug product degradation products of kinase inhibitors that contain the 4-morpholinoaniline structural motif. The detailed knowledge of the kinetics and mechanisms of the degradation reaction can also help inform the manufacturing process and storage conditions that control or prevent formation of these degradation products during drug development of the kinase inhibitors.

Despite its benefit in the design and synthesis of kinase inhibitors, 4-morpholinoaniline itself has not been a subject of extensive characterization, and literature about its degradation behavior in aqueous solution is limited to electrochemical oxidation studies primarily conducted using cyclic voltammetry.[7, 8]

Purpose

The work described herein was performed to determine the mechanism or mechanisms of 4-morpholinoaniline degradation in aqueous solution as a function of pH through the characterization of degradation kinetics and degradation products.

Experimental Materials and Methods

Materials

4-morpholinoaniline and authentic standards of proposed degradation products were used without additional purification. 4-Morpholinoaniline (≥ 98%) was purchased from Ubichem (Reddich, UK) and from VWR International (Radnor, PA); 4-(4-morpholinyl)phenol (≥ 97%), henceforth called 4-morpholinophenol, was purchased from Amatek Chemical (Zhangjiagang, China); and hydroquinone (≥ 99%) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). The following chemicals were used in preparation of buffers for kinetic and degradation studies: OmniTrace™ hydrochloric acid (34-37%, high purity) with controlled levels of iron and copper (<1 ppb each) and sodium chloride (reagent grade) were sourced from EMD Millipore (Burlington, MA); glacial acetic acid was sourced from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ); sodium acetate was sourced from Sigma-Aldrich, Inc.; cyanoacetic acid (99%) and methoxyacetic acid (98%) were sourced from Aldrich Chemical (Milwaukee, WI); and diethylenetriaminepentaacetic acid (99%), henceforth called DTPA, was sourced from Millipore Sigma (Billerica, MA). Formic acid, sourced from Thermo Scientific (Waltham, MA), and ammonium formate, sourced from Aldrich Chemical, were MS grade and used to prepare mobile phases for HPLC and HPLC-MS. HPLC grade acetonitrile sourced from Sigma-Aldrich was used for HPLC analysis and MS grade acetonitrile sourced from J.T. Baker. Water for HPLC and HPLC-MS analysis was HPLC grade and sourced from Sigma-Aldrich. Water for kinetic studies was distilled and passed through a Millipore Milli-Q water purification system. All other chemicals were reagent grade and sourced from VWR.

Determination of 4-Morpholinoaniline pK_a by Spectrophotometric Titration

The dissociation constants (pK_{a1} and pK_{a2}) of 4-mopholinoaniline were determined by spectrophotometric titration. The absorbance of 0.11 mM 4-morpholinoaniline solutions at pH 1 – 10 was measured at room temperature across a wavelength range of 200 – 400 nm using a diode array UV/Vis spectrophotometer (Agilent 8453, Santa Clara, CA, USA) equipped with a 1 cm path length quartz cell. 4-Morpholinoaniline was dissolved into aqueous solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) to achieve the desired pH range, and contained 2% (v/v) acetonitrile to prevent precipitation of 4-morpholinoaniline. The pH of 4-morpholinoaniline solutions was measured immediately after UV/Vis analysis. The pH-absorbance profiles of 4-morpholinoaniline were fitted to Equation 1 using GraphPad Prism 7 software (GraphPad Prism, Version 7.03, GraphPad Software, Inc., La Jolla, CA),

Equation 1
$$A_{(245\text{ nm or }295\text{ nm})} = A_2 + \frac{A_1 - A_2}{1 + 10^{(pKa - pH)\times(\frac{\Delta A}{\Delta pH})}}$$

where $A_{(245\text{ nm or }295\text{ nm})}$ is the absorbance of 4-morpholinoaniline at 245 nm or 295 nm, A_1 and A_2 correspond to the absorbance of the deprotonated and protonated forms, respectively, of 4-morpholinoaniline at 245 nm or 295 nm, $\frac{\Delta A}{\Delta pH}$ is the tangential slope at a given pH, and pK_a is the dissociation constant of 4-morpholinoaniline.

Preparation of Buffer Solutions

Buffer solutions at pH ≤ 2.1 were prepared by diluting concentrated hydrochloric acid to concentrations between 0.1 N and 0.01 N, adjusting to I = 0.15 M using NaCl. Cyanoacetate, methoxyacetate, and acetate buffer solutions at pH 2.5 – 4.0 were prepared at total buffer concentrations of 10 mM with I = 0.15 M (NaCl). Cyanoacetate buffer solutions (pH 2.5 and

pH 3.0) were prepared by weighing cyanoacetic acid and sodium chloride into volumetric flasks and dissolving in 80% of the target volume of water. Methoxyacetate buffer solution (pH 3.5) was prepared by adding sodium chloride and methoxyacetic acid to a volumetric flask and dissolving into 80% of the target volume of water. Acetate buffer solution (pH 4.0) was prepared by adding acetic acid, sodium acetate, and sodium chloride to a volumetric flask and dissolving into 80% of the target volume of water. The pH of each solution was adjusted to the target pH value using hydrochloric acid and/or sodium hydroxide, and the pH-adjusted solutions were brought to volume with water. A DTPA stock solution was prepared at a concentration of 2 mM by weighing DTPA into a volumetric flask and dissolving into the target volume of pH 4.0 acetate buffer. DTPA solutions in pH 4.0 acetate buffer were prepared by diluting the DTPA stock into pH 4.0 acetate buffer and adjusting the pH as necessary with sodium hydroxide and/or hydrochloric acid. Ammonium formate buffers for chromatography experiments were prepared at a 4 L scale by dissolving formic acid and ammonium formate into a 4 L bottle of HPLC grade water and measuring pH to confirm that the pH value was pH 3.5 ± 0.05. The ammonium formate buffers were filtered through a C18 extraction filter prior to use.

pH Measurements

The pH measurements were performed at ambient conditions using a Mettler Toledo SevenExcellence pH meter equipped with a Mettler Toledo InLab Expert Pro-ISM electrode. Prior to each use, the electrode was calibrated to four standards, pH 1.68, 4.01, 7.00, and 10.01.

Thermal Degradation and Kinetic Studies

Thermal degradation and kinetic studies were studied in 100% aqueous solutions at constant ionic strength, I = 0.15 M (NaCl). For all studies, the starting concentration of 4-

morpholinoaniline was 1.1 mM. Degradation kinetics of 4-morpholinoaniline in solutions at pH 1.2 – 2.1 were studied at 40 ºC, 50 ºC, and 60 ºC. Degradation kinetics of the following solutions were studied at 60 ºC only: 4-morpholinoaniline in buffers at pH 2.5 – 4.0; 4-morpholinoaniline at pH 4.0 in the presence of 0 µM, 25 µM, and 50 µM DTPA; 4-morpholinophenol in 0.1N HCl; and 4-morpholinoaniline: 4-morpholinophenol solutions prepared at molar ratios of 1:1, 1:2, and 1:4 in water adjusted to pH 4 with high purity HCl. Reaction solutions were prepared by weighing 4-morpholinoaniline into volumetric flasks, adding the buffer of desired pH, and sonicating for at least 5 minutes. When all solids were dissolved, the pH was measured and adjusted as necessary with hydrochloric acid and/or sodium hydroxide. After sampling the reaction solutions for T0 measurements, the remaining volume was transferred to foil-wrapped, type A glass media bottles, capped with butyl stoppers, and crimp-sealed. The sealed bottles were placed in temperature-controlled ovens. At pre-determined time points, aliquots of 1-2 mL were withdrawn from the reaction solutions, and the aliquoted samples were analyzed against external standard curves using the stability-indicating HPLC-UV method described in High Performance Liquid Chromatography (HPLC) Analysis.

Generation of Standard Curves

To generate standard curves for 4-morpholinoaniline, 4-morpholinophenol, and hydroquinone, concentrated stocks of each species were prepared in dimethylsulfoxide (DMSO) and then serially diluted into unbuffered water. 3-point or 5-point standard curves of 4-morpholinoaniline and 4-morphlinophenol were prepared at concentrations spanning 5.6×10^{-2} mM to 1.4 mM, and 3-point or 5-point standard curves of hydroquinone were prepared at concentrations spanning 9.1×10^{-2} mM to 2.3 mM. The amount of each species was correlated with its HPLC peak area to produce linear standard curves.

Spiking Studies for Degradation Product Identification

Stock solutions of 4-morpholinophenol and hydroquinone were prepared in water at concentrations of 0.14 mM and 0.18 mM, respectively, and spiked into a 1.12 mM solution of 4-morpholinoaniline in 0.075N HCl that was stressed for 10 days at 60 °C. For each degradation product, the spiked solution was prepared by adding 0.5 mL of stock solution to 0.5 mL of stressed solution. The spiked solution was analyzed using the HPLC method summarized in High Performance Liquid Chromatography (HPLC) Analysis. The spiked solutions were analyzed against a neat control of the reaction solution. The degradation product peaks in the spiked solutions were analyzed for peak purity using PDA purity testing in the Empower 3 software to confirm that each peak corresponded to the elution of a single chemical species. The processing method used an integration window of 5 – 15 minutes with a liftoff of 1%, a touchdown of 0.1%, and a minimum area of 1000 AU. The PDA purity testing used a wavelength limit of 200 – 400 nm, a noise interval time of 1.50 – 2.00 minutes, an active peak region of 100%, and 4 purity passes. The threshold criteria were set to auto threshold, and purity was enabled.

High Performance Liquid Chromatography (HPLC) Analysis

The HPLC-UV system used to analyze 4-morpholinoaniline and its degradation products was a Waters Acquity UPLC-UV H-Class System equipped with an H-Class quaternary pump, an autosampler, a column manager, a photodiode array (PDA) detector, and the Empower 3 software. The reverse-phase HPLC-UV method used a Waters Atlantis T3, 3µm particle size, 4.6 x 150 mm column (Waters Corporation, Milford, MA, Part# 186003729). The method parameters are presented in Table 1.

Table 1. Stability-indicating HPLC-UV method used to monitor 4-morpholinoaniline and its degradation products

Stability-Indicating HPLC-UV Method			
Detection:	UV at 240, 280 nm/ PDA 210 – 400 nm	**Mobile Phase Gradient:**	
Sampling Rate:	2 points per second	Time (min)	Mobile Phase B (%)
Resolution:	1.2 nm		
Flow Rate:	1.0 mL/min		
Run Time:	25 min	0	0
Post Run Time:	6 min	2.00	0
Injection Volume:	10 µL	3.00	5
Column Temperature:	50 °C	10.00	5
Autosampler:	10 °C	15.00	35
		21.00	70
Mobile Phase A:	20 mM ammonium formate in water, pH 3.5	23.00	100
		25.00	100
		25.10	0
Mobile Phase B:	Acetonitrile	31.00	0

U/HPLC-UV-QDa for Mass Analysis

The masses of degradation products in stressed solutions of 4-morpholinoaniline were measured using a Waters Acquity U/HPLC-UV H-Class System described above with an in-line Waters Acquity QDa single quadrupole detector mass spectrometer. The U/HPLC-UV method presented in Table 2 used a Waters BEH C18, 1.7 µm particle size, 2.1x100 mm column. The mobile phase gradient was 5% B to 90% B over 15 minutes. The QDa ionization mode was positive electrospray ionization (ESI) with a capillary voltage of 0.8 kV, a cone voltage of 15 V, and a probe temperature of 600 °C. The scan mass range was 200.00 – 1200.00 Da and the sampling rate was 10 points/sec.

Table 2. U/HPLC-UV-QDa method used to analyze degradation products of 4-morpholinoaniline in aqueous solutions

U/HPLC-UV-QDa Method	
Detection:	UV at 265 nm
Flow Rate:	0.5 mL/min
Run Time:	15 min
Injection Volume:	1 µL
Column Temperature:	30 °C
Autosampler Temperature:	10 °C
Mobile Phase A:	0.1% Formic Acid in Water
Mobile Phase B:	Acetonitrile

High-Resolution U/HPLC-UV-QTOF Mass Spectrometry

High-resolution mass spectrometric analysis was performed by U/HPLC-UV-MS using an Agilent 1290 U/HPLC-UV coupled with an Agilent 6510 Q-TOF equipped with an ESI source and the MassHunter Qualitative Analysis software. Two separate methods were used to analyze samples.

In the U/HPLC-UV-QTOF Method 1, the HPLC method described in High Performance Liquid Chromatography (HPLC) Analysis was used to separate the parent from the degradation products; however, the flow rate was reduced from 1.0 mL/min to 0.5 mL/min and the injection volume was reduced from 10 µL to 1 µL to avoid flooding the detector. Nitrogen was used as the drying gas at 45 psi. The drying gas flow and gas temperature were set to 11 L/min and 350°C, respectively. The ESI spray voltage was 3500 V, and the voltage of the fragmentor was 120V. The scan range was from 50 to 1000 Da with a scan rate of 1.00 spectra/sec.

U/HPLC-UV-QTOF Method 2 used a Waters BEH C8, 1.7 µm particle size, 2.1 x 100 mm column, which was held at ambient temperature. The injection volume was 0.5 µL. The mobile phase A was 0.1% Formic Acid in Water and mobile phase B was acetonitrile, and the gradient was 5%B to 90%B over 8 minutes at a flow rate of 0.5 mL/min. PDA data was collected at 242 nm. Nitrogen was used as the drying gas at 45 psi. The drying gas flow and gas temperature

were set to 10 L/min and 350°C, respectively. The ESI spray voltage was 3500 V, the voltage of the fragmentor was 75V, and the capillary amperage was 0.029 μA. The scan range was from 100 to 1000 Da with a scan rate of 1.00 spectra/sec.

Generation of the Major Degradation Product of 4-Morpholinoaniline at pH 4

Aqueous solutions of 4-morpholinoaniline were prepared at concentrations of 2.2 – 11.2 mM and pH-adjusted to pH 4.0 – 4.5 using HCl. The solutions were stored in a 60°C oven and aliquots were pulled from the reaction solutions at pre-determined time points. The progress of 4-morpholinoaniline degradation was monitored diluting and analyzing the aliquots by HPLC-UV-QDa against an external 4-morpholinoaniline standard curve using the method described in High Performance Liquid Chromatography (HPLC) Analysis. When at least 90% of the 4-morpholinoaniline had degraded, the reaction solutions were concentrated by lyophilization and the major degradation product was separated from other species in solution and collected using MS-guided prep-HPLC. The prep-HPLC-QDa system was a Waters Acquity H-Class system equipped with an H-Class quaternary pump, an autosampler, a column manager, an in-line Acquity QDA single-quadrupole mass spectrometer, and the Empower 3 software. The reverse-phase prep-HPLC method used a Waters CSH C18, 5 μm particle size, 19 x 150 mm column (Waters Corporation, Millford, MA, Part # 186003729). The MS-guided prep-HPLC method is presented in Table 3. The QDa ionization mode was positive ESI with a capillary voltage of 0.8 kV, a cone voltage of 15 V, and a probe temperature of 600 ºC. The scan mass range was 200.00 – 1200.00 Da and the sampling rate was 10 points/sec.

Table 3. MS-guided prep-HPLC method used to isolate and purify the 4-morpholinoaniline degradation product observed in pH 4 aqueous solutions

MS-Guided Prep-HPLC Method	
Detection:	UV at 220, 265 nm
Flow Rate:	35 mL/min
Injection Volume:	1 mL
Column Temperature:	Ambient
Autosampler Temperature:	10 °C
Mobile Phase A	0.1% Triflouroacetic Acid in Water
Mobile Phase B	0.1% Trifluoroacetic Acid in Acetonitrile

Nuclear Magnetic Resonance (NMR) Analysis

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian 400-MR 400 MHz spectrometer (Agilent Technologies Inc., Palo Alto, CA, USA). Approximately 1 mg of purified degradation product was dissolved into deuterated water. The resulting solution was aliquoted into a 3 mm tube for analysis.

Results

4-Morpholinoaniline pK_a Values by Spectrophotometric Titration

The UV spectra of 4-morpholinoaniline solutions measured under different pH conditions at room temperature are presented in Figure 4. The spectrum of 4-morpholinoaniline is pH-dependent over the pH range analyzed. The pH-dependent spectral changes are significant at the following wavelengths: 200 nm, 245 nm, and 295 nm. The pH-absorbance curves at 245 nm and 295 nm were fitted to Equation 1 (Figure 5) and yielded pK_a values of 1.8 ± 0.1 and 4.9 ± 0.3. These results are consistent with pK_a values reported for substituted anilines and aryl diamines, which are summarized in Table 4. The second pK_a is comparable to the pK_a value of 5.65 assigned to the morpholine nitrogen by Esmaili and Nematollahi.[7]

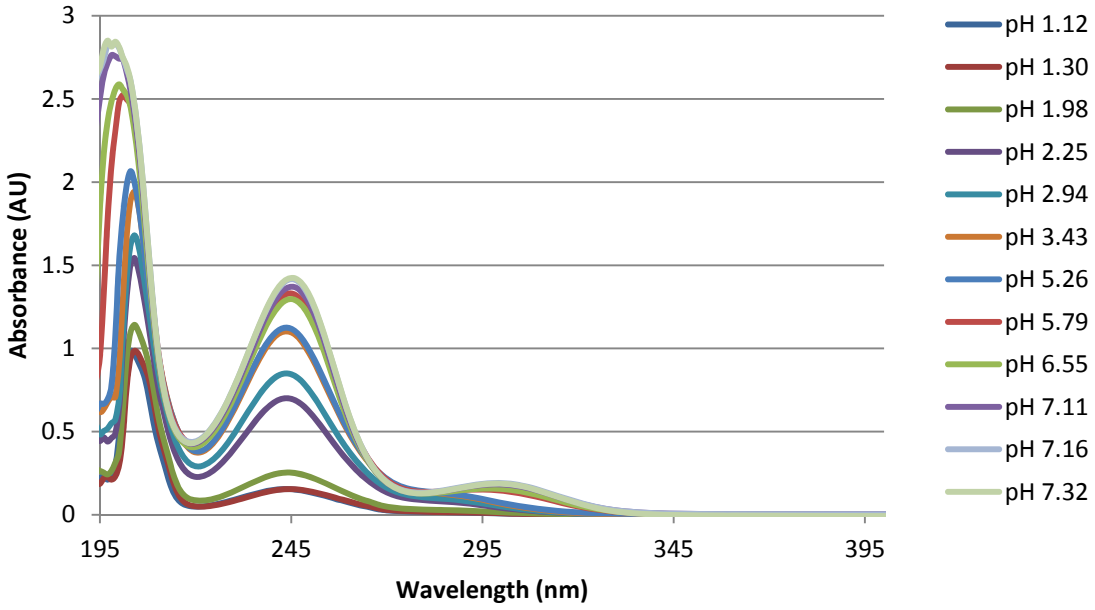


Figure 4. UV/Vis spectra of 4-morpholinoanilne in aqueous solution at pH 1 – 8 at room temperature

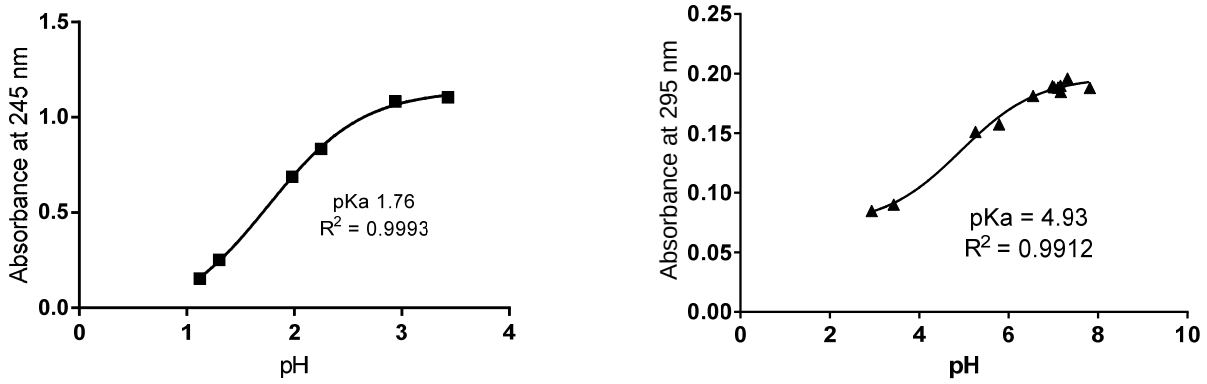
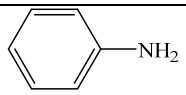
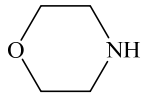
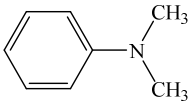
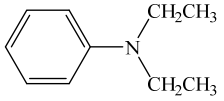
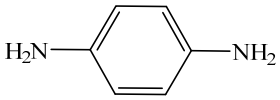
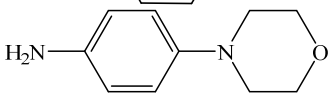


Figure 5. 4-Morpholinoaniline absorbance as a function of pH at 245 nm and 295 nm for determination of dissociation constants (pK_{a1} and pK_{a2})

Table 4. Reported pKa values for aniline, morpholine, substituted anilines, and aryl diamines

Name	Structure	pKa_1	pKa_2	Reference
Aniline		4.87	-	9
Morpholine		8.50	-	9
N,*N*-dimethylaniline		5.07	-	9
N,*N*-diethylaniline		6.57	-	9
p-phenylenediamine		6.31 6.08 – 6.44	2.97 2.80 – 3.29	9 10
4-Morpholinoaniline		5.65	Not reported	7

Identification of 4-Morpholinoaniline Degradation Products Observed in Solution in 0.1 N HCl

Two main degradation products were observed in 4-morpholinoaniline solution in 0.1 N HCl that was stressed at elevated temperature for several days. The degradation products eluted at approximately 6 minutes (RRT 0.78) and 14 minutes (RRT 1.84) (Figure 6). High resolution

mass spectrometric analysis of the product with RRT 1.84 by U/HPLC-UV-QTOF revealed an $(M+H)^+$ species with a monoisotopic mass (m/z) of 180.1011, and the Find Compound by Formula algorithm in Mass Hunter Qualitative analysis software assigned a chemical formula of $C_{10}H_{14}NO_2$ with a fit score of 98.39% and a difference of 5.30 ppm. An MS signal was not observed for the product with RRT 0.78 in high resolution mass spectrometric analysis by U/HPLC-UV-QTOF despite a strong UV signal, indicating that the product with RRT 0.78 is a UV-active compound, that either does not ionize easily in the positive ESI mode or does not contain an ionizable group. Similarly, mass spectrometric analysis by U/HPLC-UV-QDa produced a strong UV signal at RRT 0.78 with a weak MS signal that assigned a mass of 110.07.

Based on the ion with m/z 180.1011 $(M+H)^+$ and on the assigned chemical formula of $C_{10}H_{14}NO_2$, the proposed structure of the degradation product with RRT 1.84 is 4-morpholinophenol (Figure 7). Based on the ionization behavior of the species and the mass of 110.07, the proposed structure of the degradation product with RRT 0.78 is hydroquinone (Figure 7), a potential product of 4-morpholinophenol hydrolysis.

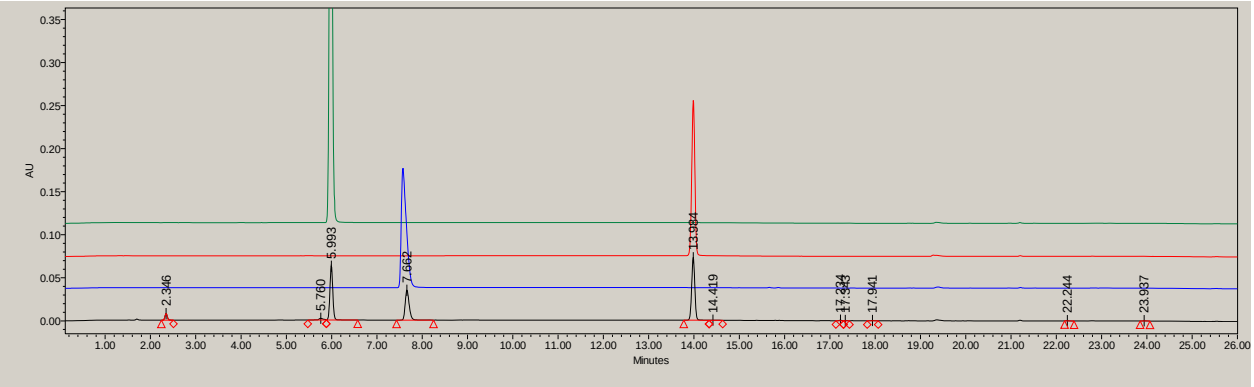
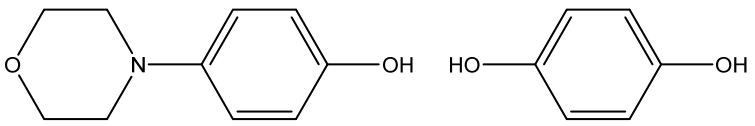


Figure 6. Chromatograms of 4-morpholinoaniline (blue), 4-morpholinophenol (red), hydroquinone (green), and a stressed 4-morpholinoaniline solution in 0.1N HCl (black)



RRT 1.84
4-morpholinophenol
Exact Mass: 179.09

RRT 0.78
Hydroquinone
Exact Mass: 110.04

Figure 7. Proposed structures of 4-morpholinoaniline degradation products generated at low pH

The identities of both degradation products were confirmed by comparison of the HPLC retention times of the products observed in stressed 4-morpholinoaniline solutions to retention times of authentic standards of 4-morpholinophenol and hydroquinone (Figure 6). The identities of the degradation products were further confirmed by spiking stressed 4-morpholinoaniline solutions with authentic standards of each proposed degradation product, and then analyzing the spectral purity of the 4-morpholinophenol and hydroquinone peaks in the spiked solutions using PDA purity testing in the Empower 3 software. The 4-morpholinophenol peak in the spiked solution had a purity angle of 0.798 and a threshold angle of 1.55 and the hydroquinone peak in the spiked solution had a purity angle of 0.118 and a threshold angle of 0.316. Because the purity angles are smaller than the threshold angles for both peaks in the spiked solutions, it can be concluded that each peak corresponds to the elution of a single chemical species. These results confirm that the degradation products with RRT 1.84 and RRT 0.78 are 4-morpholinophenol and hydroquinone, respectively.

Thermal Degradation of 4-Morpholinophenol

The degradation of 4-morpholinophenol in 0.1N HCl (pH 1.2) solution was followed at 60 ºC by monitoring changes in concentration of 4-mopholinophenol and hydroquinone over time. Mole fractions of each species in solution were calculated in reference to the starting molar concentration of 4-morpholinophenol, assuming a stoichiometric ratio of 1:1 (molar) for 4-

morpholinophenol:hydroquinone. Table 5 summarizes the concentrations of each species in µM, mole fraction, and % area. Both species demonstrate excellent agreement between mole fraction and % area at every time point, and account for more than 99% of the mass in the system through the first half-life of 4-morpholinophenol degradation. These results indicate that hydroquinone, and presumably the UV-inactive morpholine, are the primary degradation products of 4-morhpolinophenol in acidic conditions.

Table 5. **Concentrations of 4-morpholinophenol and hydroquinone in 0.1N HCl at 60 °C**

		4-Morpholinophenol			Hydroquinone			4MP + HQN	
pH	Time (Days)	µM	Mole Fraction (%)	%Area	µM	Mole Fraction (%)	%Area	Mole Fraction (%)	%Area
1.2	0	1130.3	99.3	98.83	8.1	0.7	0.64	100.00	99.5
	1	889.4	78.7	78.56	226.9	19.9	20.43	98.06	99.0
	2	712.7	63.1	62.11	409.5	36.0	36.66	98.59	98.8
	3	569.2	50.4	49.13	558.1	49.0	49.31	99.03	98.4
	4	451.6	40.0	38.98	639.9	56.2	59.27	95.89	98.3
	7	223.3	19.8	19.07	850.8	74.7	78.28	94.36	97.4

Figure 8 shows a linear semi-logarithmic plot of 4-morpholinophenol concentration versus time. The slope of the line is equal to $-k_{obs}$ and the intercept is equal to ln[4-morpholiophenol]$_0$. The data demonstrate an excellent linear fit with a correlation coefficient (R^2) of 0.9999, indicating that hydrolysis of 4-morpholinophenol to hydroquinone under acidic conditions follows pseudo-first kinetics with an observed rate constant (k_{obs}) of 0.2312 days^{-1}.

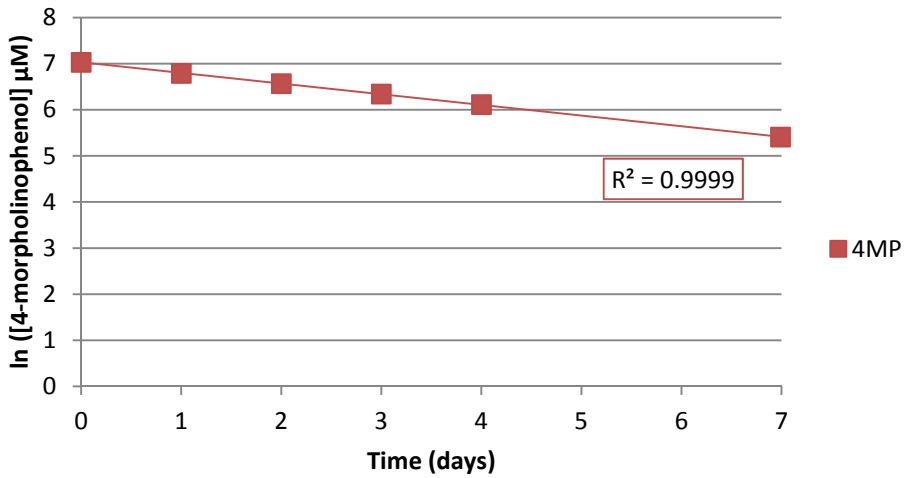


Figure 8. Concentration of 4-morpholinophenol in 0.1N HCl at 60 ºC as a function of time

Determination of Relative Response Factors

Due to significant differences in the extinction coefficients of 4-morpholinoaniline, 4-morpholinophenol, and hydroquinone (Figure 9), their relative response factors were determined to accurately estimate the amount of each species in solution by % area. Response factors were calculated from the linear slope of a standard curve for each species ($R^2 > 0.999$ for all species). The relative response factors for 4-morpholinophenol and hydroquinone, shown in Table 6, were determined by dividing the response factor for 4-morpholinoaniline by the response factors for each species.

Table 6. Response factors and relative response factors (RRF) for 4-morpholinoaniline, 4-morpholinophenol, and hydroquinone

Chemical Species	240 nm		280 nm	
	Response Factor	RRF	Response Factor	RRF
4-Morpholinoaniline	5937878	1.00	782692	1.00
4-Morpholinophenol	2274277	2.61	630941	1.24
Hydroquinone	274703	21.62	1158579	0.68

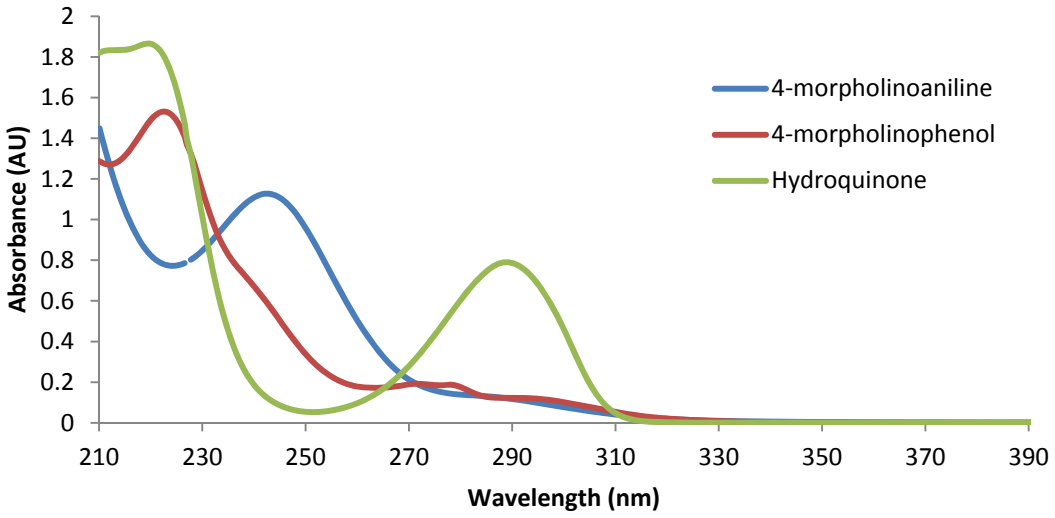


Figure 9. UV-Vis Spectra of 4-morpholinoaniline, 4-morpholinophenol, and hydroquinone

Degradation of 4-Morpholinoaniline in Aqueous Solutions as a Function of pH

The degradation of 4-morpholinoaniline and concomitant increase in concentrations of 4-morpholinophenol and hydroquinone was monitored over time in solutions at pH 1 – 4 at 60 °C. Concentrations of each species were calculated from standard curves. Mole fractions of each species in solution were calculated in reference to the starting molar concentration of 4-morpholinoaniline, assuming stoichiometric ratios of 1:1 (molar) for 4-morpholinoaniline:4-morpholinophenol, and 4-morpholinophenol:hydroquinone. The % area for 4-morpholinophenol and 4-morpholinoaniline were corrected using the relative response factors reported in Table 6. The mole fraction versus % area results are presented in Table 7 and Table 8.

In solution at pH 1.25 – 1.50, all three species demonstrate agreement between mole fraction and % area, and account for more than 90% of the species in solution at all time points. In solutions at pH 1.72 and pH 2.10, the three species demonstrate agreement between mole fraction and % area, but account for more than 90% of the species in solution only through the first half-life and t_{90} of 4-morpholinoaniline at pH 1.72 and pH 2.10, respectively. In solutions at

pH ≥ 2.5, discrepancies are observed between mole fraction and % area for all three species, and the discrepancies become more pronounced as pH increases. Furthermore, 4-morpholinophenol accounts for only a small amount of mass in the system as pH is increased above pH 2, and hydroquinone is not observed in solutions at pH ≥ 2.5.

Table 7. **Concentrations of 4-morpholinoaniline, 4-morpholinophenol, and hydroquinone in solution at pH 1.2 – 1.7 at 60 °C**

		4-Morpholinoaniline		4-Morpholinophenol		Hydroquinone		4MA + 4MP + HQN	
pH	Time (Days)	Mole Fraction (%)	Area (%)	Mole Fraction (%)	Area (%)	Mole Fraction (%)	Area (%)	Mole Fraction (%)	Area (%)
1.25	0	100	99.5	0	0	0	0	100	99.5
	0.250	97.5	96.6	2.5	2.5	0	0.0	99.9	99.1
	1	81.7	81.2	15.5	15.4	0.9	0.9	97.9	97.5
	2	66.9	66.6	26.8	26.4	3.0	3.0	96.3	96.0
	3	53.2	52.6	36.6	35.5	6.5	6.5	95.5	94.6
	4	42.5	41.8	43.6	41.7	10.7	10.6	95.2	94.1
	5	32.2	31.5	49.3	46.2	16.1	15.9	95.3	93.6
	6	24.0	23.3	53.2	48.6	21.7	21.3	95.4	93.2
	7	16.5	16.1	54.9	48.8	28.3	27.1	94.2	92.0
1.36	0	100	99.4	0	0	0	0	100	99.4
	0.250	97.9	97.4	1.8	1.9	0	0	99.7	99.8
	1	85.4	84.9	12.3	12.3	0.6	0.6	98.3	97.8
	2	72.8	72.3	22.0	22.0	2.1	2.1	97.0	96.4
	3	60.9	60.3	30.5	30.3	4.6	4.6	96.0	95.2
	4	51.4	50.8	36.5	36.2	7.4	7.4	95.3	94.4
	5	42.3	41.5	41.8	41.2	11.1	11.0	95.2	93.7
	6	35.2	34.5	45.1	44.3	14.6	14.4	94.9	93.2
	8	23.2	22.6	48.7	47.6	22.8	22.4	94.8	92.6
	10	13.0	12.2	49.2	46.7	33.8	32.2	95.9	91.1
1.50	0	100	99.5	0	0	0	0	99.7	99.2
	1	86.6	86.0	11.2	11.2	0.6	0.6	98.3	97.8
	2	75.8	75.5	19.4	19.4	1.8	1.8	97.0	96.7
	3	65.5	64.8	26.8	26.6	3.8	3.8	96.0	95.2
	4	57.1	56.3	32.1	31.8	6.1	6.1	95.2	94.2
	5	48.2	48.0	36.4	36.4	8.8	8.9	93.5	93.3
	7	35.3	34.4	43.0	42.1	15.1	14.8	93.3	91.3
	10	18.7	17.5	46.9	44.3	27.2	25.8	92.7	87.6
1.72	0	100	99.4	0	0	0	0	100	99.4
	1	92.7	92.2	6.0	6.1	0.2	0.2	98.9	98.5
	2	85.6	85.2	11.5	11.5	0.7	0.7	97.7	97.4
	4	72.1	70.8	21.0	20.7	2.8	2.8	95.9	94.3
	7	54.2	53.1	31.1	30.6	7.3	7.3	92.6	91.0
	10	38.8	37.9	37.3	36.6	13.2	13.0	89.3	87.5
	12	26.0	25.3	39.9	39.1	20.1	19.7	86.0	84.1
	15	12.6	12.7	38.7	39.4	29.8	30.6	81.1	82.7

Table 8. **Concentrations of 4-morpholinoaniline, 4-morpholinophenol, and hydroquinone in solution at pH 2.1 – 4.0 at 60 °C**

pH	Time (Days)	4-Morpholinoaniline		4-Morpholinophenol		Hydroquinone		4MA + 4MP + HQN	
		Mole Fraction (%)	Area (%)	Mole Fraction (%)	Area (%)	Mole Fraction (%)	Area (%)	Mole Fraction (%)	Area (%)
2.10	0	100	99.4	0	0	0	0	100	99.4
	1	97.5	96.9	1.8	1.9	0	0.0	99.4	98.8
	2	94.7	94.1	3.5	3.6	0.2	0.2	98.3	97.9
	4	88.2	87.1	6.6	6.6	0.6	0.6	95.4	94.3
	8	70.9	70.8	10.7	10.7	1.6	1.6	83.1	83.1
	12	45.5	50.5	12.6	14.1	2.7	3.0	60.8	67.6
	15	24.5	33.1	13.2	17.9	3.9	5.3	41.6	56.3
2.48	0	100	97.1	< LOD	< LOD	< LOD	< LOD	100	97.1
	1	93.1	93.4	0.2	0.3	< LOD	< LOD	93.3	93.6
	2	81.3	82.9	0.7	0.8	< LOD	< LOD	82.0	83.7
	3	59.3	60.7	2.2	2.3	< LOD	< LOD	61.5	63.0
	4	38.6	41.7	4.1	4.5	< LOD	< LOD	42.7	46.2
	5	23.8	28.9	4.5	5.6	0.1	0.1	28.5	34.6
3.05	0	100	97.1	< LOD	< LOD	< LOD	< LOD	100	97.1
	0.250	95.4	91.7	< LOD	< LOD	< LOD	< LOD	95.4	91.7
	1	69.1	62.3	3.0	2.8	< LOD	< LOD	72.1	65.0
	1.25	55.6	49.6	5.0	4.5	< LOD	< LOD	60.6	54.1
	2	33.5	31.1	9.6	9.1	< LOD	< LOD	43.1	40.1
	3	10.6	13.7	9.7	12.8	< LOD	< LOD	20.3	26.5
3.49	0	100	99.7	< LOD	< LOD	< LOD	< LOD	100	99.7
	0.167	92.4	83.7	< LOD	< LOD	< LOD	< LOD	92.4	83.8
	0.250	84.1	73.8	0.4	0.4	< LOD	< LOD	84.5	74.2
	1	48.5	34.5	7.0	5.0	< LOD	< LOD	55.5	39.5
	1.25	34.7	24.1	9.1	6.4	< LOD	< LOD	43.8	30.5
	2	16.1	12.7	10.0	8.0	< LOD	< LOD	26.0	20.6
4.01	0	100	96.8	< LOD	< LOD	< LOD	< LOD	100	96.8
	0.167	88.4	76.4	< LOD	< LOD	< LOD	< LOD	88.4	76.4
	0.333	74.5	58.4	0.5	0.4	< LOD	< LOD	75.0	58.8
	0.5	63.2	46.4	1.3	1.0	< LOD	< LOD	64.4	47.4
	1	38.1	26.1	4.4	3.1	< LOD	< LOD	42.5	29.2
	1.25	28.2	19.5	4.3	3.0	< LOD	< LOD	32.5	22.5

Kinetics of 4-Morpholinoaniline Degradation in Aqueous Solutions

Figure 10 shows the concentration of 4-morpholinoaniline versus time in 0.1 – 0.01 N HCl solutions (pH 1.25 – 2.10) stored at 60 ºC. The time-concentration dependence at pH 1.25 – 1.50 shows an excellent linear fit in the semi-logarithmic plot over two 4-morpholinoaniline half-lives ($R^2 \geq 0.997$), indicating that 4-morpholinoaniline degradation proceeds via pseudo-first order kinetics for 75% of the reaction. 4-Morpholinoaniline degradation in solution at pH 1.72 shows

an excellent fit to pseudo-first order kinetics over the first half-life of 4-morpholinoaniline degradation ($R^2 = 0.9992$), but deviates from linearity for the second 4-morpholinoaniline half-life. 4-Morpholinoaniline degradation in solution at pH 2.10 initially fits to a pseudo-first order kinetics model ($R^2 = 0.94$), but deviates from linearity after approximately 10% of 4-morpholinoaniline degradation. For both the pH 1.72 and pH 2.10 solutions, the deviation from pseudo-first order kinetics corresponded to an observed color change in the initially clear and colorless reaction solution.

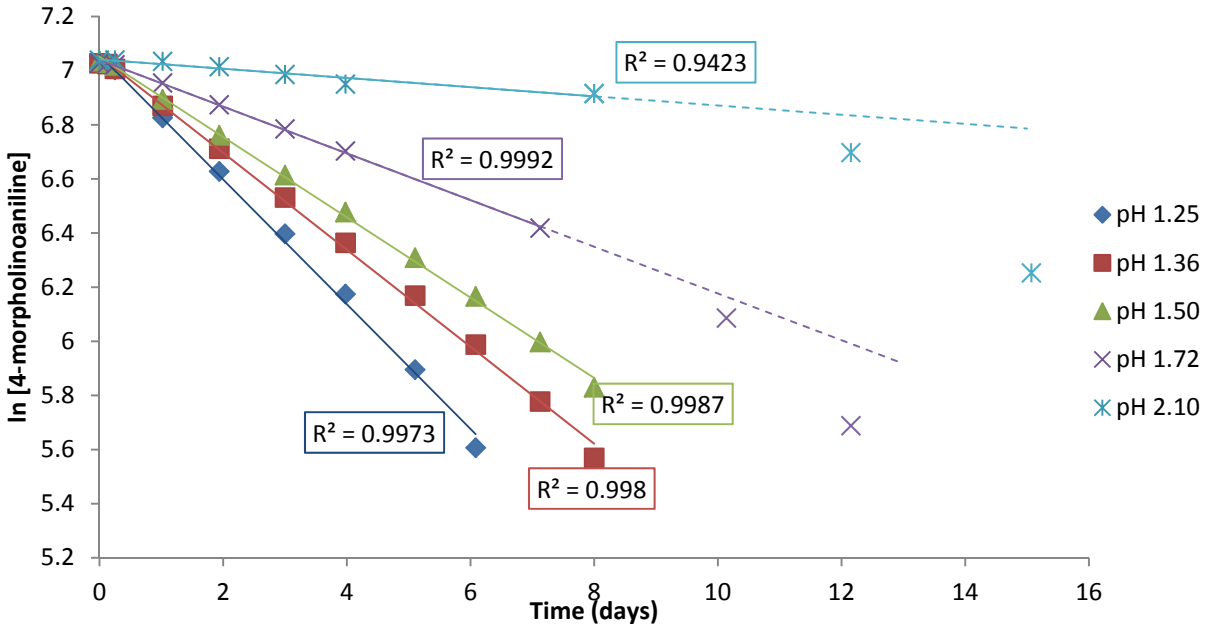


Figure 10. Decrease in 4-morpholinoaniline concentration in 0.1 – 0.01 N HCl solutions stored at 60 ºC as a function of time

Figure 11 shows semi-logarithmic plots of 4-morpholinoaniline concentration versus time in aqueous solutions at pH 2.5 – 4.0 stored at 60 ºC. 4-Morpholinoaniline degradation in these solutions proceeded more rapidly than in pH 1 – 2 solutions, and showed significant color changes that were not observed in lower pH samples (Figure 12). 4-Morpholinoaniline degradation in these solutions demonstrated poor fit to zero-, first-, or second-order kinetics. Due to the increased complexity of reaction kinetics, rate equations were not determined for 4-

morpholinoaniline degradation in solutions at pH ≥ 2.5.

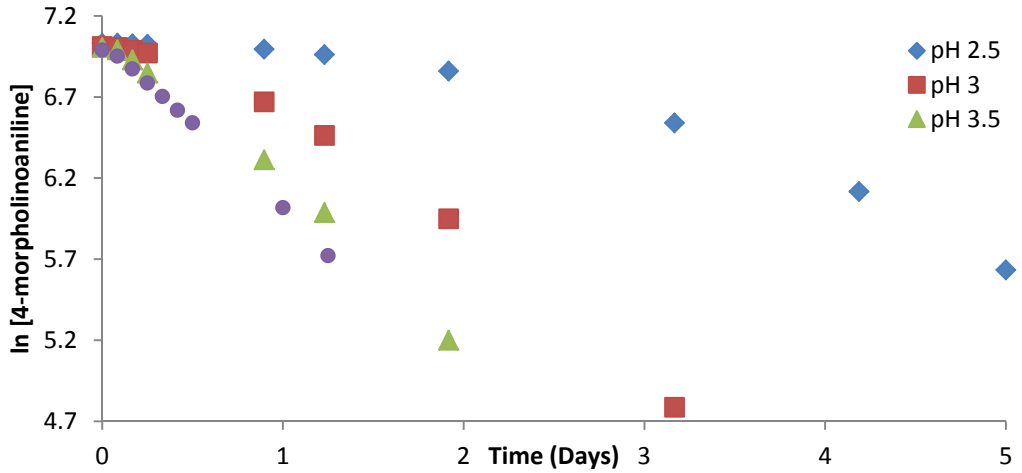


Figure 11. 4-Morpholinoaniline concentration in solutions at pH 2.5 – 4.0 stored at 60 ºC as a function of time

pH	T0	2 hr, 60 ºC	6 hr, 60 ºC	1 day, 60 ºC
2.5				
3.0				
3.5				
4.0				

Figure 12. Color changes observed in 4-morpholinoaniline solutions at pH 2.5 – 4.0

The observed first-order rate constants (k_{obs}) for 4-morpholinoaniline degradation in solutions at pH 1.25 – 2.10 at 60 ºC, 50 ºC, and 40 ºC were determined from the slope of the semi-logarithmic plot of 4-morpholinoaniline concentration as a function of time, and are summarized in Table 9. The pseudo-first order rate constants (k_{obs}) were determined from at least two half-lives of 4-morpholinoaniline degradation at pH 1.25 – 1.50, from one half-life at 4-morpholinoaniline degradation at pH 1.72, and from approximately 10% degradation of 4-morpholinoaniline at pH 2.10. The expected times required for 4-morpholinoaniline concentration to decrease by 50% (t_{50}) and 10% (t_{90}) were calculated using Equation 2 and Equation 3, respectively.

Equation 2 $$t_{50} = \frac{-\ln(0.5)}{k_{obs}}$$

Equation 3 $$t_{90} = \frac{-\ln(0.9)}{k_{obs}}$$

Table 9. Observed first-order rate constant values (k_{obs}) for degradation of 4-morpholinoaniline in solution at pH 1.25 – 2.10 stored at 60 ºC, 50 ºC, and 40 ºC.

[HCl] (N)		0.1	0.075	0.05	0.025	0.01
pH		1.25	1.36	1.50	1.72	2.10
60 ºC	k_{obs} (day^{-1})	0.2305	0.1791	0.1484	0.0863	0.0222
	t_{90} (days)	0.5	0.6	0.7	1.2	4.8
	t_{50} (days)	3.0	3.9	4.7	8.0	31.3
50 ºC	k_{obs} (day^{-1})	0.1191	0.0987	0.0772	0.0433	0.0166
	t_{90} (days)	0.9	1.1	1.4	2.4	6.3
	t_{50} (days)	5.8	7.0	9.0	16.0	41.7
40 ºC	k_{obs} (day^{-1})	0.0519	0.0407	0.0361	0.0185	0.0076
	t_{90} (days)	2.0	2.6	2.9	5.7	13.9
	t_{50} (days)	13.4	17.0	19.2	37.5	91.4
25 ºC (ext)	k_{obs} (day^{-1})	0.0151	0.0122	0.0111	0.0052	0.0033
	t_{90} (days)	7.0	8.6	9.5	20.4	31.8
	t_{50} (days)	45.8	56.9	62.2	134.3	209.3

The temperature dependence of the rate constants (k_{obs}) was determined for 4-morpholinoaniline in solution at pH 1.25 - 1.72 using a linear least-square regression fit of the data to the Arrhenius equation (Equation 4):

Equation 4 $$\ln k_{obs} = \ln A - \frac{E_a}{RT}$$

where k_{obs} is the observed pseudo first-order rate constant, A is the pre-exponential term, E_a is the activation energy, R is the universal gas constant, and T is the absolute temperature in Kelvin.

Figure 13 shows the Arrhenius plot for the degradation of 4-morpholinoaniline in solution at pH 1.25 – 2.10. The Arrhenius plots were used to extrapolate the pseudo first-order rate constants for 4-morpholinoaniline degradation to 25 °C, and the extrapolated rate constants were used to calculate 4-morpholinoaniline t_{90} and t_{50} at 25 °C. These results are included in Table 9.

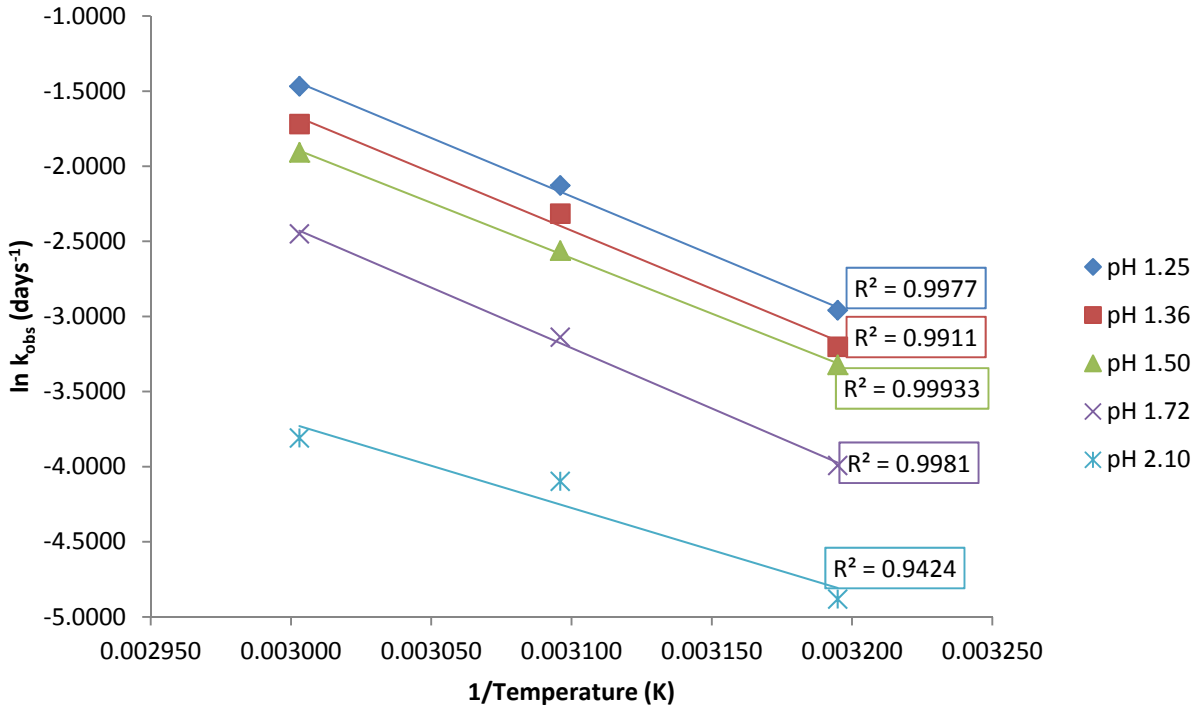


Figure 13. Arrhenius plot for 4-morpholinoaniline degradation at pH 1.25 – 2.10

The pH-dependent degradation of 4-morpholinoaniline may be described by the rate equation shown in Equation 5:

Equation 5 $$k_{obs} = k_1[H^+]f_{H_2A^{2+}} + k_2[H^+]f_{HA^+} + k_3 f_{HA^+}$$

where k_{obs} is the observed pseudo first-order rate constant, [H+] is the hydrogen ion concentration, $f_{H_2A^{2+}}$ and $f_{HA^{+}}$ are the fractions of 4-morpholinoaniline in the doubly and singly protonated states, respectively, k_1 and k_2 are the rate constants for specific acid-catalysis for the degradation of 4-morpholinoaniline in the doubly and singly protonated states, respectively, and k_3 is the rate constant for water catalysis for the degradation of 4-morpholinoaniline in the singly protonated state. Because specific acid-catalysis for the degradation of 4-morpholinoaniline in the singly protonated state should be kinetically indistinguishable from water catalysis for the degradation of 4-morpholinoaniline in the doubly protonated state, the pH-dependent degradation of 4-morpholinoaniline may also be described by the rate equation shown in Equation 6:

Equation 6 $$k_{obs} = k_1[H^+]f_{H_2A^{2+}} + k_2 f_{H_2A^{2+}} + k_3 f_{HA^{+}}$$

where k_{obs} is the observed pseudo first-order rate constant, [H+] is the hydrogen ion concentration, $f_{H_2A^{2+}}$ and $f_{HA^{+}}$ are the fractions of 4-morpholinoaniline in the doubly and singly protonated state, respectively, k_1 is the rate constants for specific acid-catalysis for the degradation of 4-morpholinoaniline in the doubly protonated state, and k_2 and k_3 are the rate constants for water catalysis for the degradation of 4-morpholinoaniline in the doubly and singly protonated states, respectively. The current data set is insufficient to identify the most correct rate equation to describe the system, and data were processed using Equation 5.

The pH-rate profile plot shown in Figure 14 was generated for 4-morpholinoaniline degradation in solution at pH 1.25 – 1.72 by plotting log k_{obs} at 60 ºC, 50 ºC, 40 ºC, and 25 ºC as a function of pH. The individual apparent rate constants were determined using nonlinear least-squares regression analysis to fit the data to Equation 5. The results were fitted using GraphPad Prism 7 software (GraphPad Prism, Version 7.03, GraphPad Software, Inc., La Jolla, CA). The

values for k_1, k_2, k_3, and goodness of fit (R^2) are summarized in Table 10. The data show good fit to Equation 5 with $R^2 \geq 0.97$, and demonstrate that the second term in Equation 5, which describes specific acid-catalysis for the degradation of 4-morpholinoaniline in the singly protonated state, is the most significant contributor to the observed rate constant.

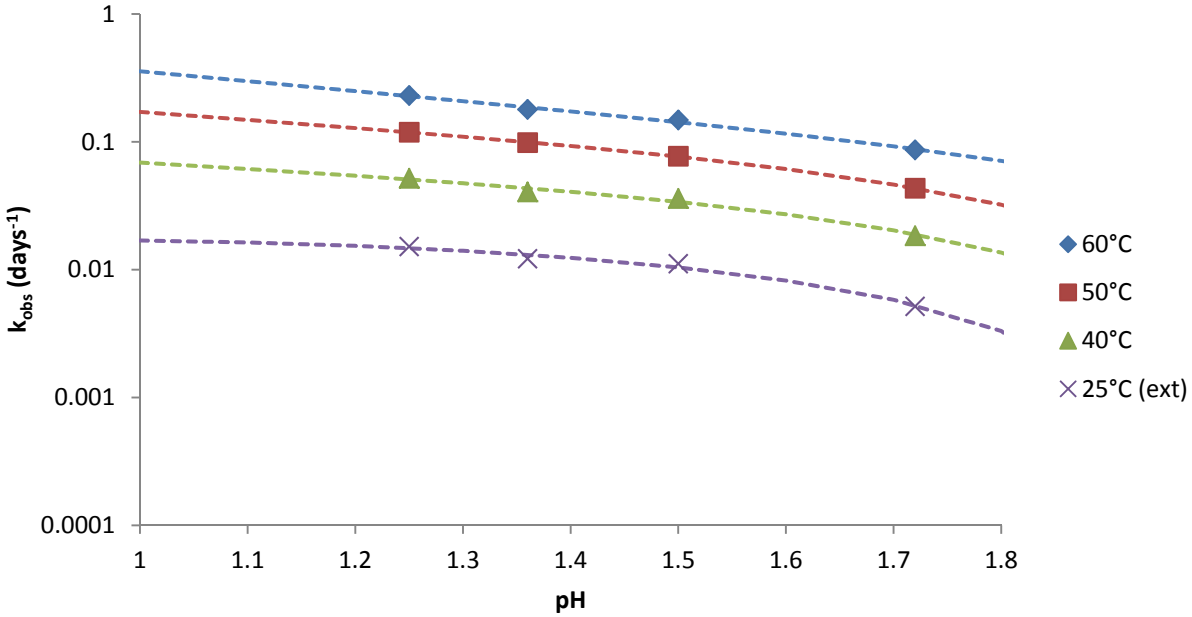


Figure 14. pH-Rate profile for the degradation of 4-morpholinoaniline in aqueous solution at 60, 50, and 40 ºC, and the extrapolated pH-rate profile for 4-morpholinoaniline degradation in aqueous solution at 25 ºC.

Table 10. Calculated specific acid-catalyzed and water-catalyzed apparent rate constants for the degradation of 4-morpholinoaniline at pH 1.25 – 1.72 determined by fitting data to Equation 5

Temperature (°C)	k_1 (Lmol^{-1}day^{-1})	k_2 (Lmol^{-1}day^{-1})	k_3 (day^{-1})	Correlation Coefficient (R^2)
25 (Extrapolated)	-0.03974	1.677	-0.01931	0.9720
40	0.2141	4.075	-0.04075	0.9769
50	0.8494	7.920	-0.07460	0.9997
60	2.596	10.32	-0.06299	0.9913

The enthalpy and entropy of specific acid-catalysis for the degradation of 4-morpholinoaniline in the singly protonated state (k_2) were determined by fitting the data to the Eyring Equation (Equation 7):

Equation 7 $$\ln\frac{k}{T} = \frac{-\Delta H^{\ddagger}}{R}\cdot\frac{1}{T} + \ln\frac{\kappa k_B}{h} + \frac{\Delta S^{\ddagger}}{R}$$

where k is the apparent rate constant for specific acid-catalysis for the degradation of 4-morpholinoaniline in the singly protonated state (k_2), T is the absolute temperature in Kelvin, R is the universal gas constant, κ is the transmission coefficient which is assumed to be unity, k_B is the Boltzmann constant, h is Planck's constant, $\Delta H^{\ddagger}$ is the enthalpy of activation, and $\Delta S^{\ddagger}$ is the entropy of activation. For each apparent rate constant, ln (k/T) was plotted against $1/T$ with an excellent linear fit in the semi-logarithmic plot ($R^2 \geq 0.999$), as shown in Figure 15. The enthalpy of reaction was derived from the slope of the line and the entropy of reaction was derived from the intercept of the line. The results are summarized in Table 11.

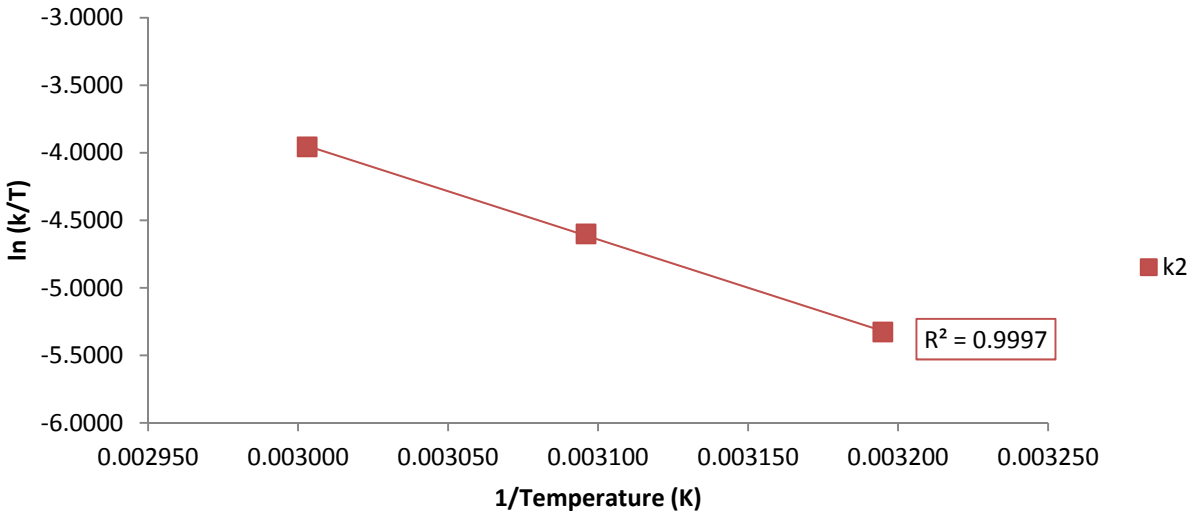


Figure 15. Eyring plot for the apparent rate constants, k_1 and k_2, from the degradation of 4-morpholinoaniline at 60 °C, 50 °C, and 40 °C

Table 11. Activation parameters for specific acid catalysis for the degradation of 4-morpholinoaniline in the singly protonated state (k_2)

Apparent Rate Constant	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/K·mol)
k_2	59.4	-146.4

4-Morpholinoaniline Degradation Products in Aqueous Solutions at pH 2 - 4

Table 12 summarizes the relative retention times and changes in % area of the major degradation products observed in 4-morpholinoaniline aqueous solutions at pH 2.10 – 4.00 at 60 ºC. The % area of 4-morpholinophenol and hydroquinone are not included in this table. Due to the large number of minor degradation products observed in these solutions, the table only summarizes degradation products that achieve at least 5% of the integrated area.

The major degradation products observed in 4-morpholinoaniline solutions at pH 2.0 – 4.0 are 4-morpholinophenol and unknown products that elute at relative retention times (RRT) of 0.75, 1.51, 2.14, 2.16, and 2.20. The distribution of these degradation products in solution is pH-dependent. The degradation product with RRT 0.75, which was also observed in small amounts (2 - 3% area) in solutions at pH < 2, is the most prominent degradation product at pH 2.10. The degradation product with RRT 1.51 is the most prominent in solutions at pH 2.5 and pH 3.0. The most prominent degradation products observed in 4-morpholinoaniline solutions at pH 3.5 and pH 4.0 are those with RRT 2.14, 2.16, and 2.20. The product with RRT 2.20 accounts for the most % area in pH 3.5 solution and the product with RRT 2.16 accounts for the most % area in pH 4.0 solution.

The Empower 3 software was used to extract the UV/Vis spectra of the unknown degradation products observed 4-morpholinoaniline solutions at pH 2 – 4. These spectra, shown in Figure 16, demonstrate that these chemical species have different absorbance behavior, and these differences can explain the discrepancies observed in 4-morpholinoaniline mole fraction and % area in the data sets for solutions at pH 2 – 4.

Table 12. Major degradation products observed in aqueous 4-morpholinoaniline solutions at pH 2 - 4 at 60 ºC

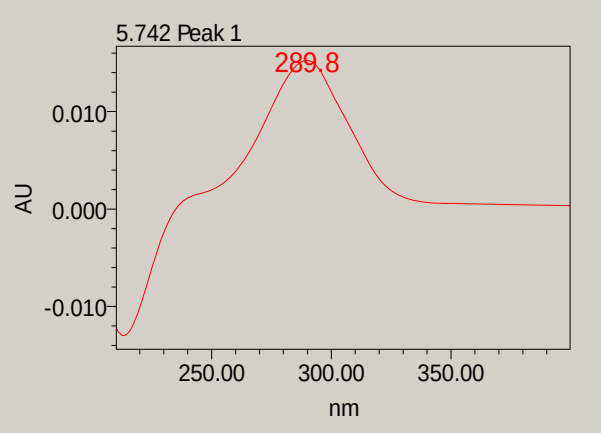
pH	Time (Days)	4MA (% of T0)	%Area at 280 nm[1,2]								
			4MA	Total Deg	RRT 0.75	RRT 1.20	RRT 1.37	RRT 1.51	RRT 2.14	RRT 2.16	RRT 2.20
2.10	0	100	99.4	0.6	-	0.02	-	-	-	-	-
	1	98.2	97.2	2.8	-	0.05	-	0.07	-	-	-
	2	95.8	94.6	5.4	0.35	0.05	-	0.17	-	0.05	-
	4	89.6	87.8	12.2	1.69	0.17	-	0.40	-	0.25	-
	8	73.4	71.6	28.4	6.72	0.12	-	1.49	-	0.68	-
	12	46.0	51.0	49.0	12.14	0.20	0.18	3.48	0.30	1.36	0.60
	15	25.8	33.3	66.8	14.10	0.35	0.20	5.81	0.42	1.82	0.69
2.48	0	100	97.1	2.9	-	-	-	-	-	-	-
	1	93.1	93.4	6.6	0.49	0.52	-	1.39	-	0.33	0.27
	2	81.3	82.9	17.1	1.34	0.62	-	5.06	0.31	1.07	1.30
	3	59.3	60.7	39.3	2.95	0.88	0.29	13.87	0.89	1.88	2.39
	4	38.6	41.7	58.3	4.67	0.93	-	22.97	-	4.75	2.69
	5	23.8	28.9	71.1	2.66	1.51	0.54	31.26	-	5.28	2.27
3.05	0	100	97.1	2.9	-	0.07	-	-	-	-	-
	0.250	95.4	91.7	8.3	-	1.91	-	0.41	0.14	0.59	1.40
	1	69.1	62.3	37.7	-	1.72	0.35	9.23	1.70	2.96	10.31
	1.25	55.6	49.6	50.4	-	1.47	0.72	13.92	2.46	3.93	13.62
	2	33.5	31.1	68.9	-	1.77	1.32	20.71	3.36	5.28	16.25
	3	10.6	13.7	86.3	-	4.39	4.11	28.79	2.71	7.40	12.46
3.49	0	100	99.7	0.3	-	0.18	-	-	-	-	-
	0.167	92.4	83.7	16.3	-	6.07	-	0.35	0.36	2.26	2.40
	0.250	84.1	73.8	26.2	-	6.19	-	1.52	0.91	4.30	6.30
	1	48.5	34.5	65.5	-	2.77	1.14	10.34	4.26	10.28	22.26
	1.25	34.7	24.1	75.9	-	3.44	2.19	11.68	5.51	12.25	24.34
	2	16.1	12.7	87.3	-	3.92	4.74	12.75	8.19	16.44	23.10
4.06	0	100	96.9	3.1	-	0.16	-	0.06	-	-	-
	0.167	89.0	76.4	23.6	-	8.52	-	1.42	0.15	4.49	1.28
	0.333	75.1	58.4	41.6	-	8.13	0.08	4.28	1.02	12.43	6.45
	0.5	63.8	46.4	53.6	-	6.19	0.22	6.08	-	17.52	11.13
	1	37.8	26.1	73.9	-	4.19	1.18	8.10	5.76	29.22	17.48
	1.25	28.1	19.5	80.5	-	4.99	1.66	8.14	8.43	33.88	16.57

Abbrevitations: 4MA – 4-morpholinoaniline; Deg = Degradation Products; RRT = Relative Retention Time (normalized to the 4-morpholinoaniline retention time)

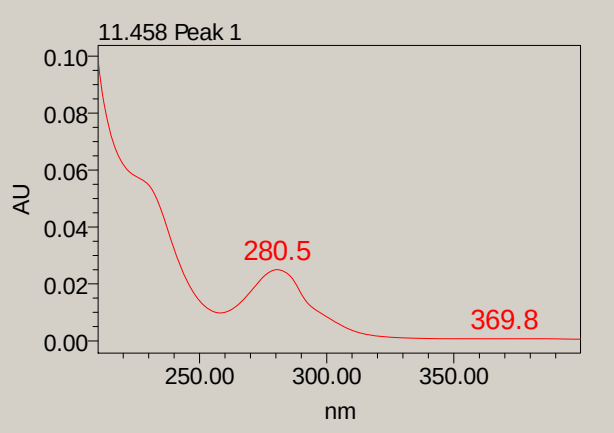
[1] This table lists only degradation products that achieve % integrated area of 5% or greater

[2] Relative resonse factors were not determined for these degradation products, thus could not be applied to correct for differences in extinction coefficients

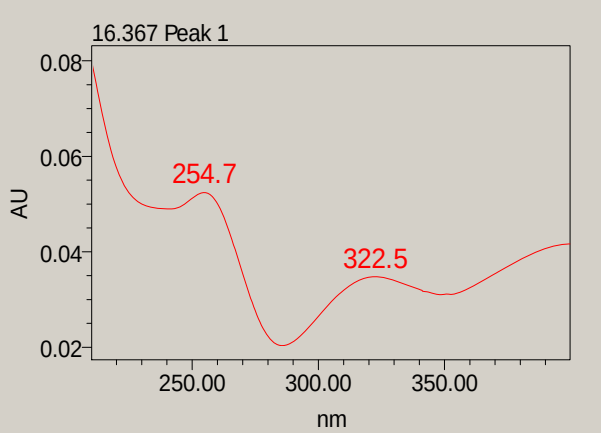
RRT 0.75



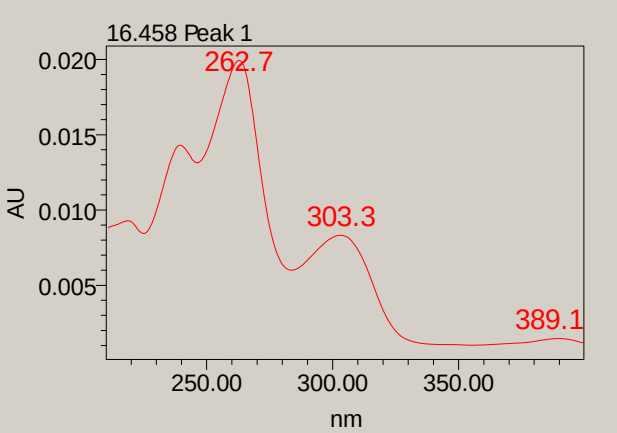
RRT 1.51



RRT 2.14



RRT 2.16



RRT 2.20

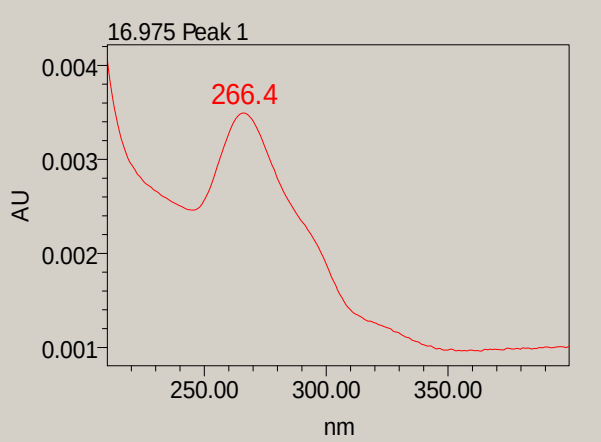


Figure 16. UV/Vis spectra of major degradation products observed in aqueous solutions of 4-morpholinoaniline at pH 2 – 4

Purification and Characterization of 4-Morpholinoaniline Major Degradation Product at pH 4

Aqueous solutions of 4-morpholinoaniline at pH 4.0 – 4.5 (pH adjusted using HCl) were prepared at concentrations of 2.2 – 11.2 mM and stressed at 60 ºC to encourage formation of degradation products. When at least 90% of the 4-morpholinoaniline had degraded, the reaction solutions were concentrated by lyophilization and MS-guided prep-HPLC was used to isolate and purify the major degradation product in solution, which was recovered in low yield. Analysis of the purified product by U/HPLC-UV-QDa revealed that the product was ~96.5% pure with a monoisotopic mass (m/z) of 352.3 and a UV/Vis spectrum consistent with that of the degradation product with RRT 2.16. High resolution mass spectrometry of the purified product by U/HPLC-UV-QTOF revealed an abundant $(M+H)^+$ peak with a monoisotopic mass (m/z) of 352.1669. The Find Compound by Formula algorithm in Mass Hunter Qualitative Analysis software assigned the chemical formula $C_{20}H_{21}N_3O_3$ to the purified product with a fit score of 93.03%.

Figure 17 shows the ^1H NMR spectrum obtained for the purified 4-morpholinoaniline degradation product with RRT 2.16. The spectrum shows five separate chemical shifts for the estimated 21 hydrogen atoms predicted by high-resolution MS, reported as chemical shifts (δ) in ppm, with multiplicity and integration in parentheses: δ 3.6 (s, 8H), δ 3.8 (s, 8H), δ 6.7 (s, 2H), δ 7.2 (s, 2H), and δ 7.4 (s, 2H). The chemical shifts at δ 3.6 and 3.8 correspond to the protons on the morpholine rings, and the integration and multiplicity results suggest that two morpholine rings are present in the degradation product. The chemical shifts at δ 6.7 – 7.4 correspond to protons in an aromatic system.

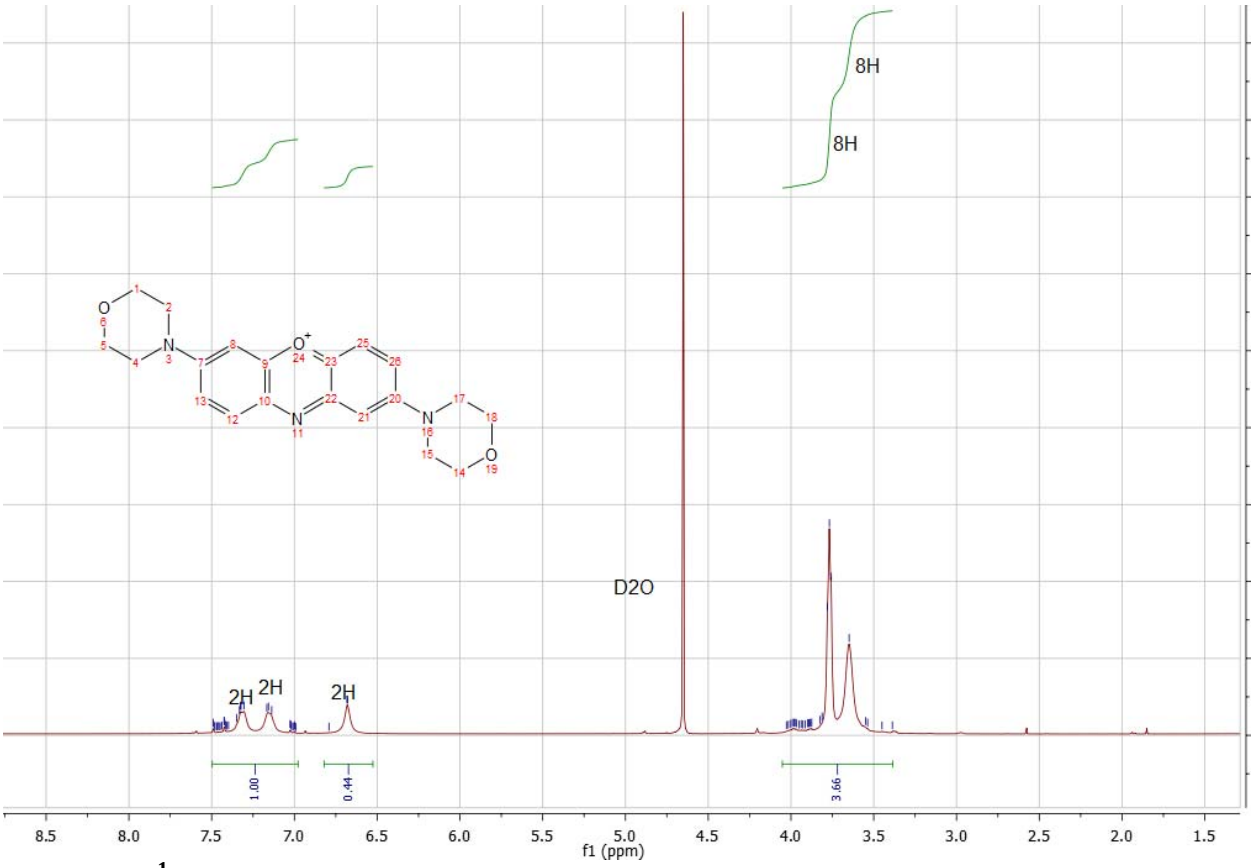


Figure 17. ^{1}H NMR spectrum for the 4-morpholinoaniline degradation product with RRT 2.16

Figure 18 shows the ^{13}C NMR spectrum obtained for the purified 4-morpholinoaniline degradation product with RRT 2.16. The spectrum shows fourteen separate chemical shifts for the estimated 20 carbon atoms predicted by high-resolution MS. The chemical shifts (δ) are reported in ppm as follows: 47.8 (4C), 66.0 (4C), 96.9, 111.9, 114.7, 117.6, 120.6, 127.1, 133.0, 133.6, 149.0, 156.8, 162.5, and 162.8. The chemical shifts are assigned to carbon atoms in the proposed degradation product structure in Figure 18.

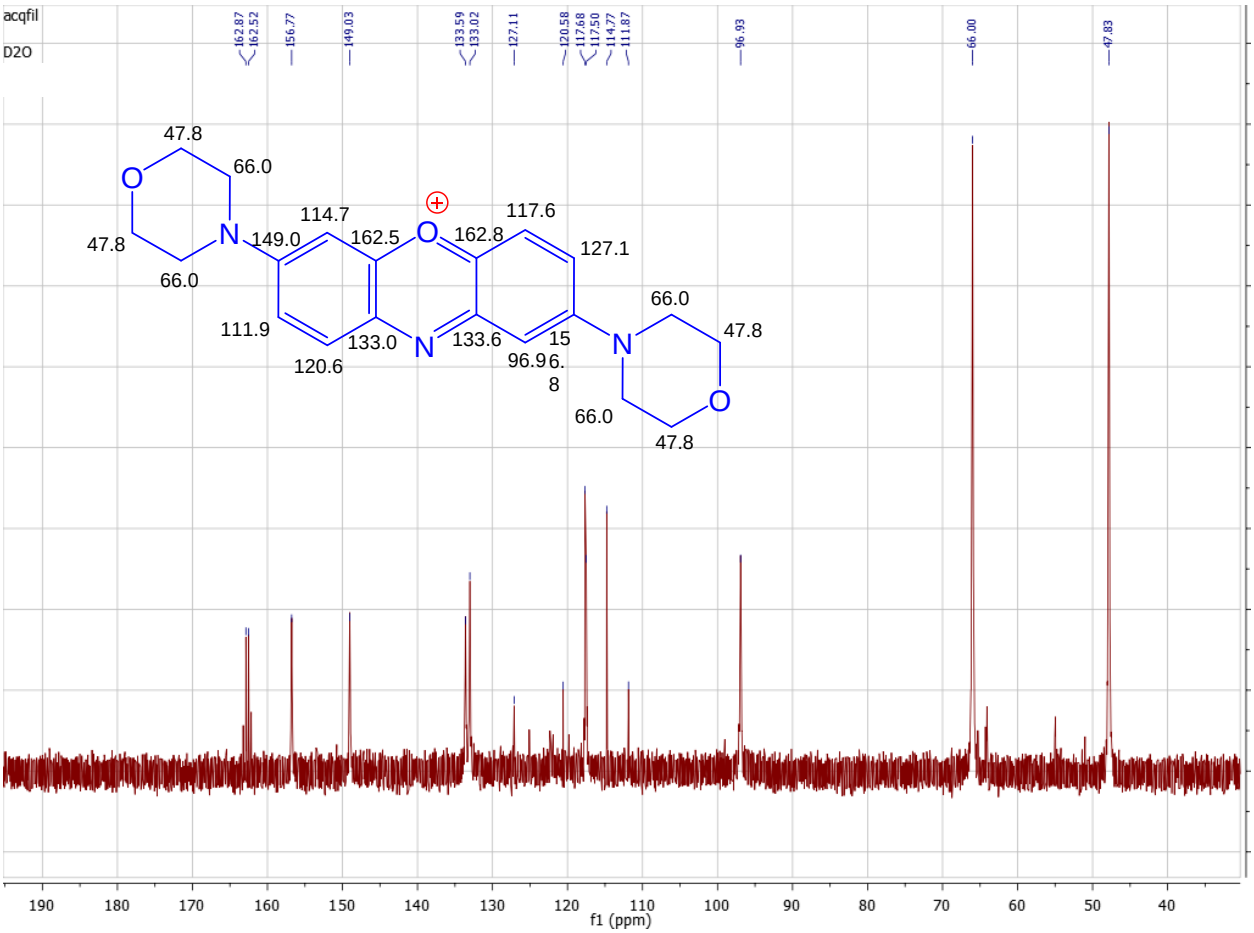


Figure 18. ^{13}C NMR spectrum for the 4-morpholinoaniline degradation product with RRT 2.16

Formation of the 4-Morpholinoaniline Degradation Product with RRT 2.16 in the Presence of DTPA

The decrease in concentration of 4-morpholinoaniline and concomitant increase of the degradation product with RRT 2.16 were monitored over time in aqueous solution at pH 4 (10 mM acetate, I = 0.15 M, NaCl) in the presence of 0, 25, and 50 µM DTPA. Figure 19 shows changes in 4-morpholinoaniline concentration and of the peak area of the product with RRT 2.16 as a function of time. The results demonstrate that the inclusion of DTPA into the solution had no effect on the rates of 4-morpholinoaniline degradation and formation of the degradation product with RRT 2.16.

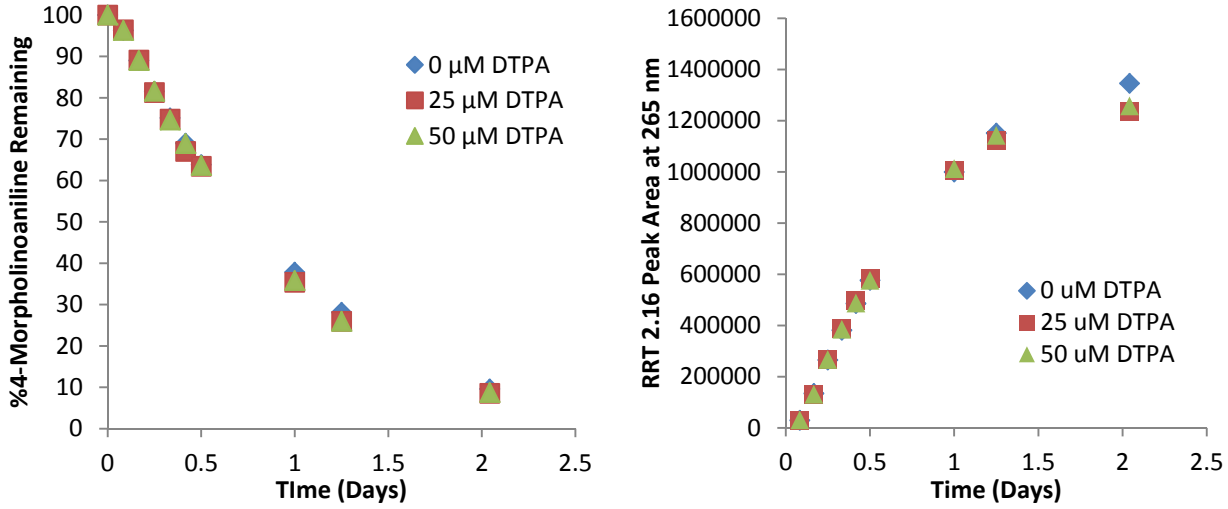


Figure 19. 4-morpholinoaniline concentration (left) and the degradation product with RRT 2.16 peak area (right) in aqueous pH 4 solution containing 0, 25, and 50 µM DTPA

Reaction of 4-Morpholinoaniline with 4-Morpholinophenol in pH 4 Solution

To determine if the degradation product with RRT 2.16 was formed by reaction of 4-morpholinoaniline with 4-morpholinophenol, 4-morpholinoaniline: 4-morpholinophenol solutions were prepared at molar ratios of 1:1, 1:2, and 1:4 in water adjusted to pH 4 with HCl and stressed at 60 ºC. Control solutions of 1.12 mM 4-morpholinoaniline and 1.12 mM 4-morpholinophenol were prepared in water adjusted to pH 4 with HCl and stressed at 60 ºC. The concentrations of 4-morpholinoaniline and 4-morpholinophenol and the peak area of the degradation product with RRT 2.16 were monitored with respect to time, and the results are shown in Figure 20 and Figure 21. The degradation of 4-morpholinoaniline and formation of degradation product with RRT 2.16 showed no trend with regard to the presence and concentration of 4-morpholinophenol in solution. In the absence of 4-morpholinoaniline, 4-morpholinophenol degraded rapidly into hydroquinone, which is consistent with the degradation pathway observed at pH 1.2. In the presence of 4-morpholinoaniline at pH 4, the degradation of 4-morpholinophenol slowed significantly and hydroquinone formation was not observed.

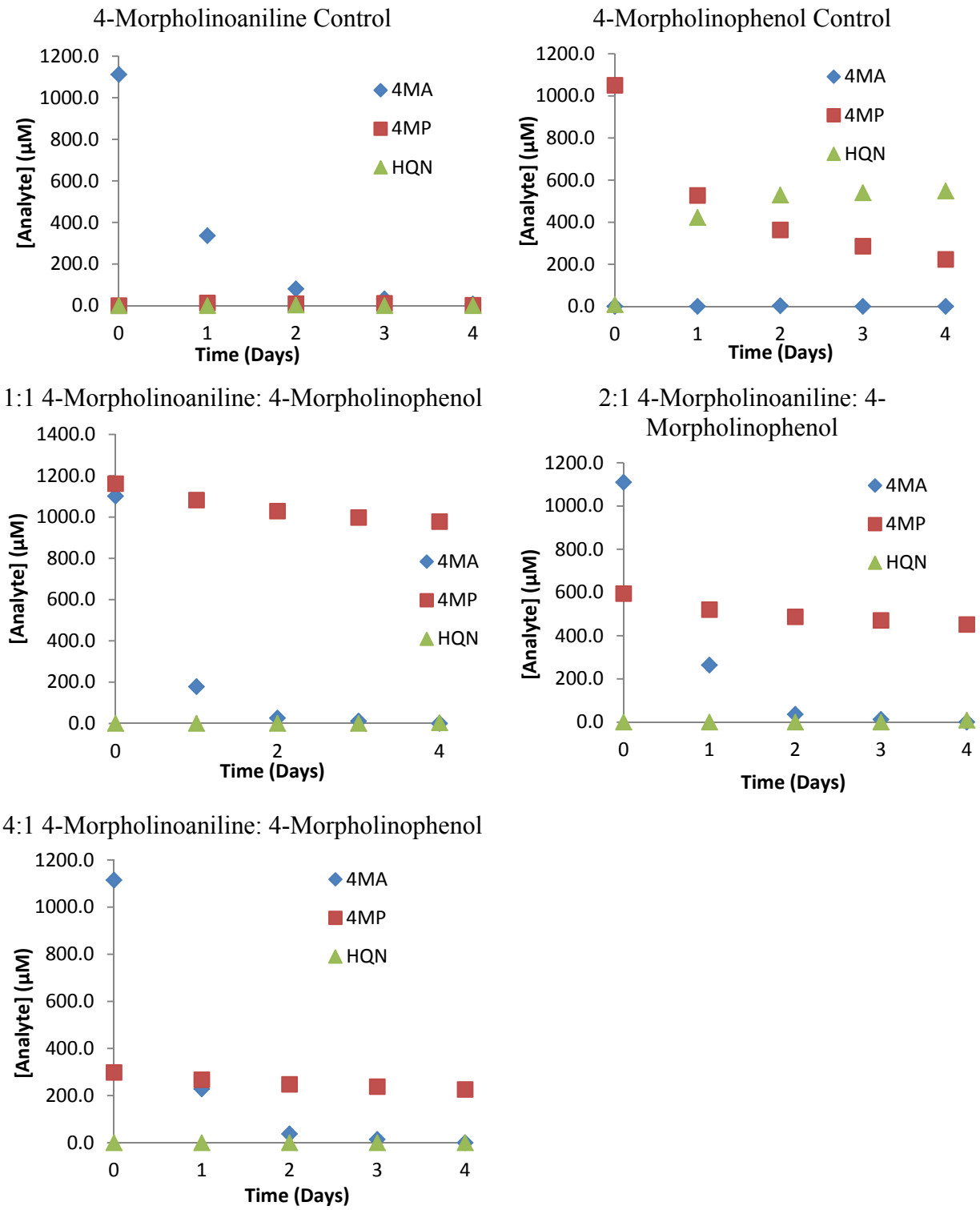


Figure 20. Concentration of 4-morpholinoaniline and 4-morpholinophenol in aqueous pH 4 solutions stored at 60 °C

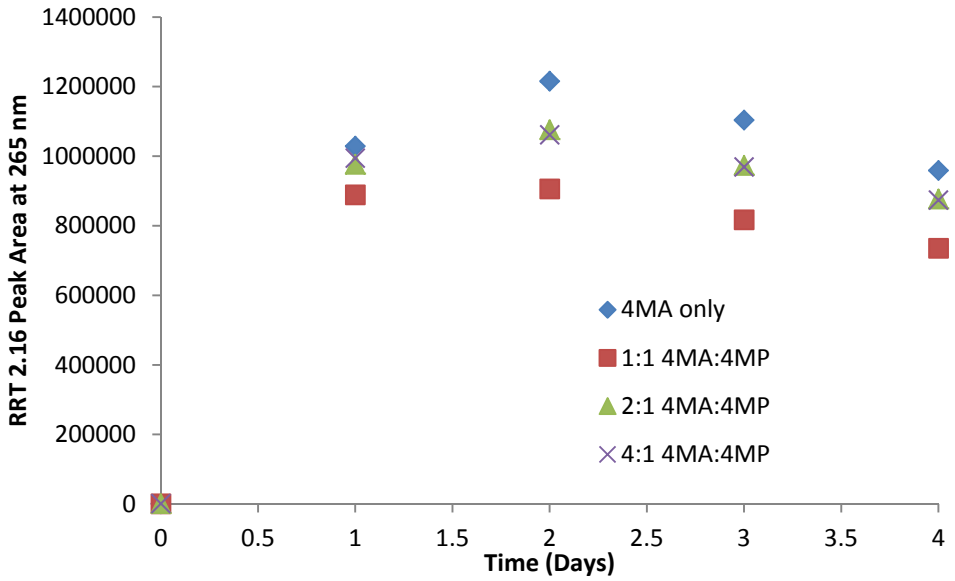


Figure 21. Peak area of degradation product with RRT 2.16 in aqueous pH 4 solutions of 4-morpholinoaniline and 4-morpholinophenol stored at 60 ºC

Discussion

In aqueous solutions at pH ≤ 1.50, 4-morpholinoaniline degradation proceeded by pseudo-first order kinetics to yield 4-morpholinophenol, which subsequently degraded by another pseudo-first order rate reaction to yield hydroquinone. Electrochemical oxidation experiments by Beiginejad, Nematollahi, and Khazalpour[8] also showed that 4-morpholinoaniline can degrade to hydroquinone in solution at low pH. Whereas their reaction required harsh oxidative conditions to produce an unstable *p*-quinone-diimine that subsequently degraded to p-quinoneimine then quinone via hydrolytic reactions, the reaction studied herein occurred in aqueous solution at low pH without oxidative catalysis and proceeded via the formation and subsequent hydrolysis of 4-morpholinophenol.

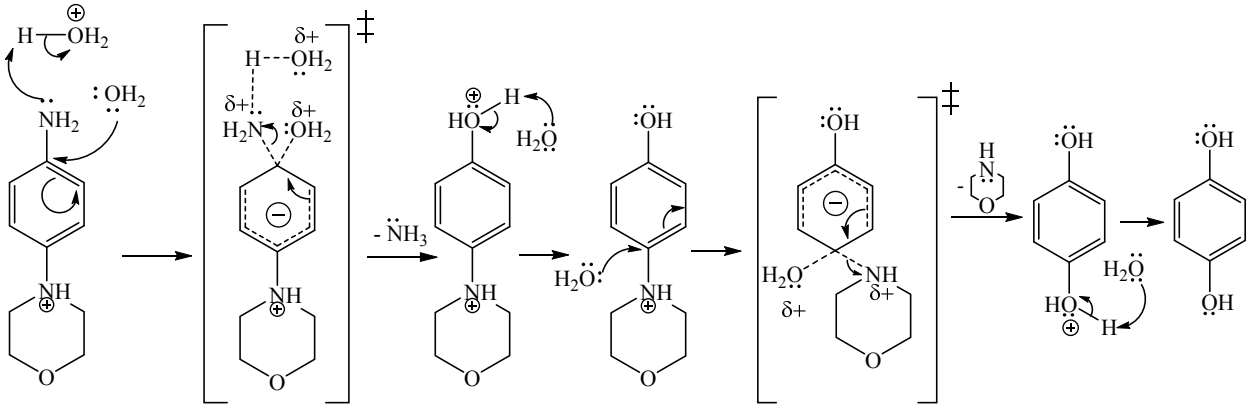
Based on the chemical structures of these degradation products, displacement of the aniline ammonia by hydroxide via nucleophilic aromatic substitution (S_NAr) is the proposed mechanism of 4-morpholinoaniline degradation in acidic aqueous solution.

Nucleophilic aromatic substitution reactions have been studied and reviewed extensively since the 1950s.[11, 12, 13] The majority of these reactions described in the literature have been studied in basic media on aromatic compounds containing strong electron-withdrawing groups. These reactions occur through one of the following mechanisms: (1) addition-elimination mechanisms via the formation of a resonance-stabilized intermediate called the Meisenheimer complex, or (2) via concerted S_N2-type mechanisms during which the addition of the nucleophile and displacement of the leaving group occur simultaneously.[14, 15] Less common nucleophilic aromatic substitution reactions have been shown to proceed by an S_N1-type elimination-addition pathway via the formation of a phenyl cation intermediate.[15, 16]

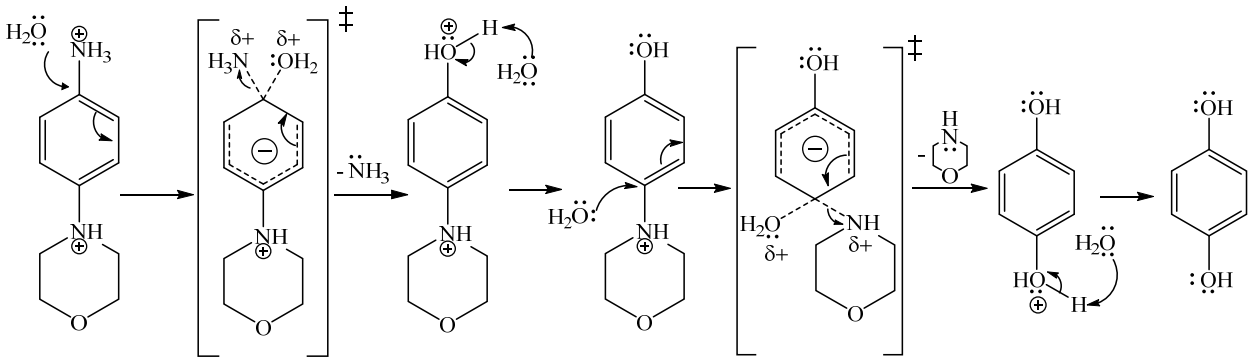
The current system provides an interesting case study, as examples of nucleophilic aromatic substitution reactions under similar reaction conditions could not be found in the literature. Whereas many published nucleophilic aromatic substitution reactions occur in basic media via the formation of a resonance-stabilized Meisenheimer complex, the displacement of the aniline ammonia by water in the current system is favored in low pH conditions. Specifically, the reaction is favored at pH $\leq pK_{a1}$ where one of the two ionizable groups of 4-morpholinoaniline is protonated and the other ionizable group is in rapid protonation/deprotonation equilibrium. This is consistent with the results summarized in Table 10, which show that specific acid-catalyzed degradation of the singly protonated species or water catalyzed degradation of the doubly protonated species contribute the most to the observed rate contstant.

The observed pH-dependence of the degradation of 4-morpholinoaniline to 4-morpholinophenol allows for the assignment of the experimentally determined dissociation constants. The degradation of 4-morpholinoaniline to 4-morpholinophenol is disfavored when pH exceeds pK_{a1}. In other words, the reaction shuts down when the majority of 4-morpholinoaniline molecules bear only one positive charge. If pK_{a1} is attributed to the aniline ammonia, the leaving group in the aromatic substitution reaction changes from NH_3 to $:NH_2^-$ at pH > 1.8, where the reaction is disfavored. As discussed by Bunnet and Zahler,[12] $:NH_2^-$ is a poor leaving group in nucleophilic aromatic substitution reactions. Furthermore, the dissociation of the negatively charged $:NH_2^-$ into the highly acidic media studied in the current system would be energetically unfavorable. Because protonation of R-NH_2 to R-NH_3^+ on the aniline ring enhances both the viability of the leaving group and the energetic favorability of the reaction, we assign pKa1 to the ammonia nitrogen on the aniline ring and pKa_2 to the morpholine nitrogen, which is consistent with similar substituted anilines and aryl diamines (Table 4).

With the pH-dependence, pK_a assignment and rate equations in mind, the reaction mechanisms in Scheme 1 and Scheme 2 are proposed for the specific acid-catalyzed degradation of the singly protonated 4-morpholinoaniline species and for the water-catalyzed degradation of the doubly protonated 4-morpholinoaniline species, respectively. In these concerted S_N2-type addition-elimination mechanisms, the reaction of water with 4-morpholinoaniline forms an intermediate structure in which the developing negative charge on the aniline ring is electrostatically stabilized by the positive charge on the morpholino nitrogen and the positive charge on the nucleophilic water molecule. In the case of the water-catalyzed degradation of 4-morpholinoaniline in the doubly protonated state, the positively charged aniline ammonia could provide additional electrostatic stabilization to the electron-rich aniline ring; however, this positive charge could also electrostatically hinder the nucleophilic attack of the water molecule. The observed pseudo-first order kinetics for this reaction provide some support for the proposed mechanisms: though S_N2-type reactions proceed via second-order kinetics, pseudo-first order kinetics have been observed when the nucleophilic species is in abundance, such as in reactions involving solvolytic displacement of the leaving group.[17, 18, 19, 20]



Scheme 1. Specific acid-catalyzed degradation of 4-Morpholinoaniline in the singly protonated state: hydrolytic displacement of the 4-morpholinoaniline ammonia by nucleophilic aromatic substitution to yield 4-morpholinophenol, which is further hydrolyzed to yield hydroquinone and morpholine



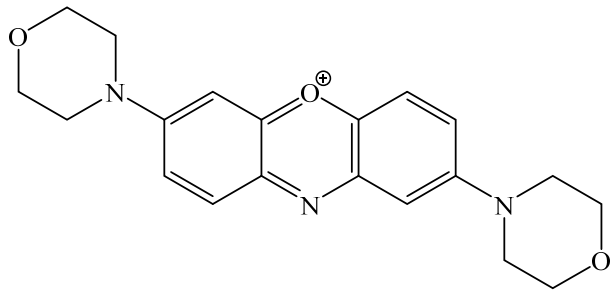
Scheme 2. Water catalyzed degradation of 4-Morpholinoaniline in the doubly protonated state: hydrolytic displacement of the 4-morpholinoaniline ammonia by nucleophilic aromatic substitution to yield 4-morpholinophenol, which is further hydrolyzed to yield hydroquinone and morpholine

One could alternatively imagine these degradation reactions proceeding via an S_N1 mechanism in which the positively charged aniline ammonia acts as a leaving group to yield a phenyl carbocation which would rapidly acquire a nucleophilic water molecule. Although the positively charged ammonia is an appropriate leaving group in S_N1 reactions, the resulting phenyl carbocation is known to be a high-energy, unstable intermediate[16]**Error! Bookmark not defined.** and its formation is not supported by the activation parameters shown in Table 11.

The mechanisms shown in Scheme 1 and Scheme 2 become a minor pathway that accounts for decreasing amounts of mass in the system as pH exceeds pKa_1 (pH 1.8). This is evidenced by reduced concentrations of 4-morpholinophenol and hydroquinone with increasing pH (Table 7 and Table 8), a change in the kinetics of 4-morpholinoaniline degradation from pseudo-first order to something more complicated than zero-, first-, or second-order (Figure 11), and the formation of new degradation products whose concentration grows with increasing pH (Table 12). These observations indicate a pH-dependent change in mechanism incurred by the deprotonation of 4-morpholinoaniline.

The formation and growth of a specific major degradation product of 4-morpholinoaniline observed at pH 2 – 4, the degradation product with RRT 2.16, corresponded to changes in the

appearance of the reaction solutions from clear and colorless to dark blue (Figure 12). The UV/Vis spectrum obtained for this product (Figure 16) was indicative of a highly conjugated structure. This product was isolated and purified, and high resolution mass spectrometry assigned a mass of 351.16 and a chemical formula of $C_{20}H_{21}N_3O_3$. ^{1}H NMR analysis (Figure 17) and ^{13}C NMR analysis (Figure 18) of the degradation product with RRT 2.16 indicate that the structure contains two morpholine rings and has several carbon atoms and protons in an aromatic system. Based on the results described above, chemical structure in Figure 22 is the proposed 4-morpholinoaniline degradation product with RRT 2.16 that is observed in aqueous solutions at pH 2 - 4.



Chemical Formula: $C_{20}H_{22}N_3O_3^+$
Exact Mass: 352.17

Figure 22. Proposed chemical structure for the 4-morpholinoaniline dimerization product observed in aqueous solution at pH 2 – 4.

Despite the elucidation of the potential structure of the degradation product with RRT 2.16, its mechanism of formation has not been solved. Considering the chemical formula and proposed structure, a dimerization reaction between 4-morpholinoaniline and 4-morpholinophenol was hypothesized and disproven when the presence of 4-morpholinophenol in aqueous pH 4 solutions of 4-morpholinoaniline had no effect on the degradation rate of 4-morpholinoaniline (Figure 20) or on the rate and extent of formation of the degradation product with RRT 2.16 (Figure 21). Metal catalysis was also ruled out when the inclusion of DTPA in

aqueous solutions of 4-morpholinoaniline at pH 4 had no impact on the rate of 4-morpholinoaniline degradation or on the rate and extent of formation of the degradation product with RRT 2.16 (Figure 19).

Though 4-morpholinoaniline dimerization has been described in the literature, the publications failed to provide much insight into the current reaction system. Hahn, *et al.*[21] implicate 4-morpholinoaniline dimerization and trimerization in color changes that were observed in their aqueous reaction solutions. The group's hypothesized dimer, an azo compound formed by the reaction of the aniline nitrogens of two 4-morpholinoaniline molecules, is based on UV-Vis λ_{max} of 254 and 412 nm and a $[M+H]^+$ of 353.1 measured by LC/MS; however, structural characterization of this compound was not performed, and there is little discussion of the conditions under which the color change was observed. Furthermore, their hypothesized dimer is not supported by the mass and chemical formula assigned to the degradation product with RRT 2.16. Similarly, the dimer produced by the Michael-type addition reaction observed in cyclic voltammetry experiments by Esmaili and Nematollahi[7] has a mass and chemical formula that are inconsistent with the results presented herein.

Conclusions

4-Morpholinoaniline is a chemical moiety that has been widely used in screening and development of small molecule kinase inhibitors (SMKIs) because of its ability to form hydrogen bonds with ATP binding pockets in kinases and because of improved solubility imparted by the morpholine ring. This moiety has been implicated in complicated degradation pathways observed for two late-stage compounds that were developed as acidic salts. Despite its wide use in SMKI screening and development, there is no literature on the degradation pathways and degradation products of 4-morpholinoaniline in aqueous solutions.

The reactivity of 4-morpholinoaniline with water was examined in aqueous solutions at pH 1 – 4. Two dissociation constant values were identified for 4-morpholinoaniline using spectrophotometric titration: pK_{a1}, assigned to the aniline ammonia nitrogen, was 1.8 and pK_{a2}, assigned to the morpholine nitrogen, was 4.9. At $pH \leq pK_{a1}$, 4-morpholinoanilne reacted with water to form 4-morpholinophenol, which further degraded to hydroquinone. The hydrolytic degradation reactions of 4-morpholinoaniline and 4-morpholinophenol exhibited pseudo-first order kinetics under the studied reaction conditions. 4-Morpholinophenol is proposed to form by nucleophilic aromatic substitution of the aniline ammonia by water via an intermediate whose electron-rich aromatic ring is electrostatically stabilized by both the positively charged protonated morpholine nitrogen and the positive charge that forms on the incoming nucleophilic water molecule.

As pH exceeded pK_{a1} and the aniline ammonia nitrogen was deprotonated, the nucleophilic aromatic substitution reaction was disfavored and accounted for less than 10% of 4-morpholinoaniline degradation at $pH \geq 2.5$. Instead, 4-Morpholinoaniline degradation in these higher pH aqueous solutions yielded multiple degradation products, and the kinetics of 4-

morpholinoaniline degradation were more complicated than zero-, first-, or second-order. Characterization and structural identification of the major degradation product observed in these higher pH solutions yielded a highly conjugated molecule whose molecular weight and proposed chemical formula suggest that it is the product of a dimerization reaction. The 4-morpholinoaniline degradation mechanisms in higher pH solutions were not resolved; however, metal catalysis and direct reaction of 4-morpholinoaniline with 4-morpholinophenol were disproven.

The findings described herein have provided some clarity to 4-morpholinoaniline degradation in acidic solutions, but have also opened several questions about the degradation mechanisms as pH is increased. Additional studies could be undertaken to resolve the mechanism or mechanisms by which 4-morpholinoaniline degrades in aqueous solutions at pH > pK_{a1}. Structural identification of the other major degradation products observed in these solutions could allow for the determination of intermediates and provide insight into the mechanism of formation of the degradation product with RRT 2.16. Potential oxidative mechanisms could be assessed through controlled experiments in the presence of different antioxidants such as oxygen scavengers, sacrificial reductants, and chain terminators. 4-Morpholinoaniline degradation at pH > pK_{a1} could be performed in ^{18}O water to determine the origin of the third oxygen observed in the degradation product with RRT 2.16. Finally, this work could be expanded into solutions at pH > 4 to determine if increased pH results in a change in rate and extent of formation of the degradation product at RRT 2.16 or if the deprotonation of the morpholine nitrogen at pH 4.9 incurs new degradation pathways.

When this work was initiated, the morpholine ring was the proposed center of chemical degradation; however, this work has shown that chemical degradation of 4-morpholinoaniline is

centered on the ammonia nitrogen on the aniline ring. Because this group becomes a secondary amino group when incorporated into larger active pharmaceutical molecules, the low-pH degradation pathways of 4-morpholinoaniline characterized herein may not apply directly to the larger molecules that contain this structural motif; however, the findings from this work can inform manufacturing processes and storage conditions of active pharmaceutical ingredients that contain 4-morpholinoaniline. For example, this work showed that 4-morpholinoaniline is prone to degradation in aqueous solutions at acidic pH; however, the rates of 4-morpholinoaniline degradation in these conditions were slower than the rates of 4-morpholinoaniline degradation observed at pH > pK_{a1}. This observation suggests development of these active pharmaceutical molecules as acidic salts is favorable rather than detrimental: instead of encouraging or accelerating degradation, the low-pH microenviroment caused by the acidic salts prevents the complicated degradation pathway or pathways that incur several degradation products including the degradation product with RRT 2.16 that was characterized as part of this work. Additionally, the reactivity of water with both 4-morpholinoaniline and 4-morpholinophenol observed under all analyzed pH conditions indicates that active pharmaceutical molecules containing this chemical moiety should be stored under desiccated conditions.

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