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Identification of degradation products occurring in acidic solutions of a 21-aminosteroid (tirilazad)

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Summary

The major degradation products occurring in pH 2–3.75 solutions of a 21-aminosteroid (tirilazad) are described. Degradation products were identified using two methods. In the first method, molecular ion information obtained from LC/MS of degraded samples implied an identity. The proposed structures of **I**, **II**, **V**, and **VI** were confirmed by comparing the mass spectra and chromatographic retention time of synthetically prepared samples to those obtained for the degraded sample by LC/MS. In the second method, degradation products (**III** and **VI**) isolated by preparative HPLC were identified by spectroscopic techniques. No structure can be assigned to compound **IV**. In acidic solutions, tirilazad degrades via oxidative and acid-catalyzed reactions. Oxidative degradation produces the C20 carboxylic acid and the amine side-chain while acid catalyzed degradation includes hydrolysis of the pyrimidine-piperidine bond and dienone-phenol rearrangement of the steroid A-ring. Quantitative data on degradation products were obtained with a gradient HPLC method.

Introduction

Tirilazad mesylate (U-74,000F), a 21-amino substituted steroid [16α-methyl-17α-hydrogen-pregna-1,4,9(11)-triene-3,20-dione-21-[2,4-bis(1-pyrrolidinyl)-6-piperazinylpyrimidine]methane sulfonate], inhibits lipid peroxidation (Braughler et al., 1987; McCall et al., 1987). Activity in experimental models of head, spinal injury (Hall, 1988; Hall et al., 1988a), and hemorrhagic shock (Hall, 1988b) were found. Tirilazad degrades in solution and the identity of degradation products has not been previously reported. Because the solubility of tirilazad decreases with increasing pH, administration in vivo uses acidic aqueous solutions (pH 3). The identification of degradation products being produced in these acidic solutions is essential to understanding the stability of tirilazad and in the development of stability-indicating methods. Fig. 1 presents structures of the tirilazad solution degradation products discussed in this article.

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Materials and Methods

HPLC method used for quantitation of degradation products and LC/MS

A 4.6 × 250 mm, octylsilane column (Zorbax

RX, Dupont, Wilmington, DE) was used with a gradient system in which mobile phases A and B were acetonitrile and 0.02 M ammonium formate adjusted to pH 4.0 with formic acid, respectively. Mobile phase A was varied from 30 to 75% from 3 to 45 min with a flow rate of 1.5 ml/min (using a low-pressure, mixing system Model 2249, LKB, Bromma, Sweden). Detection was at 254 nm with a line-source detector (UV Monitor III, LDC, Rivera Beach, FL). Drug solutions at 1.5 mg/ml were diluted 1:9 with water and 200 µl injected. A representative chromatogram is shown in Fig. 2. The relative retention and response factors (relative to tirilazad peak area) for the degradation products were **I** 0.018, 1.35; **II** 0.13, 1.82; **III** 0.32, 0.47; **IV** 0.44, 1.0; **V** 0.47, 0.81; **VI** 0.62, 0.76 and **VII** 0.92, 1.00. A photo-diode array HPLC detector (Model 990, Waters Assoc., Milford, MA) was used to obtain UV spectra of the degradation products.

Preparation of solutions for degradation studies

Unless otherwise specified, tirilazad mesylate was dissolved at 1.5 mg/ml in a 0.02 M citric acid/sodium citrate buffer at pH 3 containing 4.5 mg/ml of sodium chloride. The pH of citric acid solutions was modified by varying the ratio of citric acid and sodium citrate. When specified, reduction in oxygen content was accomplished by placing solutions within a nitrogen purged glove-bag, purging for 30–60 min with He and sealing the container before removal from the glove-bag. Samples degraded at 100°C were placed in 3 ml reaction vials sealed with a teflon-faced closure. A headspace of 1 ml of air was left for those solutions specified as containing ambient levels.

HPLC method for tirilazad mesylate

A 4.6 × 250 mm, octylsilane column (Nucleosil C-8, Alltech, Deerfield, IL) was used with a mobile phase of acetonitrile:potassium monobasic phosphate buffer, 6.8 g/l:phosphoric acid:*N*,*N*-dimethyloctylamine (400:600:1.2:2.0) at a flow rate of 0.5 ml/min. The 'apparent' pH of the mobile phase was 3.5 ± 0.05. Adjustments to mobile phase pH were made using either phosphoric acid or ammonium hydroxide as required. Detection was UV at 254 nm using a mercury-line source detector. The injection volume was 10 µl. Standards were prepared by: accurately weighing about 10 mg of standard, dissolving in 20.0 ml of Internal Standard Solution and adding 10.0 ml of mobile phase. The internal standard solution was propiophenone in mobile phase at a concentration of 0.5 µl/ml.

Samples were prepared by adding 3.0 ml of internal standard for each milliliter of sample and diluting approx. 1:3 with mobile phase. The solutions at pH 3.75 had a substantial amount of precipitate after heating at 100°C. To analyze the solutions, a known amount of ethanol was added to the samples to re-dissolve drug and degradation products.

The retention times of the tirilazad and the propiophenone are approx. 16 and 27 min, respectively. Degradation products are separated from tirilazad and the propiophenone. Retention relative to tirilazad for degradation products was **I** 0.03, **II** 0.24, **III** 0.44, **IV** 1.9, **V** 1.3, **VI** 4.2 and **VII** 1.3. Standard and sample preparations are stable for 48 h when stored in amber vials at room temperature.

Isolation of degradation products

Compound **III** [pregna-1,4,9(11)-triene-3,20-dione-21-piperazine] was isolated from a solution containing 20 mg/ml tirilazad mesylate in 0.9% NaCl (adjusted to pH 2.5 with HCl) stored for 12 months at 4°C. (Although subsequent studies showed more efficient conditions to produce **III**, a large quantity of the above solution was available with **III** present at 8% (w/w) of the tirilazad mesylate content.) Preparative HPLC was used to first separate **III** and other degradation products from tirilazad. The preparative HPLC system consisted of a 41.4 × 250 mm octylsilane column (Dynamax C-8, Rainin, Woburn, MA). A mobile phase of acetonitrile:0.1 M ammonium formate (55:45) adjusted to pH 4 with ammonium hydroxide was used at a flow rate of 60 ml/min. House vacuum and heating (50°C) were used to remove partially acetonitrile from fractions collected from the preparative system containing **III**. After adjusting the pH of the remaining solution to 8 with 1 M NaOH, the solution was extracted twice with an equal volume of methyl ethyl ketone. During the evapor-

ation of the methyl ethyl ketone, **III** precipitated from the remaining water (solubility of water in methyl ethyl ketone is 10%). Acetonitrile was added to re-dissolve **III** for re-purification using a 4.6 × 250 mm octylsilane column (Dynamax C-8, Rainin, Woburn, MA) and the gradient elution conditions described for the LC/MS method. Acetonitrile was partially removed from fractions collected with this system by evaporation with nitrogen at ambient temperature. The remaining solution was extracted with chloroform. Evaporating the chloroform layer to dryness under nitrogen produced enough pure **III** for NMR experiments. Preparative separations used a gradient high-pressure mixing system (Prep 350, Beckman, Fullerton, CA).

A sample containing about 8% of **VII** (1-methyl-3-hydroxyestra-1,3,5(10),9(11)-tetraene analog of tirilazad), was generated by preparing a 80 mg/ml solution of tirilazad mesylate in water, adjusting the solution to pH 1.5 with phosphoric acid, and heating for 2 h at 120°C in a sealed vial. About 2 ml of the degraded sample were injected into a semi-preparative HPLC system consisting of a 9.4 × 250 mm octylsilane column (Zorbax C-8, Dupont, Wilmington, DE) and acetonitrile: water:*N*,*N*-dimethyloctylamine:acetic acid (500: 500:3.0:1.6) mobile phase flowing at 8 ml/min. Acetonitrile was partially removed from collected fractions by evaporation under nitrogen. To remove the *N*,*N*-dimethyloctylamine, a 7.0 × 300 mm poly(styrene-divinylbenzene) co-polymer column (PRP-1, Hamilton, Reno, NV) was used with a mobile phase of acetonitrile:10 mM ammonium formate (75:25) adjusted to pH 5.8. Acetonitrile was partially removed from fractions collected with this system by evaporation under nitrogen. The remaining solution was freeze-dried to remove the remaining solvents and ammonium formate and yield the purified degradation product.

Reagents

All solvents were HPLC grade (Burdick and Jackson, Muskegon, MI). Other reagents were analytical reagent grade.

Spectroscopy

Mass spectra were acquired with a quadrupole MS with thermospray interface (Model 4600, Finnigan, San Jose, CA) using the analytical HPLC system described above. The instrument was operated with a vaporizer temperature of 100°C, a jet temperature of 250°C and a repeller voltage of 80 V. Hydrogen NMR spectra were obtained with several different instruments. Field strength and solvent are specified for each sample. IR spectra were acquired using 3 mm KBr discs and an FTIR spectrometer (Model 605X, Nicolet, Madison, WI).

Synthetically prepared degradation products

Synthetic **I** [2,4-bis(pyrrolidinyl)-6-piperazinylpyrimidine] was prepared and characterized as previously described (McCall, 1988). Melting point 177–178°C. Hydrogen NMR (400 MHz, $CDCl_3$): δ 5.31 (s,1H), 4.87 (s,1H), 3.5 (m,12H), 3.1 (m,4H), 1.9 (m,8H).

Synthetic **II** [2,4-bis(pyrrolidinyl)-6-(**N**-formylpiperazinyl)pyrimidine] was prepared by formylation of **I** (Djuric, 1984). Compound **I** (1.03 g) was reacted with *t*-butyldimethylsilyl chloride (0.56 g), 4-dimethylaminopyridine (0.02 g) and triethylamine (0.52 ml) in dimethylformamide (5 ml) at 20°C for 24 h. The product was extracted into dichloromethane, washed with 5% $NaHCO_3$ and H_2O, dried over $MgSO_4$, concentrated and chromatographed on silica (40 g, 70–230 μm) with 80% EtOAc/hexane. Eluate containing pure product was concentrated to give a yellow solid (0.92 g, 82% yield, m.p. 147–150°C). Hydrogen NMR (300 MHz, $CDCl_3$): δ 8.10 (s,1H), 4.87 (s,1H), 3.66–3.57 (m,4H), 3.57–3.48 (m,6H), 3.48–3.36 (m,6H), 1.96–1.92 (m,4H), 1.92–1.86 (m,4H). IR (KBr): 3450, 2960, 2860, 1675, 1560, 1430, 1230, 1000, 780 cm^{-1}. MS (EI) *m/e* 330, 302, 246, 218, 121. Further detailed spectroscopic work (^{13}C NMR, ^{15}N NMR and UV) confirmed the structure assignment.

Synthetic **V** [2,4-bis(pyrrolidinyl)-6-hydroxypyrimidine] was prepared by partial reaction of 2,4-bis(pyrrolidinyl)-6-chloropyrimidine (4.0 g) (McCall, 1988) with potassium *t*-butoxide (8.9 g) in THF (40 ml) and toluene (32 ml) under reflux for 18 h. The resulting *t*-butyl ether was hydrolyzed by addition of water (225 ml) and sulfuric acid to pH 1.4. The aqueous phase was separated, ex-

tracted with ethyl acetate (4 × 100 ml) to remove unreacted starting material, adjusted to pH 7.0 with 50% aqueous sodium hydroxide and extracted with dichloromethane (2 × 200 ml). The combined organic phases were washed with a mixture of brine (100 ml) and water (500 ml), dried over sodium sulfate, and concentrated. The residue was recrystallized from ethanol (75 ml) to give a white solid (0.9 g, 24% yield, m.p. 268.5–270.5°C). Hydrogen NMR (400 MHz, $CDCl_3$): δ 10.9 (s,1H,60H), 4.7 (s,1H,5H), 3.55 (m,8H,2′,2″,5′,5″H), 1.9 (m,8H,3′,3″,4′,4″H). MS (50 eV FAB ionization with glycerol matrix): MH^+=235. MS/MS (Ar collision gas at 0.4 Torr): *m/z*=70 (pyrrolidine), *m/z*=95,96 (6-hydroxypyrimidine), *m/z*=164 (6-hydroxypyrrolidinylpyrimidine).

Synthetic **VI** [20-β-carboxy-16α-methylandrosta-1,4,9(11)-triene-3,20-dione] was prepared by oxidation of 21-hydroxy-16α-methylpregna-1,4,9(11)-triene-3-one (7.3 g) (McCall, 1988) with sodium metaperiodate (6.0 g) in methanol:water (200 ml/500 ml) at 50°C for 2 h. The product was extracted into dichloromethane, concentrated and recrystallized from 57% EtOAc/hexane to give a tan solid (4.05 g, 58% yield, m.p. 255–262°C). Hydrogen NMR (90 MHz, $CDCl_3$): δ 7.20 (dd, 1H, *J*= 13 Hz), 6.3 (dd, 1H, *J*=13 Hz), 6.1 (bs,1H), 5.55 (m,1H), 2.80–0.9 (m,13H, 1.45 (s,3H), 1.1 (d,3H), 1.1 (d,3H), *J*=7 Hz), 0.85 (s,3H). IR ($CDCl_3$): 3500–2500, 1685, 1642, 1605, 1590 cm^{-1}.

Results and Discussion

The proposed degradation scheme is shown in Fig. 1. Acid-catalyzed reactions are shown above the structure of tirilazad mesylate (**VIII**) and result in the formation of compounds **III**, **V** and **VII**. Oxidative reactions are shown below the structure of tirilazad and result in the formation of compounds **I**, **II** and **VI**. A chromatogram of a solution degraded for 24 h at 100°C is shown in Fig. 2. The numerical assignment follows the order of elution with tirilazad eluting last.

Development of preparative isolation methods

A previously reported chromatographic method used octylsilane columns with a mobile phase of acetonitrile:water:acetate buffer. Triethylamine was used as a competing-amine modifier to improve chromatographic efficiency (Cox and Pullen, 1988a,b). Because of the low chromatographic resolution between **VII** and tirilazad, a more effective competing amine (*N*,*N*-dimethyloctylamine) was used to increase the capacity of the column while maintaining resolution. The *N*,*N*-dimethyloctylamine was removed by using a poly(styrene-divinylbenzene) column which did not need a competing amine to attain good peak shape.

Identification

A chromatogram of a degraded sample is shown in Fig. 2. Table 1 shows data obtained for each peak: UV absorption maxima, HPLC retention time, calculated and observed molecular ions. The UV spectra readily identify which impurities contain the aromatic amine side-chain which has an absorption band at about 295 nm.

Peaks **I** and **II** had LC-Thermospray mass spectra, UV spectra and retention time equivalent to those of synthetically prepared samples.

Peak **III** was isolated by preparative HPLC and identified by MS and NMR as a product of the hydrolysis of the piperazine-pyrimidine bond. The protonated molecular ion at mass 409 was consistent with loss of the 2,4-(bispyrrolidino)pyrimidine group. Hydrogen NMR of **III** was analyzed relative to that of tirilazad (free base). For tirilazad in $CDCl_3$, hydrogen NMR clearly identified the

TABLE 1

Analytical data for degradation products

Compound	UV absorption maxima (nm)	RT[a]	MW	LC/MS ion
I	245,295	3	302.43	303 (M+H)
II	248,296	5	330.4	331 (M+H)
III	242	17	408.6	409 (M+H)
IV	242	19		339 (M+H)
V	238,278	24	234.3	235 (M+H)
VI	244	28	326.43	327 (M+H)
VII	251,293	41	624.87	625 (M+H)
Tirilazad (**VIII**)	244,295	46	624.87	625 (M+H)

[a]Retention time for the LC system used for LC/MS (min).

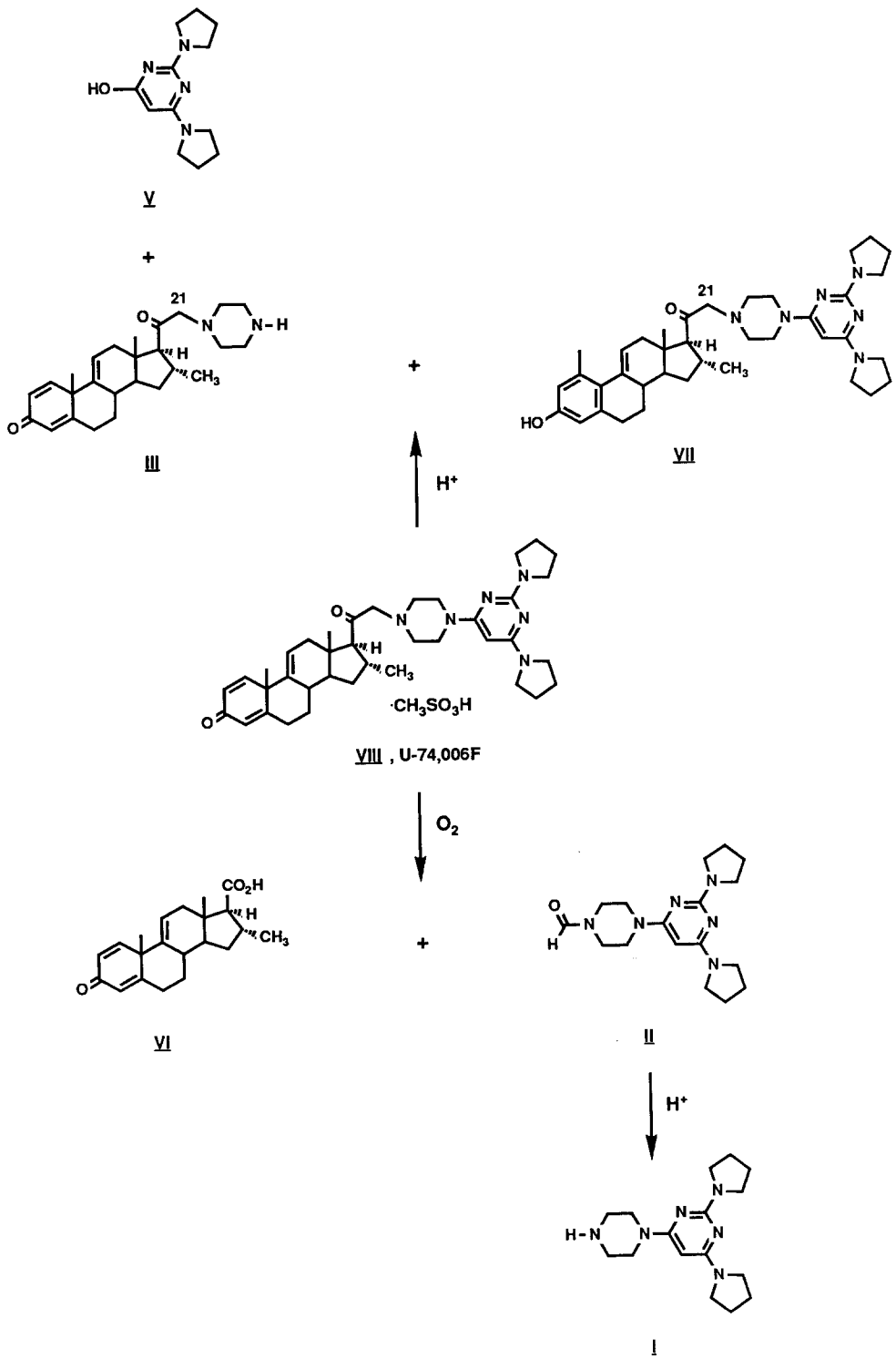

Fig. 1. Degradation scheme for tirilazad.

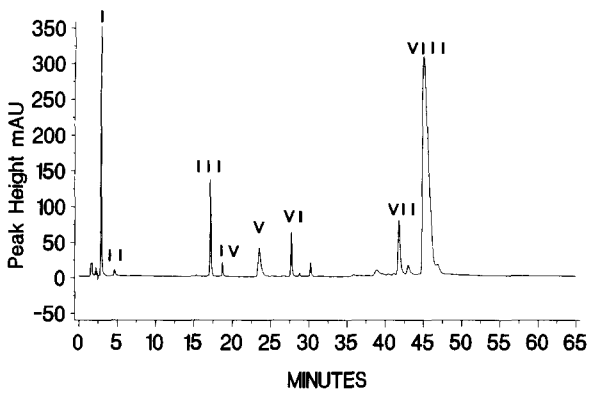

Fig. 2. Chromatogram of a degraded sample of tirilazad in 0.02 M citric acid at pH 3.0 (degraded for 2 days at 100°C; HPLC system was the same as used for LC/MS – see Materials and Methods).

eight pyrrolidinyl α-CH_2s at 1.9 ppm, one pyrimidinyl CH at 4.8 ppm, four piperazinyl CH_2s at 2.5 ppm, and a distinctive AB splitting at 3.2 ppm due to the 21-CH_2. All pyrrolidinyl and pyrimidinyl hydrogens were absent from the spectrum of **III** under identical conditions. The 21-CH_2 AB pattern altered due to more equivalent chemical shifts, indicating a less bulky substituent at C21. In addition, the piperazinyl 3-CH_2s and 5-CH_2s shifted 0.4 ppm upfield indicating a less electronegative group on 4-N. The spectrum indicated no significant changes to the steroidal resonances, other than at C21. The UV spectra were consistent with a steroid without the aromatic amine side-chain (Table 1). Compound **III** is formed from the hydrolysis of the pyrimidine-piperidine bond. The other fragment from hydrolysis is **V**.

Peak **IV** has not been assigned a structure. The UV spectra were consistent with a steroidal compound as the 290 nm band which is observed with compounds containing the amine side-chain was not observed (Table 1). LC/MS data indicated a possible molecular ion (M+H) of 339. From the molecular weight, the compound was tentatively hypothesized to be the 21-aldehyde (21-formyl-16α-methyl-17α-hydrogenpregna-1,4,9(11)-triene-3,20-dione). However, when the 21-aldehyde was synthesized (Palmer, 1988) and structure spectroscopically confirmed, the retention time and UV spectra did not match the unknown. The generally low levels of this compound did not justify further efforts at this time.

Peak **V** had mass spectra, UV spectra and retention time equivalent to that of a synthetically prepared sample. This compound, along with **III**, is a result of the hydrolysis of the piperazine-pyrimidine bond.

Peak **VI** has mass spectra, UV spectra and retention time equivalent to those of a synthetically prepared sample. This compound, along with **II**, is a result of oxidation of the 20-keto group.

Peak **VII** was isolated by preparative HPLC and identified by MS, NMR and IR spectra as a dienone-phenol, re-arrangement product. Thermospray mass spectra showed the same molecular ion for tirilazad and the isolated material. IR spectra of the isolated material showed the absence of a carbonyl band at 1665 cm^{-1} and the appearance of strong bands at 3217 and 3137 cm^{-1}, consistent with a phenolic OH group. The hydrogen NMR of the isolated compound, when compared to tirilazad, was very similar except for two major features: (i) In the region from 6.0 to 7.2 ppm, tirilazad shows an AMX three-spin coupled system with $J_{1,2}$=13 Hz, and $J_{2,4}$=2 Hz, while the product was an AX two-spin coupled system with J=2 Hz. (ii) In the methyl region, the C18-CH_3 singlet and C16-CH_3 doublets remained constant at 0.71 and 0.98 ppm in both compounds, while the C19-CH_3 singlet shifted from 1.41 ppm in tirilazad to 2.37 ppm. This data is consistent with a dienone-phenol re-arrangement reaction of tirilazad to a 1-methyl-3-hydroxyestra-1,3,5(10),9(11)-tetraene analog of tirilazad.

Degradation reactions

From the identity of the degradation products, we conclude that tirilazad degrades by a combination of oxidative and acid-catalyzed reactions (Fig. 1). Other supportive evidence for the proposed degradation reactions is discussed below.

The oxidative decomposition products of tirilazad (**II**, **VI**) are also formed readily in methylene chloride solution. Over 48 h at 20°C about 30% of tirilazad is lost. In ethyl acetate, about 50% decomposition occurs. The analogous products were reported for the reaction of hydrogen peroxide with α-(*N*,*N*-dialkyl)aminoketones in methanol

TABLE 2

Effect of pH and oxygen level on degradation products (citric acid, sodium citrate buffer; 1 day at 100°C, % w/w)

pH	Oxygen[a]	I	III	IV[c]	V	VI	VII
2.0	L	1.0	18.2	0.4	10.3	0.4	20.2
3.0	L	1.8	5.2	0.1	2.5	0.4	3.5
3.75[b]	L	0.6	0	0	0	0	0.9
2.0	H	2.8	15.2	0.3	8.3	0.7	9.1
3.0	H	4.0	6.1	0.1	4.5	1.5	3.3
3.75	H	1.5	3.3	0.2	0.5	2.5	0.4

[a]L, He purged solution; H, ambient oxygen level.
[b]A precipitate was observed in pH 3.75 solutions after heating.
[c]Quantitated based on an assumed response factor of 1.0.

(Wenkert et al., 1983). The proposed mechanism of degradation is addition of hydrogen peroxide to the ketone followed by Grob fragmentation to give a carboxylic acid and an iminium intermediate which is further oxidized to an amide. Although **II** and **VI** were formed in equivalent amounts in methylene chloride, levels of **II** were only a small fraction of the amount of **VI** in acidic aqueous systems. Hydrolysis of the formyl group was confirmed by the appearance of **I** when **II** was spiked into aqueous pH 3 solutions.

The dienone-phenol rearrangement reaction has been extensively studied for steroids (Kirk and Hartshorn, 1968; March, 1985) and is initiated by proton attack on the ketonic carbonium ion followed by rearrangement of the intermediate carbonium ion. The *meta* and *para* isomers of the hydroxy and methyl groups (originating from C10) are produced whose ratio depends on the solvent and structural features of side-groups. In aqueous mineral acids, similar to the solutions used here, the *meta* isomer is produced in greater abundance (Dreiding et al., 1953). The *para* isomer is likely present in degraded tirilazad but at lower concentrations.

Data for the effect of oxygen and pH on levels of degradation products produced at 100°C are shown in Table 2 and are consistent with the proposed degradation reactions. Decreased pH increases the level of **III**, **V**, and **VII** (**III** and **V** are produced from the hydrolysis of the piperazine-pyrimidine bond and **VII** is from the dienone-phenol rearrangement). Increased oxygen level increased the amount of **VI** and **I** (20-carboxylic acid and 21-amino side-chain) while decreasing the amount of **VII**. Originally, **I** was thought to be exclusively produced via **II** (formyl derivative of 21-amino side-chain) from hydrolysis of the formyl group. However, there was insufficient **VI** produced to explain the large amount of **I** found. Either **I** is also produced by another oxidative mechanism or **VI** is unstable. If **I** were produced by another oxidative mechanism, then the question arises as to what is the other steroid-containing fragment. Compound **IV** is one possibility under the presumption that **IV** is unstable.

TABLE 3

Degradation product data for pH 3.0 tirilazad mesylate solutions (1.5 mg/ml, 0.02 M citrate, % w/w)

Lot	Months	Temperature (°C)	I	III	IV	V	VI	VII
A	12	4	0.42	0.94	0.12	0.14	0.02	0.05
	12	30	1.78	8.7	0.9	4.2	0.09	0.17
B	9	4	0.34	0.98	0.08	0.26	0.02	0.05
	9	30	1.33	7.3	0.6	3.43	0.06	0.16

Lot	Months	Temperature (°C)	Total degradation (%)	Tirilazad mesylate (mg/ml)	Mass balance (%)
A	12	4	3.06	1.49	101.0
	12	30	17.5	1.25	99.5
B	9	4	3.13	1.50	101.8
	9	30	14.4	1.32	101.1

TABLE 4

Fraction of drug loss accounted for by identified degradation products on a mole basis (solution heated at 100°C; pH 3.0)[a]

Day	Tirilazad	I	III	V	VI	VII	Mass balance
0	1.00						
1	0.83	0.02	0.06	0.05	0.01	0.03	0.93
2	0.58	0.10	0.09	0.17	0.01	0.03	0.88
3	0.39	0.22	0.08	0.23	0.02	0.02	0.86
6	0.15	0.26	0.08	0.44	0.09	0.02	0.87

[a]For mole basis, **I**, **V** and **VII** were summed together.

Data for the long-term accumulation of degradation products in samples stored at 4 and 30°C (9, 12 months) is shown in Table 3. The largest amount of degradation products, in order, are **III**, **V** and **I**. **III** and **V** together account for 68 and 38% of the amount of tirilazad lost at 30 and 4°C, respectively.

To determine if any other important degradation routes are undetected, mass balance on a mole basis was evaluated by summing the molar amounts of one product from each proposed route of degradation (i.e. the molar amounts for **I**, **V** and **VII** and tirilazad were summed, see Table 4). Mass balance > 85% was observed for degradation to 15% of the starting concentration, despite the lack of consideration of steroidal impurities that have undergone the dienone-phenol re-arrangement. The four reactions discussed here explain the degradation of tirilazad.

Conclusions

Tirilazad degrades from four reactions: hydrolysis of the piperazine-pyrimidine bond, oxidation of the C-20 ketone, dienone-phenol rearrangement of the A ring and oxidation to produce the amine side-chain.

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