Autoxidation of Tetrazepam in Tablets: Prediction of Degradation Impurities from the Oxidative Behavior in Solution

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Abstract □ The major route of degradation of tetrazepam (1) is oxidation to 7-chloro-5-(3-keto-cyclohexen-1-yl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (3) via the stable 7-chloro-5-(3-hydroperoxy-cyclohexen-1-yl)-1,3-dihydro-1-methyl-2*H*-1,4 benzodiazepin-2-one (2). Minor degradation products are 7-chloro-5-(1,2-epoxycyclohexan-1-yl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (5) and 7-chloro-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2,5-dione (4), resulting from cleavage of the C—C bond between the cyclohexene ring and the benzodiazepine ring. After 48 h, AIBN (2,2'-azobis[2-methylpropanenitrile]) in acetonitrile at 40 °C produced qualitatively the same impurities as those observed in the stability study of tablets of 1. Other stress tests (thermal stress at 80 °C, heavy metal oxidation, hydrogen peroxide, acid-catalyzed oxidation) caused qualitatively different profiles of degradation.

Tetrazepam [7-chloro-5-(cyclohexen-1-yl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one; 1] is a benzodiazepine with skeletal muscle relaxant properties.[1] Although 1 is quite a stable drug in solid pharmaceutical forms, it is susceptible to autoxidation. The purpose of this investigation was to elucidate the degradation pattern of 1 and to evaluate the predictiveness of the results of different stress tests applied to solutions of 1.[2]

In our opinion, a predictive study of the stability should be able to anticipate within a few days the profile of degradation impurities which could be found in long-term studies. The pharmaceutical literature describes investigations of the sensitivity to oxidation using various oxidizing agents, such as hydrogen peroxide, heavy metal ions, acids, bases, high oxygen pressure at high temperature and, in some instances, strong oxidants such as potassium permanganate.[3] In most autoxidations involving a C(sp^3), the initiation does not involve oxygen, which is only the final electron acceptor.[4] In this case, the result of a study of the stability in a high oxygen atmosphere may be not reproducible because the autoxidation process is initiated by minute quantities of impurities. According to Burton and Ingold,[5] a spontaneous aleatory initiation step was the cause of the confusion and the poor reproducibility of the data in the literature on the antioxidant potency of phenolic compounds. They obtained good reproducibility by using 2,2'-azobis[2-methylpropanenitrile] (AIBN) as a radical-chain initiator.[5,6] Surprisingly, this technique is not normally reported in papers on studies of drug stability. Besides the other tests listed above, we carried out AIBN-catalyzed oxidation of 1 in an inert solvent (acetonitrile) at a temperature of 40 °C. The degradation profile obtained under the different conditions was compared with the analytical results of a highly degraded sample from a long-term stability study of 1 tablets (stored for 6 months at 55 °C and 85% relative humidity).

Experimental Section

Materials and Reagents—Tetrazepam (batch 5ARS039 or 7ARS026) and 3 were obtained from Sanofi Chimie (Paris). All other chemicals were reagent or HPLC grade.

Instruments—The IR spectra were obtained with a Perkin-Elmer model 881 dispersive spectrophotometer; the resolution was 2.4 cm^{-1}. The NMR spectra were acquired on a Bruker WP 250 or on a Varian XL-200 instrument, with tetramethylsilane as an internal standard. The UV spectra were obtained with a Beckman model DU6 spectrophotometer. The MS spectra were acquired on a VG model 70-70EQ spectrometer at 70 eV, and FAB spectra (Xenon atoms beam) were obtained in the positive-ion mode, using glycerol as the matrix.

High-Performance Liquid Chromatography—The HPLC instrument consisted of a Perkin-Elmer series 3 pump, a Rheodyne 7161 injector with a 20-μL loop, a RoSil C18 HL (4.6 mm × 25 cm) 5-μm column (R. S. L., Eke, Belgium), a Hewlett-Packard 1040A diode array UV detector, and a Hewlett-Packard 79994A data station. The diode array detector was used to acquire UV spectra from the beginning, middle, and end of each peak in a chromatogram. The identity of each impurity in the chromatogram of mixtures was confirmed by comparison of the retention time and of the UV spectrum at the peak maximum with the corresponding data obtained from an authentic sample of the impurity. The analytical wavelength was 254 nm. The isocratic mobile phase consisted of a mixture of 50 vol of a 0.01 M aqueous solution of potassium dihydrogen phosphate (pH 4.65) and 50 vol of acetonitrile. The flow rate was 1.1 mL/min.

Sample and standard solutions were diluted to ~1 mg/mL with the mobile phase before injection. Sample solutions for tablet analysis were prepared according to the following procedure: tablets were powdered, a quantity of this powder containing 10 mg of 1 was dispersed in 10 mL of the mobile phase, and the dispersion was sonicated (~5 min) and filtered.

Characterization of Tetrazepam—^{1}H NMR ($CDCl_3$): δ 1.5–2.7 (8H, m), 3.25 (3H, s, NCH_3), 3.6 (1H, d, J = 11 Hz, H-3), 4.6 (1H, d, J = 11 Hz, H-3), 5.95 (1H, bs), and 7.1–7.5 (3H, m); ^{13}C NMR ($CDCl_3$): δ 21.8, 22.3, 25.4 (C-6'), 26.1 (C-3'), 34.4, 56.4, 122.2 (C-9), 128.8, 129.8 (C-6), 130.1, 130.6 (C-8), 138.1 (C-1'), 138.2 (C-2'), 142.1, 169.8, and 170.2; EI-MS: *m/z* 288/290 ($^{35}Cl/^{37}Cl$, $M^{\cdot+}$), 253 ($M^{\cdot+}$-Cl); IR (KBr): 1690 (C=O), 1640 (C=C), and 1605 (C=N) cm^{-1}; UV(EtOH): λ_{max} 227 (29 000), 250 (sh, 16 100), and 307 (2200) nm.

Anal.—Calc. for $C_{16}H_{17}ClN_2O$: C, 66.55%; H, 5.93%; N, 9.70%; Cl, 12.28%. Found: C, 66.85%; H, 6.04%; N, 9.65%; Cl, 12.10%.

Isolation of 7-Chloro-5-(3-hydroperoxy-1-cyclohexen-1-yl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (2)—Tetrazepam (100 mg) was dissolved in acetonitrile (7 mL), and a 330-mg/mL iron (II) sulfate heptahydrate aqueous solution (3 mL) was added. The solution was stored for 48 h in the dark at 30 ± 2 °C, then concentrated and purified by preparative TLC using silica gel 1-mm plates and a hexane:acetone (5:2) mixture as the eluant. A temperature not exceeding 30 °C was maintained during the workup of the reaction. Compound 2 was obtained as an amorphous solid (HPLC purity 95%); ^{1}H NMR ($CDCl_3$): δ 1.5–2.5 (6H, m, H-4', 5', 6'), 3.25 (3H, s, NCH_3), 3.5 (1H, d, H-3), 4.5 (1H, d, H-3), 4.5 (1H, m, H-3'), 5.9 (1H, m, H-2'), and 6.9–7.5 (3H, m, H-6, 8, 9); FAB-MS: *m/z* 413 $(M+H+Gly)^+$, 321 $(M+H)^+$, 305 $(M+H-O)^+$, 303 $(M+H-H_2O)^+$.

7-Chloro-5-(3-keto-cyclohexen-1-yl)-1,3-dihydro-1-methyl-2*H*-1,4 benzodiazepin-2-one (3)—Compound 3 was obtained from

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Sanofi Chimie (Paris); ^{1}H NMR ($CDCl_3$): δ 2.1–2.95 (6H, m, H-4′, 5′, 6′), 3.28 (3H, s, NCH_3), 3.68 (1H, d, J = 11 Hz, H-3), 4.78 (1H, d, J = 11 Hz, H-3), 5.95 (1H, s, H-2′), 7.25 (2H, m, H-6,9), and 7.45 (1H, dd, H-8), NOE effect at 7.52 ppm (+12%) by irradiating at 5.95 ppm; ^{13}C NMR ($CDCl_3$): δ 22.1, 25.6, 34.6 (NCH_3), 37.6 (C-6′), 57.3 (C-4′), 122.6, 128.2, 128.9, 129.4, 131.6 (C-8 or C-2′), 133.1 (C-2′ or C-8), 142.0, 157.2 (C-1′), 168.5, 169.1, and 200.0 (C-3′); IR(KBr): 1672 cm^1; UV(EtOH): λ_{max} 245 (26 700), 260 (sh,14 900), and 314 (1550) nm.

Anal.—Calc. for $C_{16}H_{15}ClN_2O_2$: C, 63.47%; H, 4.99%; N, 9.25%. Found: C, 63.16%; H, 4.97%; N, 9.07%.

Isolation of 7-Chloro-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2,5-dione (4)—Tetrazepam (1 g) and 2,2′-azobis[2-methylpropanenitrile] (2 g) were dissolved in acetonitrile (30 mL), and the solution was saturated with oxygen and placed in a sealed vessel. The vessel was stored 4 h at 65 °C. The solution was concentrated and purified by column chromatography using ethyl acetate as the solvent. Fractions were monitored with TLC on alumina plates using ethyl acetate as the eluant. Fractions containing a spot at *Rf* 0.17 were collected and further purified by preparative TLC using alumina 1.5-mm plates and ethyl acetate as eluant. A 50-mg yield of 4 was obtained, with an HPLC purity of 95%. The HPLC retention time (2.9 min), UV spectrum obtained with diode-array detector, and ^{1}H NMR spectrum were identical to those of a sample of 4 obtained as reported;[7] ^{1}H NMR ($CDCl_3$): δ 3.32 (3H, s, NCH_3), 3.78 (2H, d, J = 6 Hz, s after D_2O, H-3), 6.68 (1H, d, J = 7 Hz, H-9), 7.38 (1H, dd, J_1 = 7 Hz, J_2 = 2 Hz, H-8), 7.74 (1H, d, J = 2 Hz, H-6), and 7.4 (1H, m, deuterable, N*H*); IR (KBr): 3257, and 1664 cm^{-1}; UV (HPLC mobile phase): λ_{max} 216, 247, and 298 nm; EI-MS: *m/z* 224 ($M^{\cdot+}$).

Preparation of 7-Chloro-5-(1,2-epoxycyclohexan-1-yl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (5)—Tetrazepam (2 g) was dissolved in 100 mL of a 6:3 mixture of ethanol and 35% hydrogen peroxide. The solution was kept at room temperature. At day 4, hydrogen peroxide (10 mL) was added. At day 7, the solution was diluted to 500 mL with water and extracted with ethyl acetate (250 mL). The organic phase was washed with water (5 × 100 mL), dried over anhydrous sodium sulfate, and evaporated. The residue was dissolved in acetone (10 mL) and evaporated. Then, 1.68 g of 5 as an off-white amorphous solid with 98.0% HPLC purity was obtained; IR (KBr): 1677 and 1626 cm^{-1}; UV (95% ethanol): 229 (30 700), 249 (sh, 8000), 310 (1700); ^{1}H NMR (DMSO-d_6, room temperature): δ 1.2–2.6 (8H, m, H-3′, 4′, 5′, 6′), 2.9 (0.7H, bs, H-2′), 3.6 (0.3H, bs, H-2′), 3.23 (2.1H, s, N-CH_3), 3.26 (0.9H, s, N-CH_3), 3.60 (1H, s, J = 11 Hz, H-3), 4.40 (1H, d, J = 11 Hz, H-3), and 7.6–7.8 (3H, m), NOE effects at 7.5 ppm (+5%) and 1.9 ppm (+8%) by irradiating at 2.9 ppm; ^{1}H NMR (DMSO-d_6, 413 K): δ 1.2–2.4 (8H), 3.14 (1H, bs), 3.28 (3H, s), 3.9 (1H, bd), 4.16 (1H, bd), and 7.4–7.8 (3H, m); ^{13}C NMR ($CDCl_3$): δ 18.8, 19.7* (relative intensity 70%) and 20.0 (relative intensity 30%), 24.0* and 24.27, 26.6* and 27.6, 34.7 (NCH_3), 55.9 and 56.4* (C-3), 56.9 and 58.2* (C-2′), 61.2 and 62.6 (C-1′), 122.2 (C-9), 127.0, 127.8 and 128.4* (C-6), 129.0* and 129.7 (C-7), 131.2* and 131.5 (C-8), 141.7 (C-9a), 169.1 and 169.7*, and 170.4; EI-MS; *m/z* 304 ($M^{\cdot+}$), 303, 287, 276, 248, 233, 261.

Anal.—Calc. for $C_{16}H_{17}ClN_2O_2$: C, 63.05%; H, 5.62%; N, 9.19%. Found: C, 62.88%; H, 5.74%; N, 9.20%.

Preparation of 7-Chloro-5-(3,3-[^{2}H]cyclohex-1-en-1-yl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (8)—Tetrazepam (0.5 g) was dissolved in deuterated dimethyl sulfoxide (5 mL), and deuterium oxide (2 mL) and deuterated trifluoroacetic acid (0.5 mL) were added. The solution was stored for 8 h in the dark, and then aqueous 2.5% sodium hydrogen carbonate (200 mL) was added. After filtration, the residue was washed with water (300 mL) and dissolved in methylene chloride (50 mL). The solvent was evaporated, and the solid was dissolved with 2 M HCl (20 mL). The suspension was rapidly neutralized (to avoid deuterium removal) with 5% aqueous sodium carbonate. The aqueous phase was filtered and extracted with methylene chloride (50 mL). The organic phase was washed with water (2 × 50 mL), desiccated over anhydrous sodium sulfate, and evaporated. The residue was purified by crystallization from acetone: 140 mg of 8 with 99.0% HPLC purity was obtained, with a retention time identical to that of 1; ^{1}H NMR ($CDCl_3$): δ 1.5–2.7 (6H, m, H-4′, 5′, 6′), 3.26 (3H, s, NCH_3), 3.56 (1H, d, J = 10 Hz, H-3), 4.55 (1H, d, J = 10 Hz, H-3), 5.9 (1H, sb, H-2′), and 7.0–7.4 (3H, m, H-6, 8, 9); IR (KBr): 2978, 2854, 2160 and 2082 (C-^{2}H), 1674, and 1632 cm^{-1}; ^{13}C NMR ($CDCl_3$): δ 21.8, 22.5, 25.6 (C-6′), 34.6, 56.6, 122.4, 129.1, 130.0, 130.6, 130.8, 138.4 (C-1′), 138.3 (C-2′), 142.3, 169.8, and 170.5; EI-MS: *m/z* 290 ($M^{\cdot+}$), 253 (M-Cl).

Oxidation Tests (Table I)—Tetrazepam (50 mg) was dissolved in 5.0 mL of the same solvent used to dissolve the appropriate catalyst. The solution was placed in a 25-mL pyrex vial fitted with a screw cap, and the vials were stored at 40 ± 0.5 °C in the dark. After 48 h, each solution was diluted with HPLC mobile phase to a suitable concentration and injected. (Warning: do not dissolve AIBN in acetone, where it can explode.[8])

Results and Discussion

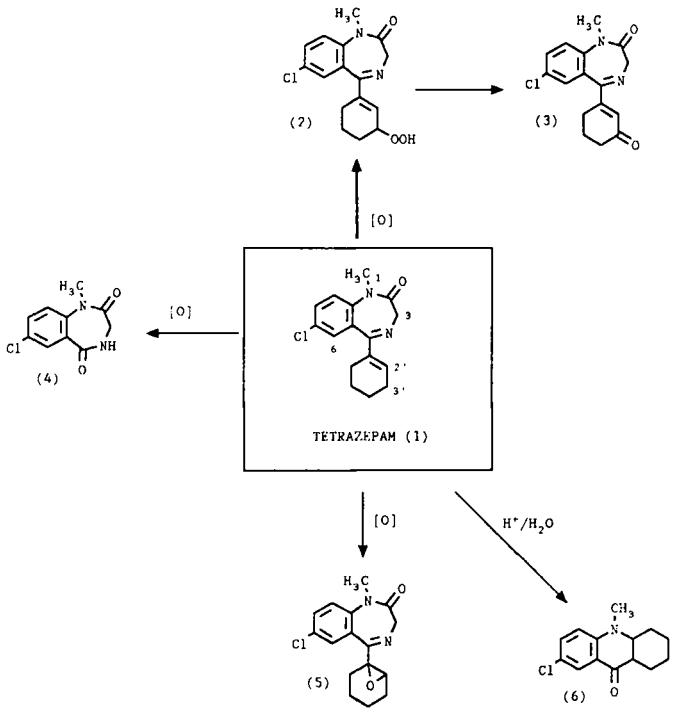
Degradation Products—Tetrazepam degrades by hydrolysis and oxidation (Scheme I). It is hydrolyzed in HCl to give 7-chloro-1,3,4,4*a*,9*a*,10-hexahydro-10-methyl-9(2*H*)-acridinone (6).[9] Products of this reaction were never detected in the present study, since it only occurs under strong acidic conditions. Oxidation of the 3′ carbon atom was the major site of degradation, and the corresponding oxo-derivative 3 was the main marker of 1 degradation. The same product is a known metabolite in rhesus monkey.[9] The intermediate hydroperoxide 2 was prepared by iron(II)-catalyzed oxidation of 1. Product 4 was isolated from the reaction mixture of the AIBN-catalyzed oxidation, and it is identical to an authentic sample prepared according to the literature.[7] In the presence of an excess of hydrogen peroxide, 1 was oxidized to the epoxide 5. The ^{1}H and ^{13}C NMR spectra of 5 in deuterated dimethyl sulfoxide at 313 K showed the presence of two distinct rotational isomers, whereas at 413 K, their signals coalesce. Epoxides of azadienes are commonly obtained only with alkaline hydrogen peroxide and are not stable,[10] whereas 5 was found to be a stable product.

Tests of Tetrazepam in Solution (Table I)—*AIBN-Initiated Oxidation*—The AIBN-catalyzed oxidation in acetonitrile of 1 yielded all the degradation products listed above and a number of minor impurities. A typical HPLC chromatogram after 48 h at 40 °C is shown in Figure 1B. No degradation was observed in acetonitrile in the absence of AIBN. The reaction was not very sensitive to the polarity of the solvent, as shown by the data for

Table I—Variation of Product Distribution for Tetrazepam[a] Degradation after Various Stress Tests

Catalyst	Solvent	Temperature, °C	Time, h	Composition, %[b]				
				1	2	3	4	5
None	CH_3CN	40	48	99.5	0.1	0.3	—	—
AIBN (60 mM)	CH_3CN	40	48	65.8	8.4	11.2	1.9	2.8
AIBN (60 mM)	CH_3CN (80%) H_2O (20%)	40	48	68.1	9.6	11.4	1.4	2.8
AIBN (60 mM) + BHT (4.5 mM)	CH_3CN	40	48	96.4	2.3	0.4	0.04	0.13
Citric acid (2.5 mM)	CH_3CN	40	48	11.3	64.5	16.8	1.0	0.3
$FeCl_3$ (1.5 mM)	CH_3CN	40	48	53.4	1.5	18.4	3.4	4.5
$CuSO_4 \cdot 5H_2O$ (1.5 mM)	CH_3CN	40	48	66.2	0.4	22.8	2.8	—
Silica gel[c]	None	55[d]	—[e]	37.1	3.9	40.9	7.7	4.5
Heat	None	80	16	94.7	0.2	3.6	0.3	0.3
Heat	None[f]	55[d]	—[g]	41.8	14.6	28.1	3.3	0.9

[a] Tetrazepam (35 mM). [b] Only known impurities are reported. [c] Chromatographic grade. [d] 85% relative humidity. [e] Fifteen days. [f] 50-mg tablets. [g] Six months.



Scheme I—Tetrazepam (1) and its proposed degradation pattern.

the oxidation in a mixture of acetonitrile and water (8:2). Repeated experiments showed very good reproducibility of the reaction. With aliphatic alcohols as solvents, the reaction is slower (especially with 1-propanol) than in acetonitrile and quantitatively not very reproducible because of competitive oxidation of the solvent. 2,6-Bis(dimethylethyl)-4-methylphenol (BHT), chosen as a typical phenolic antioxidant, enhanced the stability of 1 in the presence of AIBN, as expected.

Acid-Catalyzed Oxidation—In the presence of a weak acid, such as citric acid, the oxidation of 1 occurred even in the absence of radical-chain initiators. The oxidation of many substances is pH sensitive, but usually both acidic and basic drugs degrade more rapidly under neutral and alkaline conditions than acidic ones.[4] One important exception is compounds that undergo acid-catalyzed enolization (e.g., Schiff bases of α,β-unsaturated ketones which undergo autoxidation via direct interaction of the corresponding enamines with triplet oxygen[11]). This mechanism could explain the acid-catalyzed autoxidation of 1, provided that 1 equilibrates with the enamine 7 (Scheme II). Indeed, H:D

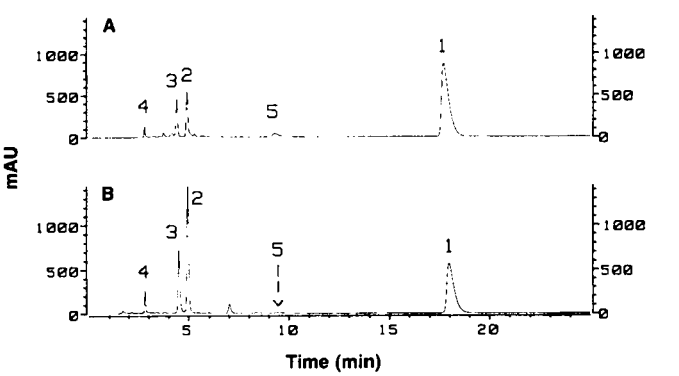
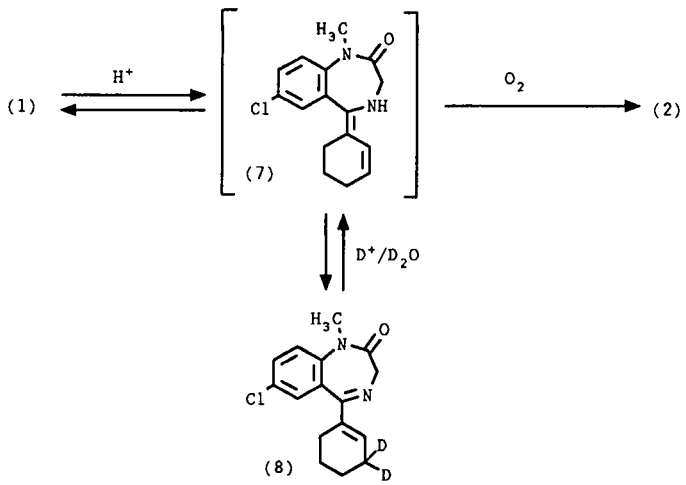


Figure 1—The HPLC chromatograms of (**A**) 35 mM tetrazepam (1) in acetonitrile after a 48-h degradation at 40 °C in the presence of AIBN, and of (**B**) 1 in tablets stored for 6 months at 55 °C and 85% relative humidity.



Scheme II—Proposed mechanism of the acid-catalyzed autoxidation of 1 and of its deuteration.

exchange in a mixture of deuterated dimethyl sulfoxide, water, and trifluoroacetic acid yielded the 3′,3′-dideuterotetrazepam (8), indicating a tautomerization to the enamine 7.

Other Tests—The stability of 1 is decreased by hydrogen peroxide, iron (III), and copper(II) ions in solution and by high temperatures (80 °C) and silica gel in the solid state. Some of these reactions were useful on the preparative scale, as described above.

Degradation of Tetrazepam Tablets—The degradation of 1 in tablets stored for 6 months at 55 °C and 85% relative humidity was found to be qualitatively very similar to that by AIBN. In particular, the AIBN-initiated reaction products were predictive of the main impurities and roughly of the same relative amounts. Certainly, the main reason is that AIBN oxidation occurs with the same radical-chain mechanism as the natural autoxidation of the drug.

One other important point is that the mild and neutral conditions of the AIBN oxidation made it possible to identify the hydroperoxide 2, which is quite a labile substance but likely to be found in the natural degradation of 1. Oxidation by Fe(III) and Cu(II) ions and by the thermal stress (80 °C) of the bulk substance did not reveal the importance of the hydroperoxide 2 in the degradation pattern of 1. The hydrogen peroxide oxidation, largely used in the preformulation studies, gave the least predictive results, although it was useful for the synthesis of the minor impurity 5. For our preformulation studies, we consider AIBN oxidation to be the best test to anticipate the profile of autoxidation impurities.

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