Direct Evidence of 2-Cyano-2-Propoxy Radical Activity During AIBN-Based Oxidative Stress Testing in Acetonitrile–Water Solvent Systems

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ABSTRACT: Oxidative susceptibility testing was performed on a drug substance containing a methoxy-naphthalene moiety. 2,2′-azobisisobutyronitrile (AIBN) was employed to initiate peroxy radical oxidation to mimic autoxidation processes. In acetonitrile (ACN)–water solvents, three major degradation products are formed. However, addition of small amounts of methanol to the solvent system completely eliminated the observed degradation products. To understand this effect, the structures of the three degradants have been elucidated using nuclear magnetic resonance, liquid chromatography–tandem mass spectrometry, and accurate mass Fourier transform ion cyclotron resonance mass spectrometry. One degradant structure definitively proves the degradation resulted from *alkoxy* radicals (2-cyano-2 propoxy radical) arising from the disproportionation of the tertiary AIBN-derived peroxy radicals, rather than from the intended action of the AIBN *peroxy* radicals themselves. The reaction occurs over a wide range of AIBN and drug substance concentrations. This "protective effect" of several percent methanol by volume is rationalized by known methanol H atom donation rates to similar *tert*-butoxy and cumyloxy radicals (ca. 10 M^{-1} s^{-1}) and the high methanol concentration relative to the dilute substrate being investigated. This work confirms recent proposals for addition of at least about 10% methanol to the standard ACN–water AIBN stress testing diluent to insure that only the desired peroxy radical activity is present during the oxidative stress test. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:1554–1568, 2013

Keywords: analysis; antioxidants; chemical stability; forced conditions; free radicals; oxidation

INTRODUCTION

Pharmaceutical stress testing has become an integral part of the drug development process, and numerous literature reports[1–5] and several book contributions have been dedicated to this subject.[6,7] A major aspect of such stress testing is carrying out simple solution-based studies, which can reveal potential hydrolytic and oxidative liabilities of the active pharmaceutical ingredient (API). Predicting the oxidative behavior of formulated API's in solid dosage forms under long term stability conditions from the solution oxidative stress test results has remained challenging. The primary reason for this is that the causative oxidants in the solid dosage form may not be well understood, in combination with a less than complete understanding of the actual "oxidants" generated in oxidative stress testing systems. Thus improving the predictive power of oxidative stress testing methodologies continues to be a subject of interest[8–16].

The work herein is rooted in this latter topic, and in particular in understanding the actual oxidants present during early stage, solution phase oxidative screening experiments designed to expose drug molecules to peroxy radicals. Peroxy radical-based oxidation (chain oxidation or sometimes referred to as free radical autoxidation) is a common oxidative pathway as demonstrated by the ubiquitous use of phenolic chain breaking antioxidants such as 2,6-di-*tert*-butyl-4-methoxyphenol and 2,6-di-*tert*-butyl-4-methylphenol, which quench peroxy

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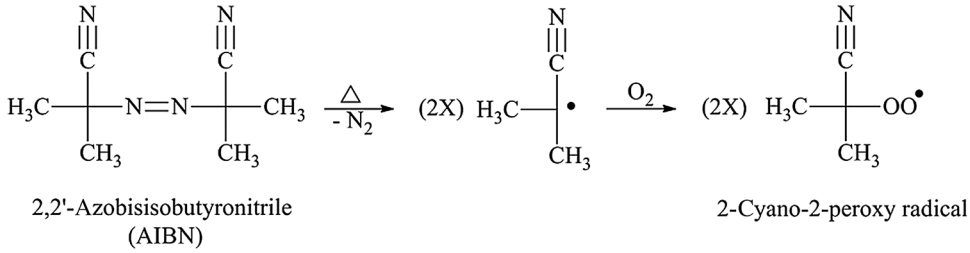


Figure 1. Thermal degradation of AIBN to form peroxy radicals in solution.

radicals.[10,17–19] A survey of stress testing industry practices nearly 10 years ago found a growing trend in the use of azonitrile "radical initiators" such as AIBN (2,2′-azobisisobutyronitrile) to create peroxy radical mediated oxidation in the oxidative screening experiment.[20] This trend has likely continued since that time. These experiments rely on thermal decomposition of an azonitrile compound to generate a peroxy radical oxidant (2-cyano-2-peroxy radicals) as shown in Figure 1 for AIBN. Generation of significant degradant products from this type of AIBN oxidative screening experiment is then interpreted as signaling the drug molecule's susceptibility toward autoxidation. In this regard, the absence of degradant peaks in this oxidative test is a highly desired outcome, as it proves no chemical bonds are reactive with the highly selective peroxy radical.[16,17,21] This, in turn, portends no peroxy radical driven oxidation will be observed in formulated dosage forms over longer term drug product ICH stability studies.

The desire to appropriately signal "nonreactivity" in the subject selective oxidative screening test is the central motivation for the current work. The potential problem comes from the way the azonitrile-based experiment has evolved in the early phase, pharmaceutical oxidative screening context. Dilute API concentrations are typically used (ca. 0.1–1 mg/mL) along with 20–100 mol % (or higher) of the azonitrile compound. Typical solvents used historically were acetonitrile (ACN)–water mixtures. Many of the compounds being screened will *not* be reactive with peroxy radicals. Under these conditions, the disproportionation of the 2-cyano-2-peroxy radicals formed in Figure 1 cannot be neglected. The issue is that the 2-cyano-2-peroxy radicals in Figure 1 are tertiary, and as such disproportionation will result in formation of a new, much stronger oxidant as shown in Figure 2.[21] The 2-cyano-2 propoxy radical thus formed is an alkoxy radical. Similar alkoxy radicals such as *tert*-butoxy and cumyloxy radicals have reaction rates with organic substrates which are about 10^4–10^5 times faster than the analogous peroxy radicals.[22] These rates reflect the ~20 kcal/mol higher bond dissociation energies (BDEs) of the RO—H bonds relative the ROO—H bond as shown in Table 1. The presence of this alkoxy radical in solution could destroy the intended peroxy radical selectivity of this oxidative screening test giving "false positive" results. This problem is not unique to AIBN but is general for all of the azonitrile compounds commonly used for oxidative susceptibly studies in that they all generate tertiary peroxy radicals similar to that shown for AIBN in Figure 2. Werber et al.[23] recently analyzed the degradation products of 2,2′-azobis(2-amidinopropane) dihydrochloride in aqueous solution in the absence of any added substrate, and concluded that the alkoxy radical (analogous to that shown in Figure 2 for AIBN) was the prominent radical species in solution. Nelson et al.[15] have recently reported AIBN and ACVA [4,4′-azobis(4-cyanovaleric acid)] oxidative screening of cumene (used as a model drug substance). Through several lines of indirect evidence, they attributed oxidative degradation products of cumene to the activity of 2-cyano-2-propoxy radical—rather than the expected peroxy radicals. Similar to our observations with developmental drug compounds,[24,25] Nelson and coworkers showed a 5–10-fold reduction in the cumene oxidation products attributed to alkoxy radical activity upon the addition of only a few volume percent methanol to the ACN solvent-based AIBN experiment.

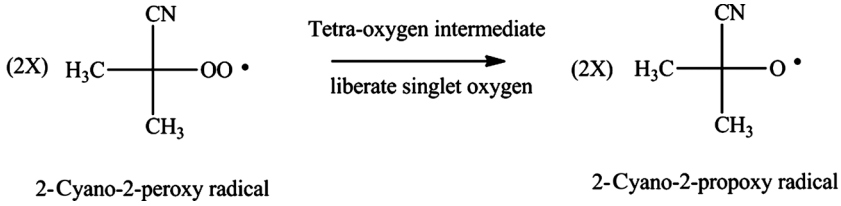


Figure 2. Disproportionation of tertiary 2-cyano-2-peroxy radical.

Table 1. Calculated (∗) and Experimental Bond Dissociation Energies (BDEs)

Compound	Bond	BDE (kcal/mol)
2-Cyano-2-hydroperoxide	$NC(CH_3)_2COO—H$	83[a]
2-Cyano-2-propanol	$NC(CH_3)_2CO—H$	106[a]
tert-Butanol	$(CH_3)_3CO—H$	105[b]
Cumyl alcohol	$C_6H_6(CH_3)_2CO—H$	∼103[c]
Methanol	$HO(CH_2)—H$	96[d]
Acetonitrile	$NC(CH_2)—H$	94[b]

[a] Calculated as described in *Experimental* section.
[b] Ref. 28.
[c] Ref. 29.
[d] Ref. 30.

The current work for the first time unambiguously proves the presence and activity of the 2-cyano-2-propoxy radical in ACN–water solvent systems during pharmaceutically relevant oxidative screening experiments. AIBN and ACVA oxidative stressing of compound 1 (Fig. 3) in ACN–water mixtures show about 20% degradation of compound 1, which is eliminated by addition of only a few volume percent methanol. Full structural elucidation of the degradation products provides direct evidence of 2-cyano-2-propoxy radical activity. The dependence of degradant yields on AIBN and drug concentration (in the absence of methanol) is examined and discussed. The protective effect of methanol is rationalized, and the potential impact of methanol addition to the wide range of mole percent AIBN levels commonly used in oxidative screening experiments is discussed and recommendations made.

EXPERIMENTAL

Materials and Reagents

The drug substance compound 1 was synthesized in 98% purity by Merck and Company, Inc. (Rahway, New Jersey). AIBN was obtained in 98% purity from Sigma–Aldrich (St. Louis, Missouri) and was used as received. Nuclear magnetic resonance (NMR) solvents CD_3CN and d_6-DMSO were obtained in 99.96%D isotopic purity from Cambridge Isotope Labs. All other reagents were of analytical grade quality or higher.

METHODS

High-Performance Liquid Chromatography and Oxidation Conditions

2,2′-azobisisobutyronitrile and ACVA oxidative stresses were typically performed in 50:50 ACN–water solvents with 0%–20% methanol by volume added unless otherwise indicated. Azonitrile compound was present at 5 mM (0.8 mg/mL) and compound 1 present at 0.1 mM (0.05 mg/mL) unless otherwise specified. The stress solutions were held at 40°C for 3 days to provide adequate time for oxidation to occur. An Agilent 1100 HPLC equipped with a photodiode array detector and a thermally controlled column compartment and autosampler was used for all chromatographic analysis. Chromatographic analysis was performed using an isocratic mobile phase [29/71 (v/v) 0.1% formic acid–ACN] using an Agilent Zorbax 4.6×100 mm, 1.8 μm SB-C18 column held at 60°C with a mobile phase flow rate of 1.5 mL/min and a run time of 4 min.

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR–MS) and NMR analyses of each degradation product were conducted using isolated and purified samples of each degradation product prepared by preparatory scale high-performance liquid chromatography (HPLC). Preparatory HLPC was performed with an Agilent 1100 preparatory HPLC system. Degradant 1 was isolated using a Phenomenex Luna 21.2 × 250 mm, 5 μm C8 column held at ambient temperature with an isocratic

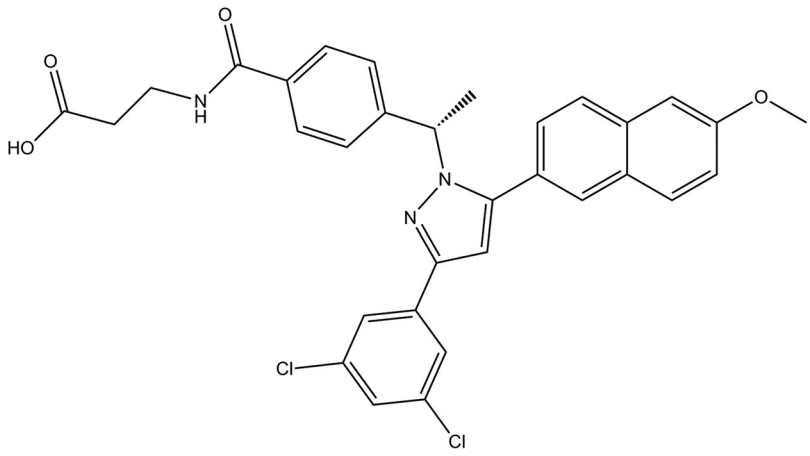


Figure 3. Compound 1 - (*S*)-3-(4-(1-(3-(3,5-dichlorophenyl)-5-(6-methoxynaphthalen-2-yl)-1*H*-pyrazol-1-yl)ethyl)benzamido)propanoic acid; an API containing a methoxy-naphthalene moiety.

mobile phase consisting 60/40 ACN–0.1% formic acid pumped at a flow rate of 5 mL/min with a 5 mL injection volume. Degradants 2 and 3 were isolated using an Agilent Zorbax 9.4 × 250 mm, 5 μm SB-CN column held at ambient temperature with an isocratic mobile phase consisting 50:50 ACN–0.1% formic acid pumped at a flow rate of 5 mL/min with a 5 mL injection volume. Solvent was removed from the preparatory fractions by rotary evaporation followed by lyophilization.

Liquid Chromatography–Mass Spectrometry

A similarly equipped Agilent 1100 HPLC system was interfaced with a Finnigan LCQ Deca-XP quadrupole ion trap for nominal mass and MS^n analysis of degradation products. A Bruker Daltonics 7T-Actively Shielded BioApex II FT-ICR/MS equipped with electrospray ionization (ESI) for accurate mass analysis of degradation products and MS–MS fragment ions. Ions of interest were radio-frequency isolated in the Infinity ICR cell before infrared multiphoton dissociation for structural analysis. External/internal calibration was accomplished using polypropylene glycol oligomers. The Finnigan LCQ mass spectrometry method for LC–MS–MS analysis employed positive ion ESI with data dependent scanning (most abundant ion was selected) for MS–MS analysis with a collision-induced dissociation energy maintained at 20% (relative collision energy) while scanning the instrument from 100 to 1000 *m/z*. The ion source conditions are as follows: ESI emitter voltage held at 5 kV, capillary voltage of 5 V heated to 200°C with a sheath gas flow rate of 70 (arbitrary units) and an auxiliary gas flow rate of 5 (arbitrary units).

Nuclear Magnetic Resonance

Nuclear magnetic resonance analyses were conducted on Varian^{UNITY} INOVA 600 MHz spectrometers, one equipped with an indirect detection, "carbon enhanced," cryogenically cooled, HCN triple resonance probe and the other with a standard, indirect detection, HCN triple resonance probe. Compound 1 and degradant 1 were analyzed by NMR as CD_3CN solutions. Because of solubility reasons, degradants 2 and 3 were analyzed as d_6-DMSO solutions. The NMR solutions were each spiked with tetramethylsilane to serve as a chemical shift reference. The samples were maintained at 25°C for all NMR experiments. Standard Varian pulse sequences (1D, HMQC, gHMBC, and ROESY) were used to acquire the NMR data.

Theoretical Computations

Bond dissociation energy values for the RO—H bonds of 2-cyano-2-hydroperoxide and 2-cyano-2-propanol were determined using density functional theory calculations (BLYP/AUG-cc-pVDZ level of theory) performed using a Linux High-Performance Computing cluster and the Gaussian 09 computational package.[26] An isodesmic cycle with the experimentally determined BDE of *tert*-butanol was used as a reference.

RESULTS

Solvent Dependence of AIBN-Induced Degradation Products

Three major degradation products are generated from the AIBN oxidative stress of Compound 1 in ACN–water solvent systems as shown in the upper chromatogram in Figure 4 (5 mM AIBN, 0.05 mg/mL compound 1). Degradant 1 is the most abundant and elutes just before compound 1; the total compound 1 degraded is near 20% over the 3-day experiment. The lower chromatogram in Figure 4 is from an identical experiment except using a 50:50 (vol %) methanol–water solvent system. No degradants are observed. Figure 5 demonstrates just how little methanol needs to be present to significantly quench the formation of degradants 1–3. Adding 1% methanol has a large effect, whereas 10% methanol has essentially eliminated all degradant formation. A different Azonitrile initiator, 4,4′-azobis (4-cyanovaleric acid) (ACVA), was also used in 50:50 ACN–water solvent to determine if the observed oxidation was specific to AIBN. Even larger yields of degradant 2 and 3 were observed, degradant 1 was not observed and there were several new lower level peaks throughout the chromatogram. All of the ACVA-induced degradants disappeared upon addition of methanol at levels similar to that shown for the AIBN case shown in Figure 5.

Degradation Time-Course and Drug–AIBN Concentration Study

The analysis of compound 1 degradation over time was performed by preparing a 5 mM AIBN/0.05 mg/mL compound 1 in 50:50 ACN–water stress solution, adding this solution to multiple HPLC vials stored on an HPLC autosampler heated to 40°C and analyzing one HPLC vial every 5 h for nearly 100 h. The degradation time-course data in Figure 6 show no interdependence of reaction rate for any of the degradation products. In a separate experiment, each degradation product (degradants 1, 2, and 3) was isolated in abundance by preparative HPLC and was subsequently subjected to a 3 day, 5 mM AIBN stress. None of the degradation products observed during the AIBN oxidative stressing of compound 1 were formed from stressing isolated degradation products.

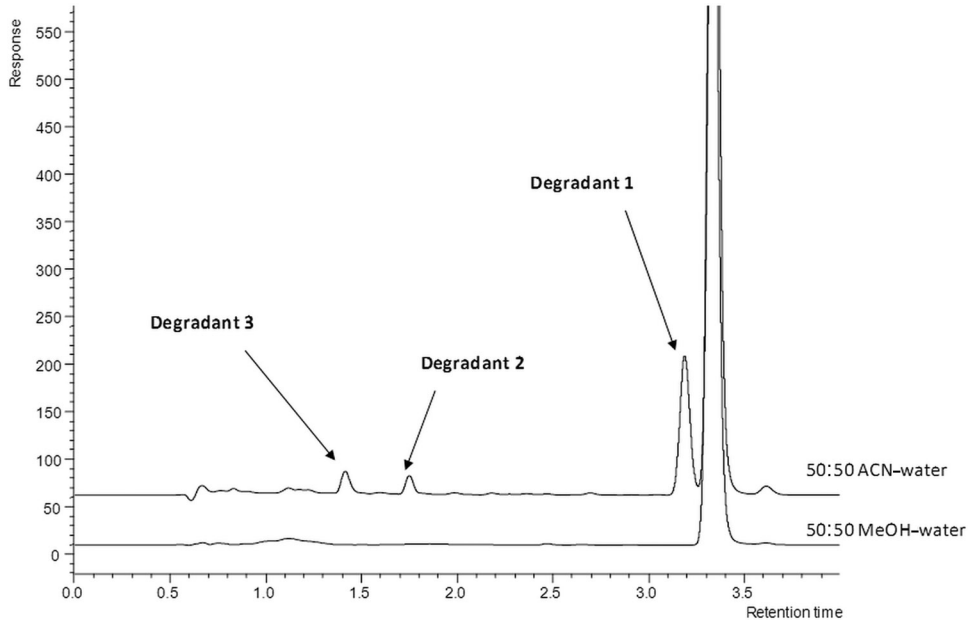


Figure 4. AIBN stress (5 mM) of 0.05 mg/mL compound 1 in ACN–water and MeOH–water diluents.

Figure 7 shows the effect of ranging the AIBN concentration from 1 to 5 mM while holding drug constant at 0.05 mg/mL (solvent 50:50 ACN–water). The upper portion of Figure 7 shows the percent compound 1 degraded, ranging from near 20% with 5 mM AIBN to near 4% at approximately 1 mM AIBN. The lower portion of Figure 7 shows the individual degradant area percent (relative to the 0.05 mg/mL compound 1 standard peak area) formed during the same experiments. Overall, the responses in Figure 7 appear linear. In Figure 8, the compound 1 concentration was ranged from 0.05 to 1.0 mg/mL (Upper, using 50:50 ACN–water solvent system) and from 0.10 to 10 mg/mL using 100% ACN solvent (lower portion of Fig. 8). After stressing, the samples were diluted to 0.05 mg/mL compound 1 and assayed as previously. The *y*-axis shows the summed area percent (relative to 0.05 mg/mL compound 1 standard peak area) for all three degradants. The decreasing summed degradant area percent data in Figure 8 clearly show that there is a limit to the absolute amount (mg/mL) of compound 1 degradants that can be formed.

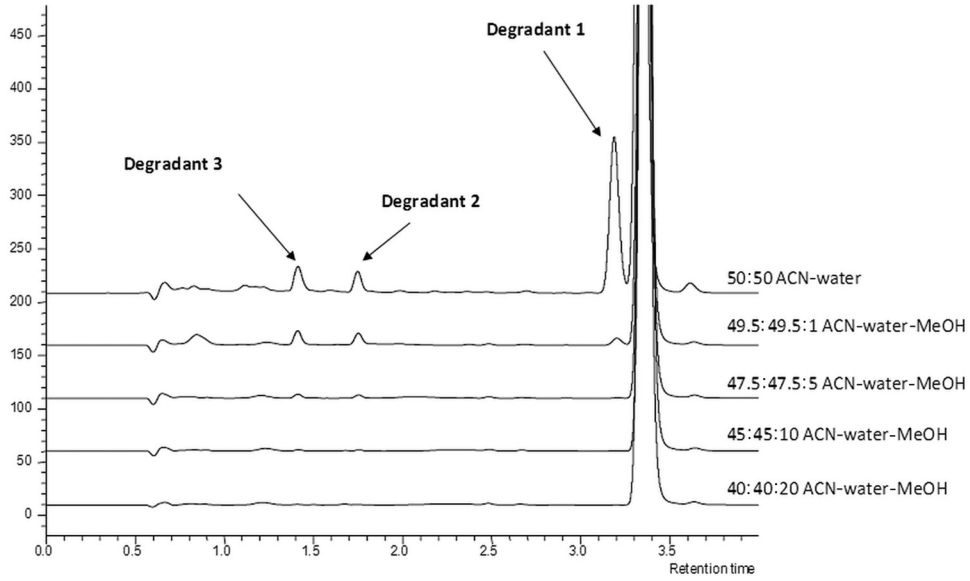


Figure 5. AIBN stress (5 mM) of 0.05 mg/mL compound 1 in ACN–water–MeOH diluents.

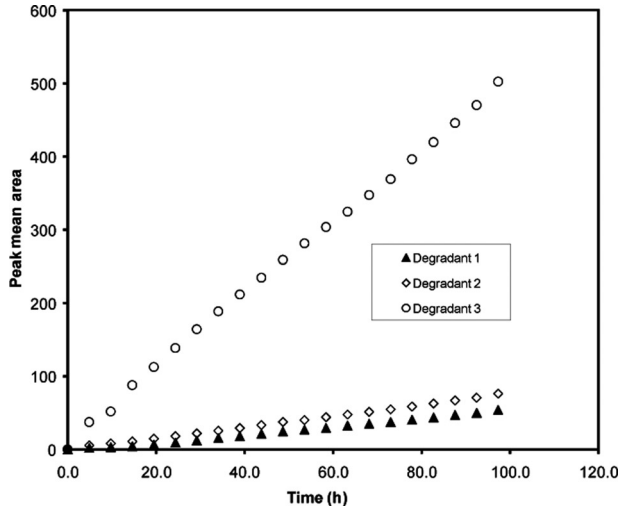


Figure 6. AIBN degradation time course for compound 1. Each degradant possess a linear abundance–time relationship.

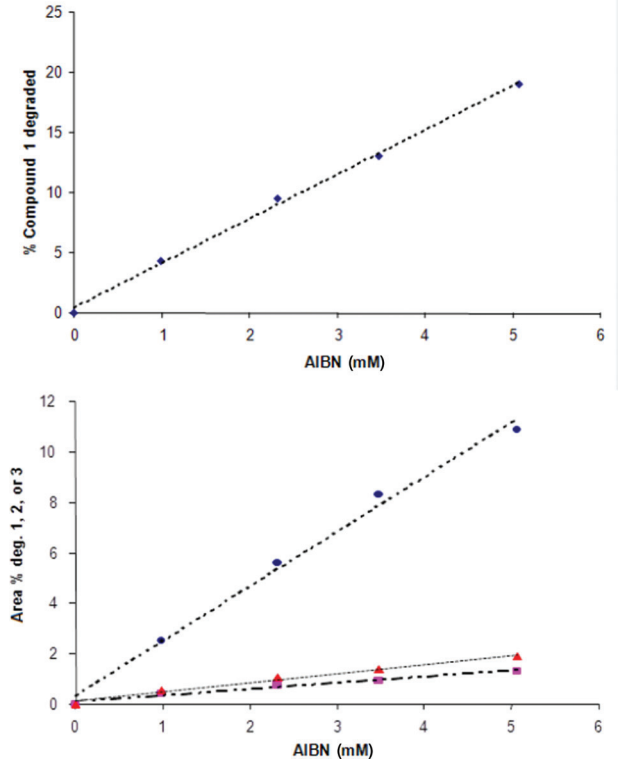


Figure 7. Upper, % compound 1 degraded at 0–5 mM AIBN (compound 1 at 0.05 mg/mL). Lower, area% (relative to 100% compound 1 peak area) of Degs. 1 (circles), Deg. 2 (squares), or Deg. 3 (triangles) in same experiment. Solvent = 50:50, ACN–water.

Structural Elucidation of the Degradants

LC–MS

The nominal molecular weight of the degradation products was obtained by positive ion ESI/LC–MS analysis of the AIBN stressed solution using a quadrupole ion trap mass spectrometer. The LC–MS data revealed the mass (relative to the mass of Compound 1) of degradant 1 to be +83 Dalton (Da), the odd number indicating the net gain of an odd number of nitrogen atoms. Nominal masses indicated that degradant 2 and 3 were compound 1 plus 30 and 48 Da, respectively, indicating the possibility of adding two oxygen atoms (followed by elimination of two hydrogen atoms) and the addition of three oxygen atoms, respectively.

Degradant 1 was further examined with tandem mass spectrometry. Nominal mass losses of 27 Da followed 41 Da provide evidence that degradant 1 contains an added nitrogen. The unusual 27 Da fragment is indicative of the loss of HCN from a nitrile-containing species. A definitive molecular formula was obtained for degradant 1 through FT-ICR–MS accurate mass measurement. The molecular formula of the API was found to have increased by C_4H_5NO.

Nuclear Magnetic Resonance

Degradant 1 NMR. The proton NMR spectrum of Degradant 1 was compared with that of the API. Significant differences were found only in the resonances of the naphthyl system. In particular, the 1-position proton of the naphthyl moiety was missing and a new singlet, integrating for 6 h, was observed at 1.79 ppm. The naphthyl moiety carbon and proton resonances were assigned via analysis of the two- and three-bond C—H couplings (gHMBC experiment). The chemical shift of the 1-position carbon was found to be 137.7 ppm, consistent with heteroatom substitution (likely oxygen). No C–H or H–H couplings were observed between the naphthyl moiety and the group attached at the 1-position. Fortunately, NOEs (ROESY) were observed between the 1.79-ppm protons and the 8-position naphthyl proton resonance at 8.13 ppm and between the 1.79-ppm protons and the methoxymethyl group resonance at 3.99 ppm. Several lines of evidence point to the 1.79-ppm resonance being due to a geminal pair of methyl groups. First, the 1.79-ppm resonance integrates for six protons. Second, an autocorrelation in the gHMBC experiment (1.79 ppm H to 28.3 ppm C) is consistent with three-bond C–H coupling from one methyl to the other methyl. The methyls have degenerate proton and carbon chemical shifts. A carbon chemical shift of 28.3 ppm is consistent with a methyl group that has several beta substituents. The only other long-range C–H correlations involving the geminal methyl groups are between the methyl protons and the carbon resonances at 75.4 and 122.4 ppm. Because a correlation was not observed to a second downfield carbon, it is likely that the 122.4-ppm carbon is not olefinic and the methyl groups are therefore attached to the 75.4-ppm carbon. The intensity of the gHMBC correlation

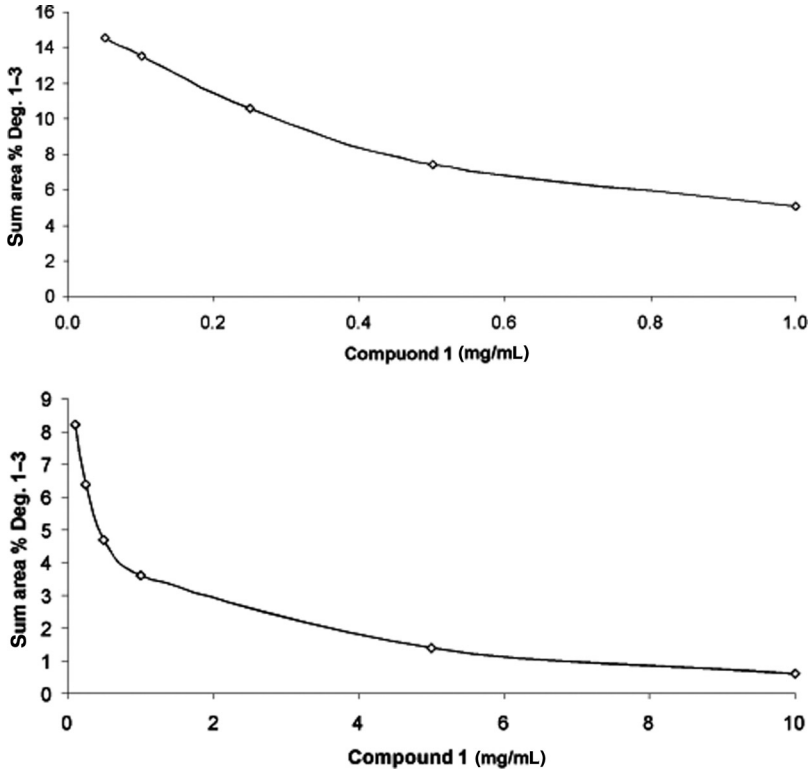


Figure 8. Upper, 5 mM AIBN, compound 1 ranges from 0.05 to 1.0 mg/mL (50:50, ACN–water solvent). Summed area% (relative to 100% 0.05 mg/mL compound 1) decreases from near 14% to 5%. Lower, similar plot except ranging 10× higher in compound 1 (10 mg/mL) but in 100% ACN solvent. Summed degradant area decreases to 0.6% at 10 mg/mL compound 1.

from the methyl protons to the 122.4-ppm carbon strongly suggests that it is due to a carbon three bonds away from the methyl protons. Giving additional consideration to chemical shift, the 122.4-ppm resonance is likely due to a nitrile group. The substantial downfield shift of the 75.4-ppm carbon suggests that its fourth attachment is to an oxygen atom. Thus, the moiety at the 1-position of the naphthyl system is the 2-hydroxy-isobutylnitrile group shown in Figure 9, nicely in accord with the added molecular formula identified by accurate mass measurement.

Degradant 2 NMR. Degradant 2 was dissolved in d_6-DMSO and its proton NMR spectrum compared well except for the resonances in the naphthalene region. A 1,2,4-trisubstitued aryl group was found attached to the pyrazole by observation of NOEs from 7.35 ppm to protons at 7.94 and 7.87 ppm. The proton–proton couplings easily established the 1,2,4 relationship of the substituents. The methoxyether moiety of the parent compound was found to be intact, but from the ROESY data, adjacent only to a isolated aromatic proton at 6.41 ppm (singlet). HMQC and gHMBC experiments were next used to assign the carbon resonances of the degradant. The carbon framework of the naphthalene was found preserved in the degradant structure, although two carbons of the naphthalene ring system were found shifted substantially downfield, one to 178.9 ppm and the other to 183.8 ppm. Using the ring attached to the pyrazole as a starting point, the proton at 7.94 ppm showed a three-bond gHMBC correlation to the 183.8-ppm carbon. The proton on the opposite side of the ring at 8.11 ppm showed a similar correlation to a carbon at 178.9 ppm. Both of these downfield carbons exhibited long-range correlations to the olefinic proton at 6.41 ppm. The correlation from 6.41 to 183.8 ppm was found to be rather weak, as one would expect for a two-bond correlation. The two downfield carbons must be carbonyls based on their chemical shift. The only structure that fits all the NMR data and the nominal mass increase of 30 Da over compound 1 is the *para*-quinone structure shown in Figure 10.

Degradant 3 NMR. d_6-DMSO was used as the solvent for NMR analysis of this degradant as it was not fully soluble in CD_3CN. Proton NMR resonances of the degradant 3 were compared with those of compound 1. Some chemical shift differences were expected because of the solvent change. Significant differences were found in the resonances of the naphthyl system even after accounting for the change of solvent. The most significant change in the proton spectrum was the appearance of a vicinal set of protons

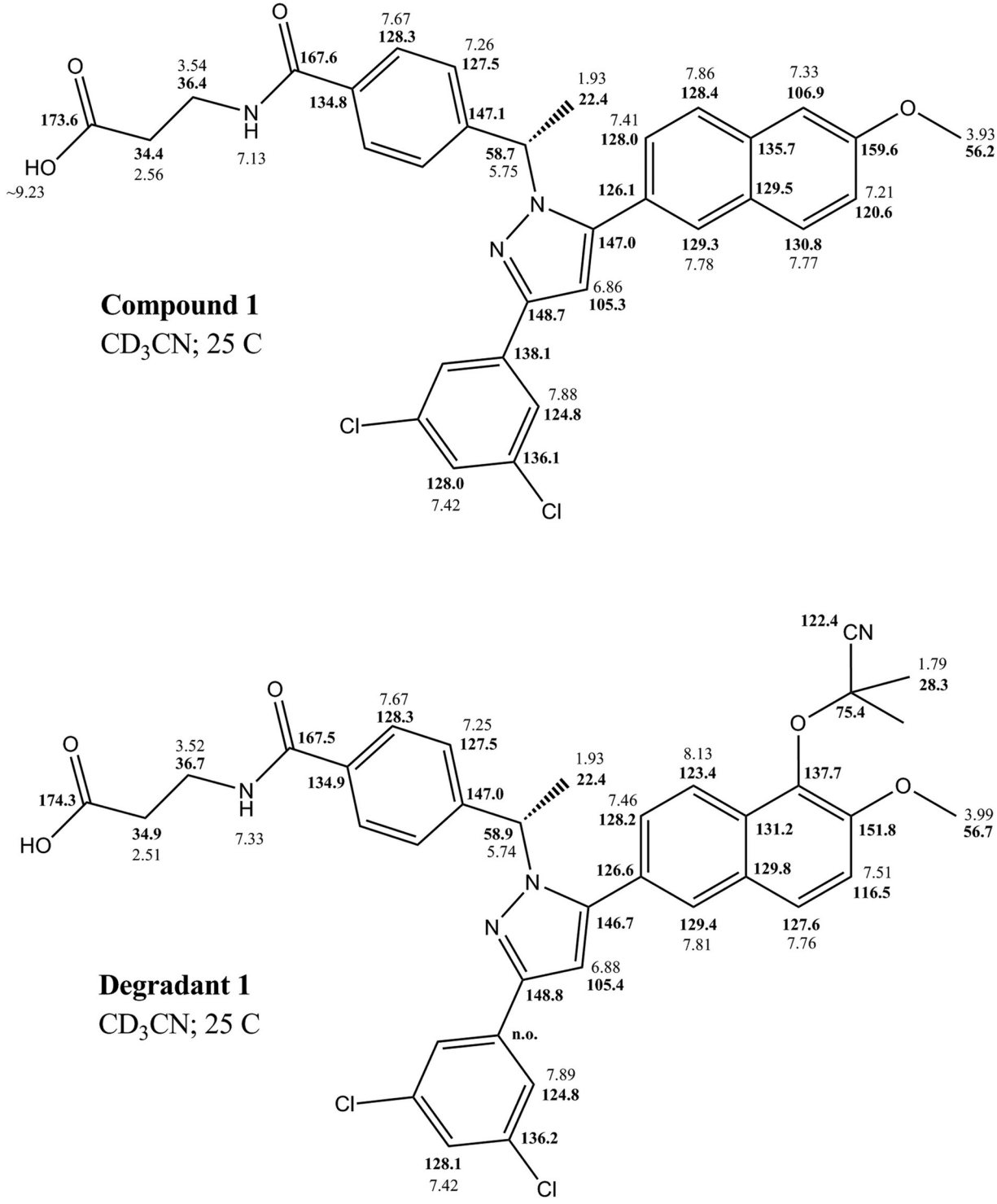


Figure 9. NMR chemical shift assignments for compound 1 and degradant 1, (*S*)-3-(4-(1-(3-(3, 5-dichlorophenyl)-5-(6-methoxynaphthalen-2-yl)-1*H*-pyrazol-1-yl)ethyl)benzamido)propanoic acid and (*S*)-3-(4-(1-(5-(5-((2-cyanopropan-2-yl)oxy)-6-methoxynaphthalen-2-yl)-3-(3, 5-dichlorophenyl)-1*H*-pyrazol-1-yl)ethyl)benzamido)propanoic acid, respectively.

at 8.54 and 6.22 ppm that share a 16.2-Hz coupling. These resonances are suggestive of a transoid α,β-unsaturated carbonyl structure. The 0.24-ppm upfield shift of the methoxynaphthalene protons from 3.93 to 3.69 ppm also signified a major structural change. HMQC and gHMBC experiments were next used to assign most of the degradant carbon resonances. Not all resonances were observed because of the limited amount of sample. The methoxy group was found to be part of a methyl ester based on a three-bond

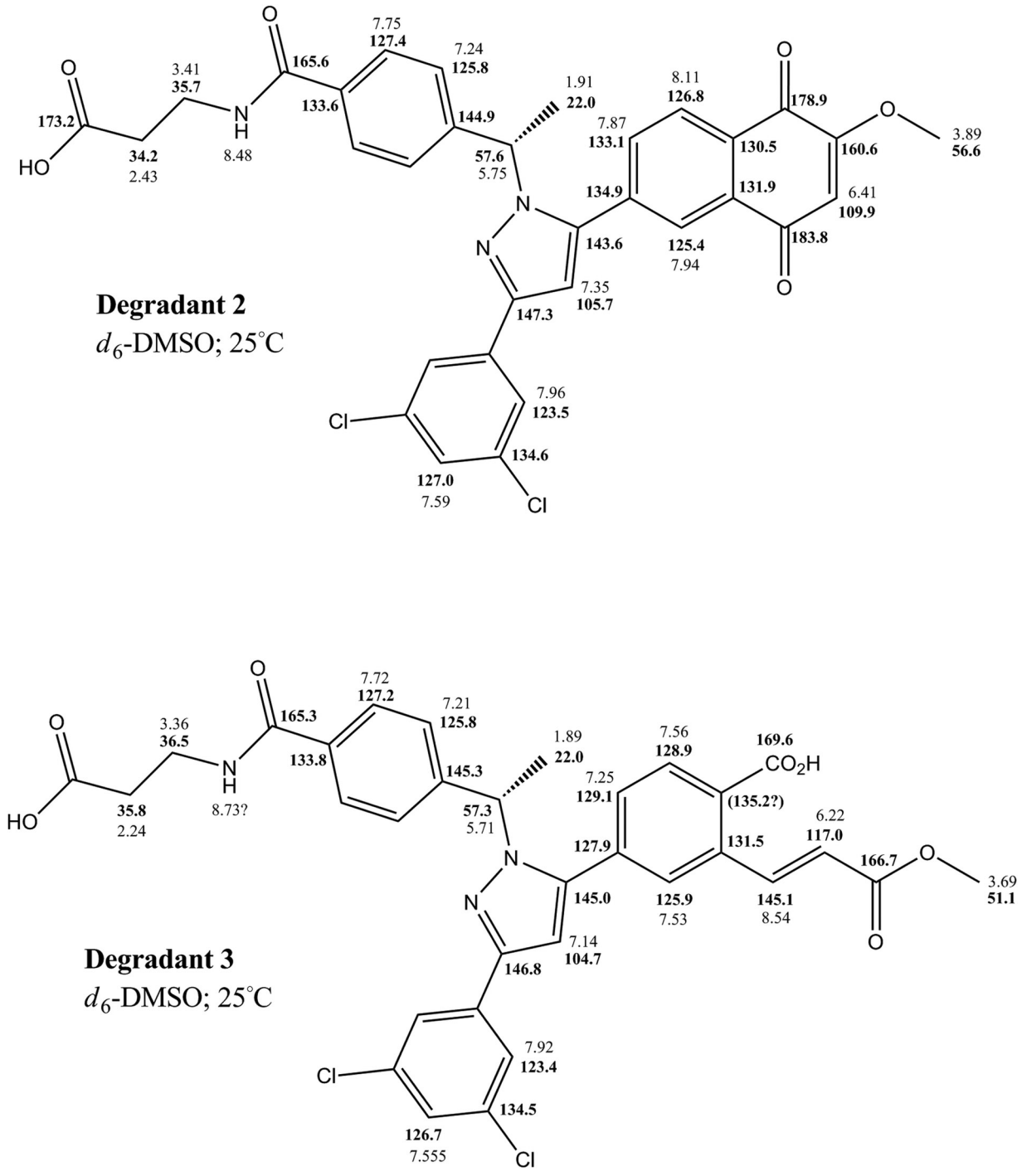
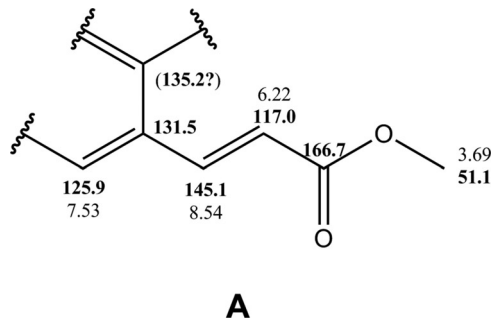


Figure 10. NMR chemical shift assignments for degradants 2 and 3, (*S*)-3-(4-(1-(3-(3,5-dichlorophenyl)-5-(6-methoxy-5,8-dioxo-5,8-dihydronaphthalen-2-yl)-1*H*-pyrazol-1-yl)ethyl) benzamido)propanoic acid and (*S,E*)-4-(1-(1-(4-((2-carboxyethyl)carbamoyl)phenyl)ethyl)-3-(3,5-dichlorophenyl)-1*H*-pyrazol-5-yl)-2-(3-methoxy-3-oxoprop-1-en-1-yl)benzoic acid, respectively.

coupling from the methyl protons to a carbonyl carbon at 166.7 ppm. Also found to be coupled to the 166.7-ppm carbonyl are the 8.54 and 6.22-ppm resonances mentioned above. This observation established substructure A (Fig. 11). A three-bond coupling from the 6.22-ppm proton resonance to 131.5 ppm assigned the carbon γ to the carbonyl. An additional long-range correlation from the 8.54-ppm proton to the protonated carbon at 125.9 ppm further defined the attachment point. The 135.2-ppm carbon shown in substructure A was not observed via correlation from 7.53 ppm, indicating that the resonance for the carbon at that position may be sufficiently broad to not show up in the low signal-to-noise gHMBC data set.



A

Figure 11. Degradant 3 substructure A.

Extremely weak correlations (possibly noise peaks) were observed coming from the 8.54-ppm and the 7.25-ppm resonances to the tentatively assigned 135.2-ppm carbon (see Fig. 10). Additional gHMBC and HMQC correlations were used to establish that the left side of substructure A is a phenyl moiety that remains after fragmentation of the parent naphthalene ring structure. Evidence for a carboxyl group being attached to the tentatively assigned 135.2-ppm carbon is a strong three-bond correlation from the proton resonating at 7.56 ppm to a carbonyl carbon at 169.6 ppm. A carboxyl group is also the only moiety at this position that gives a structure consistent with the observed nominal mass increase of 48 Da over compound 1.

DISCUSSION

Degradant 1: Definitive Proof of 2-Cyano-2-Propoxy Radical Formation and Activity

The incorporation of the 2-cyano-2-propoxy radical into the methoxy-napthalene ring moiety of compound 1 (Fig. 9, degradant 1) is the first definitive proof of the formation and activity of this AIBN derived alkoxy radical under pharmaceutically relevant oxidative susceptibility screening conditions (if methanol is not used as a cosolvent). Figures 5 and 6 show that degradant 1 (and the related degradants 2 and 3, below) persist at 1–5 mM AIBN levels and throughout the entire 0.05–10 mg/mL compound 1 range studied. Recently, the potential presence of the 2-cyano-2-propoxy radical was implicated[15] when studying the AIBN-induced oxidation of cumene (as a simple model for a drug molecule) under similar conditions as described here. However, in that case, all oxidation products observed derive from an initial H atom abstraction of the single benzylic C—H bond of cumene. Thus, the oxidation product structures formed do not unambiguously distinguish between an initial H atom abstraction by an alkoxy radical or by a peroxy radical. In the present case, it is clear that degradant 1 results from an initial addition reaction of the 2-cyano-2-propoxy radical to the C_{10} position of compound 1. Figure 12 depicts our view that degradant 1, 2, and 3 all in fact derive from the same initial addition reaction to C_{10}. A detailed mechanistic rationalization of each degradant is not central to the main thrust of the current work. Figure 12 provides a general rationalization, and highlights that after 2-cyano-2-propoxy addition at C_{10}, oxygenation at either C_9 (giving peroxy radical intermediate A) or C_7 (peroxy radical intermediate B) is possible. Intermediate A could lead to degradant 1 if elimination (of water or hydrogen peroxide) across the C_9C_{10} bond occurs, whereas C_9C_{10} bond scission leads ultimately to degradant 3. Intermediate B leads to degradant 2 and requires additional oxidation at C_{10} and C_7.

Note ACVA similarly produced degradants 2 and 3, but the ACVA analogue of degradant 1 (containing an additional $-CH_2COOH$ group compared with degradant 1) did not appear to be stable enough to accumulate under the experimental conditions utilized.

Mechanism of Methanol Quenching

Figure 5 and the degradant 1 structure show explicitly that low levels of methanol serve to eliminate the ability of the 2-cyano-2-propoxy radical to diffuse long enough to encounter compound 1. The effect of only 1% methanol in Figure 5 is remarkable. A chemical reaction with methanol is implicated rather than a decrease in yield of 2-cyano-2-propoxy radical (Fig. 1 followed by Fig. 2). A minor solvent composition change in this system is expected to have a weak impact on 2-cyano-2-propoxy radical yield.[27] The only plausible methanol chemical reactivity to account for this effect would be a hydrogen atom donation from methanol to the 2-cyano-2-propoxy radical to yield acetone cyanohydrin as shown in Figure 13. Note that methanol radical would be formed, which would be expected to oxygenate rapidly in oxygen-saturated solution, giving a methanol peroxy radical (see Fig. 13). In this way, the 2-cyano-2-propoxy radical activity would be "converted" to methanol peroxy radical activity. In fact, Nelson et al.[15] showed that low levels of methanol oxidation products formaldehyde and formic acid where found in their cumene experiments, consistent with Figure 13.

The protective effect of a few percent methanol by volume in Figure 13 is readily rationalized by concentration and reactivity arguments. The H atom abstraction rate of 2-cyano-2 propoxy radical from methanol can be estimated from analogous data for known alkoxyl radicals of similar structure and RO–H BDE values. The *tert*-butoxy and cumyloxyl radicals provide convenient comparisons. Table 1 shows the relevant RO–H BDE values are within a few kcal/mole.[26–28] Table 2 shows the H atom abstraction rates of *tert*-butoxy and cumyloxy radicals from methanol are very similar[31,32] and a similar rate for the 2-cyano-2-propoxy radical is expected. Table 2 also

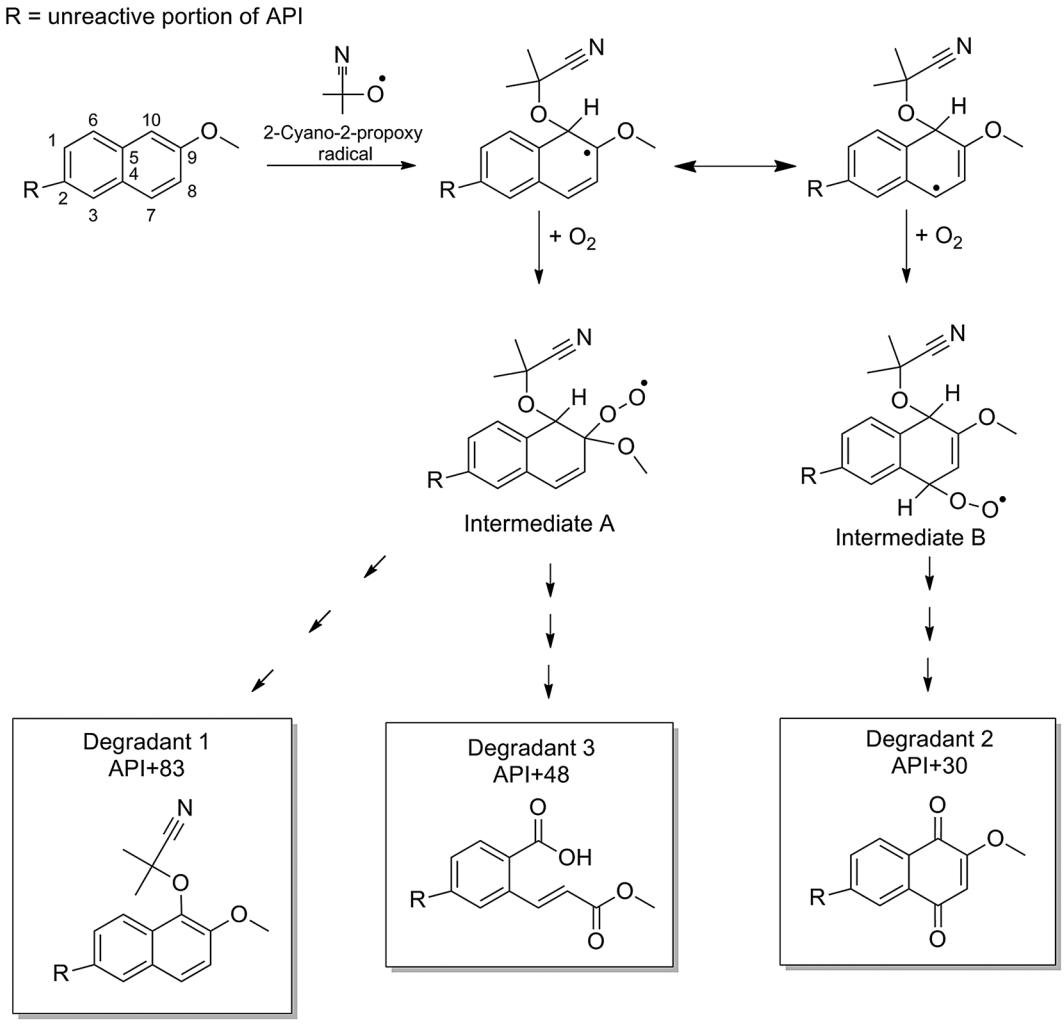


Figure 12. General mechanistic rationalization of degradants 1–3. All three degradants are formed by addition of the 2-cyano-2-propoxy radical at C_{10}. Oxygenation at C_9 leads to degradant 1 and degradant 3, whereas oxygenation at C_7 ultimately leads to degradant 2.

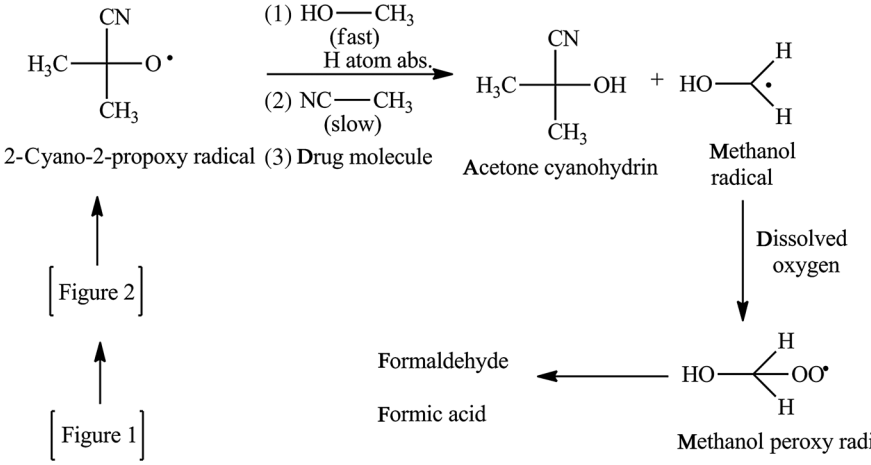


Figure 13. 2-Cyano-2-propoxy reaction with methanol to form acetone cyanohydrin and methanol radical. Note methanol, ACN, and dilute drug molecules all are potential substrates.

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Table 2. *Tert*-butoxy and Cumyloxy Radical (Parenthesized) H Atom Abstraction Rates from Methanol and Other More Reactive Organic Substrates

Compound	K_{RH} (M^{-1} s^{-1})	Compound	K_{RH} (M^{-1} s^{-1})
Methanol	2.9×10^5 (3.0×10^5)	2-Propanol	18×10^5
Toluene	2.3×10^5	Cyclohexene	57×10^5 (101×10^5)
Cumene	8.7×10^5 (22.7×10^5)	Tetrahydrofuran	83×10^5 (104×10^5)

All *tert*-butoxy values are from Paul et al.[31] Cumyloxy data are from Baingnee et al. [29] with the exception of the methanol value which derives from Banks et al. [32]

compiles similar data for substrates with activated C—H bonds such as those found in tetrahydrofuran and the allylic C—H bond in cyclohexene.[27,31] These more reactive substrates should be viewed as surrogates for dissolved drug molecules in the oxidative test. These substrates show 20–30-fold higher reaction rates (compared with methanol) with *tert*-butoxy and cumyloxyl radicals (Table 2) and by analogy with the 2-cyano-2-propoxy radical. However, given that methanol even at 2% by volume corresponds to a concentration of approximately 500 mM, the protective effect of 2%–10% added methanol on dilute submillimolar substrates with a wide range of C—H bond reactivities is readily rationalized.

It is interesting to note that ACN solvent on a volume basis does not appear able to be as effective an H-atom donor as methanol. This would not appear generally consistent with the relevant BDE values shown in Table 1. However, this relatively "inert" property of ACN has been consistently observed and reported for *tert*-butoxy and cumyloxy radical reactions.[29–35] In this context ACN is considered more comparable to benzene. A rate constant for *tert*-butoxy radical reaction with ACN of about 8.3×10^3 M^{-1} s^{-1} can be estimated from Paul et al.[31] and the B-scission rate for *tert*-butoxy radical in ACN reported by Tsentalovich.[33] This is approximately 35 times slower than the rates shown for methanol in Table 2. The B-scission product distribution of cumyloxy radical was recently used[15] to similarly estimate an approximately 35-fold slower H atom abstraction rate from ACN compared with methanol. This work suggests that 2-cyano-2-propoxy radical also exhibits a similarly slow rate of reaction with ACN. The strikingly different reaction kinetic profile for H atom abstraction from methanol and ACN by 2-cyano-2-propoxy radical, even though they have nearly identical H atom bond energies, points to factors that influence the reaction transition state. Transition state polarity is one such factor that can influence barrier height and thus the reaction kinetics of these systems.[15] This relative decrease in H atom abstraction reaction rate for 2-cyano-2-propoxy radical toward solvent in ACN–water systems give it a long enough diffusional lifetime to encounter and react with mM levels of dilute, more reactive drug substances.

Linear and Nonlinear Behaviors in Figures 5 and 6

Although not central to the primary focus of this work, the data in Figures 5 and 6 have practical and mechanistic interest to practitioners of the oxidative screening experiment and will be discussed very briefly. The linear behavior in Figure 7 might not at first seem expected, given the species reacting is the 2-cyano-2-propoxy radical which derives from disproportionation (Fig. 2) and might increase with the square of the initial AIBN concentration used. However, application of steady state equilibrium considerations reveals that the steady state concentration of the peroxy radicals in Figure 2 will only increase by the square root of the factor increase in initial AIBN concentration being used. Thus, the linear behavior in Figure 7 is expected.

Figure 8 highlights that the absolute amount (mol/L) of total degradation products being observed reaches a maximum value as the drug concentration increases at fixed AIBN levels. The total moles of degradants being formed at the 10 mg/mL compound 1 data point in Figure 8 can be estimated. The total moles of the 2-cyano-2-propoxy radical formed can be estimated from the AIBN Arrhenius parameters in our experiments as described in detail by Boccardi[36] if an efficiency of disproportionation in Figure 2 is assumed. Using an efficiency value of approximately 50% in Figure 2 (allowing for peroxide formation for example) then the moles of 2-cyano-2-propoxy radical formed is only a factor of about 2 greater than the moles of compound 1 degradants formed. These estimations show that the yield of the 2-cyano-2-propoxy radical itself is clearly the "limiting reagent" in Figure 8.

Implications for Azonitrile-Based Oxidative Susceptibility Screening Experiments: Addition of 10% Methanol to the Solvent System and Higher AIBN–Drug Molar Ratios

This work confirms the addition of $\geq$10% by volume methanol to all azonitrile compound-based oxidative susceptibility screening test solutions as recommended by Nelson et al.[15] to quench any 2-cyano-2-propoxy radical activity that may be present. In Figure 5, the true reactivity of compound 1 with peroxy radical—the original point of the AIBN or ACVA oxidative screening test—is revealed only after the addition of methanol. Without methanol added, and without full structural elucidation of all degradants, the 20% of compound 1 degraded in Figures 2 and 3

would be interpreted as a molecule with significant peroxy radical reactivity that might require careful excipient/formulation choices. However, with methanol added, Figure 5 shows there is no reactivity of compound 1 with the 2-cyano-2-peroxy radicals generated from Figure 1. The methanol results better predict the subsequent actual long-term stability data, which showed that conventional solid dosage formulations of compound 1 show no detectable oxidative degradation over long term accelerated stability conditions. Use of methanol will insure the appropriate peroxy radical activity of the subject test, facilitate better correlations with real stability data, and will allow for better delineation between oxidatively "reactive" and "nonreactive" molecules.

This last delineation is worthy of some consideration, and has some bearing on the use of methanol as described here. What is a reasonable definition of a "reactive" molecule in this context? Boccardi[36] suggested that if a molecule reacts with *every* AIBN (or ACVA) derived peroxy radical produced by Figure 1 during the experiment, that would be classed as "reactive," and further propagation of the drug-peroxy radical thus formed (i.e., chain lengths greater than 1) would indicate a "very reactive" drug substance. However, such a "reactive" molecule would be hard to observe under the some of the oxidative screening test conditions being utilized in the industry. Currently, in the early oxidative screening paradigm, there is a large range of mol % azonitrile compound used, ranging from approximately 10 to 5000 mol %.[8,11,13,15] Drug concentrations are typically in the 0.1–1 mg/mL range, whereas AIBN or ACVA are used from approximately 0.2–5 mM. Many practitioners use 20 mol % or less, and 5–20 mol % values have been recently recommended.[37] However, using even 20 mol % AIBN in a 40°C experiment for 3 days would lead to degradation of only 1.5% of the drug substance present for a compound thus classified as "reactive."[36] Very reactive compounds would degrade more than 1.5%, and "nonreactive" compounds would not degrade any drug. This is a relatively poor "dynamic range" for oxidative classification in our view. This type of practical consideration is what lead Boccardi[12] to recommend approximately 100 mol % azonitrile levels. The only "danger" from using higher and higher azonitrile compound levels, in our view, is possible creation of the strong alkoxy radical oxidants from the disproportionation in Figure 2. However, as demonstrated here, that danger is completely obviated with the use of ≥10% methanol. Compound 1 is clearly very reactive with the 2-cyano-2-propoxy radical. Yet Figure 5 shows complete elimination of the 2-cyano-2-propoxy derived degradants, even at the 5000 mol % AIBN (or ACVA) used. Addition of methanol thus allows for larger, more convenient dynamic ranges. For example, for a 3-day AIBN experiment at 40°C, use of approximately 750 mol % AIBN would correlate to 50% drug lost for a reactive compound (chain length = 1). Similarly, a 24 h experiment with three fold higher AIBN levels (~2300 mol % AIBN) would also correlate to 50% drug loss for a reactive molecule as defined above. In our laboratories, we routinely use ≥50% methanol as solvent, drug at 0.1–1 mM, and AIBN (40°C) at 1000–5000 mol % over a 1–3-day period. Many compounds are found to degrade a few percent or less under these conditions, and seldom do we encounter a 50% drug loss over the 3-day test period. In our view, these results reflect the well known selectivity of the peroxy radical reaction.

CONCLUSIONS

Oxidative susceptibility screening of compound 1 using AIBN or ACVA in an ACN–water solvent has been shown to generate alkoxy radical activity. Structure elucidation of degradant 1 (Fig. 9) proves, unequivocally, the presence and activity of the 2-cyano-2-propoxy radical as the oxidizing species, which is formed from the disproportionation of the 2-cyano-2-peroxy radical (Fig. 2). The absolute amount of degradants (1–3) formed is limited by the amount of the 2-cyano-2-peroxy radical, which can form given Figure 2. The generation of the 2-cyano-2-propoxy radicals is a general problem for the subject oxidative test, as this alkoxy radical is more reactive, and less selective, than the desired peroxy radical. This situation has the potential to yield misleading information by giving the appearance that the drug substance is susceptible to chain oxidation processes when in fact that may not be the case. Small amounts of methanol were found to quench the undesired 2-cyano-2-propoxy radical activity in the present study (Fig. 5), similar to that reported previously.[15] The protective methanol effects can be rationalized by large relative concentrations and competitive H atom donation rates relative to dilute drug substance substrates. It is strongly recommended that all azonitrile-based oxidative stress testing experiments maintain at least 10% methanol (by volume) in the reaction cosolvent. The addition of methanol should lead to a reexamination of commonly used azonitrile mol % levels in the early oxidative screening paradigm. Higher azonitrile mol % values of 100%–1000% relative to the drug substance being screened are readily warranted.

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