Autoxidation of 3-Hydroxyanthranilic Acid

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3-Hydroxyanthranilic acid in aqueous solution can be autoxidized to yield two different products depending on the pH at which the oxidation is performed. At acidic pH the formation of cinnabarinic acid is favored while at alkaline pHs the major product is a newly characterized *p*-quinone dimer. Both of these oxidation products are formed at pH 7. A mechanism to account for these pH-dependent oxidations is proposed.

A number of aromatic compounds have been shown to be involved in the tanning of proteins in insect cuticle and cocoon; however, the detailed reaction mechanisms are poorly understood.[1] One such compound, 3-hydroxyanthranilic acid (**1**), a normal metabolite of the amino acid tryptophan, is responsible for the tanning of cocoon protein in some species of moths, for example, *Samia cynthia*[2] and *Bombyx mori*.[3] Interestingly, 3-hydroxyanthranilic acid is also involved in the formation of the colored pigments of several Australian marsupials.[4] For instance, cinnabarinic acid (**2**) is the principal component of the red sternal patch of the Red Kangaroo (*Megaleia rufa*). It is not clear, however, if these pigments arise from an enzyme-catalyzed process in the apocrine glands or by autoxidation of **1** that has been deposited externally on hair follicles. 3-Hydroxyanthranilic acid is very sensitive to oxidation and it is an oxidized form of **1** which is responsible for initiating the tanning of protein and the formation of **2**.

As part of a program to investigate the mechanism of protein tanning we have examined the autoxidation of **1** under neutral and alkaline conditions.

Results and Discussion

Autoxidation of 3-Hydroxyanthranilic Acid at pH 11.7. Butenandt[5] and co-workers reported the formation of the monohydrate of either **3** or **4** from the oxidation of **1** with molecular oxygen at pH 11.7. The structure **3** or **4** was assigned on the basis of UV and microanalytical data and from the formation of the phenoxazone **5** upon basic hydrolysis. We have repeated this experiment and obtained in 45% yield a compound, $C_{14}H_{10}N_2O_7$, that had an identical UV spectrum to that reported for **3** or **4** by Butenandt.[5] The ^1H and ^{13}C NMR spectral data for this compound clearly indicated the *p*-benzoquinone structure **6**. The ^1H NMR spectrum of **6** showed a single olefinic resonance as a singlet at δ 5.08 (DMSO-d_6). The ^{13}C NMR spectrum of **6** showed four carbonyl resonances and four olefinic resonances, one of which appeared as a doublet in the off-resonance ^{13}C NMR spectrum (Table I). The ^{13}C NMR assignments for **6** were based on those values reported for actinocin.[6] Base hydrolysis of **6** gave, as expected, the phenoxazone **5**.[5]

Additional support for structure **6** came from the isolation of **7a** (65% yield) from the high pH oxidation of **1** in the presence of aniline (5 equiv). Compound **7a** was identical in all respects (UV, TLC, NMR, MS, IR) to an authentic sample of **7a** prepared by a modified route according to Schäfer.[7,8] Schäfer has observed **7a** as a minor product in the preparation of B from A with primary aliphatic amines in refluxing ethanol. We assume **7a** arises from B via an S_N2 dealkylation mechanism (Scheme I). In our modified procedure **7a** was obtained in nearly quantitative yield when A was treated with benzylamine (2:2 equiv) in refluxing ethanol. The exclusive formation of **7a** over B and the isolation of *N*-benzylaniline is clearly consistent with an S_N2 dealkylation mechanism. The ^{13}C NMR spectrum of **6** and **7a** showed a very good correlation (Table I). The aliphatic amines, benzylamine, 2-phenylethylamine, and *N*-α-*t*-Boc-L-lysine, gave analogous adducts **7** (**b–d**) in 50–60% yield.

Autoxidation of 3-Hydroxyanthranilic Acid at pH 7. The autoxidation of **1** at pH 7 and pH 6.5, however, was very sluggish and gave a mixture of **6** and cinnabarinic acid (**2**) (**6**:**2**, 4:1 and 2:3, respectively) in about 20% yield after 7 days. The remaining material was unreacted **1**. The

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(5) Butenandt, A.; Keck, J.; Neubert, G. *Liebigs Ann. Chem.* **1957**, *602*, 61.
(6) Hollstein, U.; Breitmaier, E.; Jung, A. *J. Am. Chem. Soc.* **1972**, *94*, 8036.
(7) Pardo, M.; Joos, K.; Schäfer, W. *J. Chem. Res., Miniprint* **1978**, 2201.
(8) An authentic sample of **7a** was kindly supplied by Prof. W. Schäfer.

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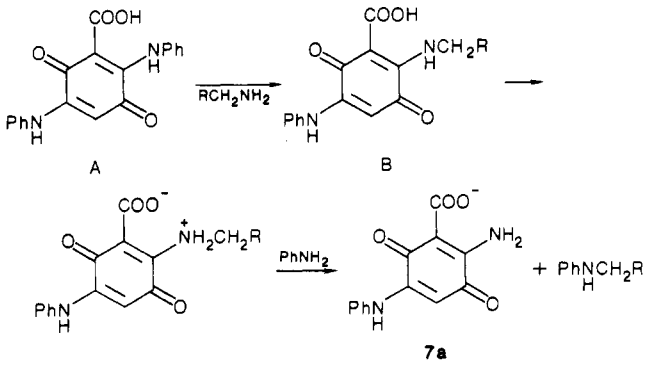
Table I. ^{13}C NMR Spectral Data for 6 and 7a in DMSO-d_6

compd	C-1[a]	C-2	C-3	C-4	C-5	C-6	aromatics	CO_2H
6	94.8 (s)	175.0 (s)[d]	149.1 (s)[e]	98.4 (d)	180.1 (s)[d]	157.6 (s)[e]	b	168.3 (s)
7	94.7 (s)	175.1 (s)[d]	148.7 (s)[e]	96.6 (d)	179.8 (s)[d]	156.7 (s)[e]	c	167.8 (s)

[a] Refer to structure 6 for numbering system. [b] 152.3 (s),[e] 129.1 (d), 128.5 (s), 123.6 (s), 122.4 (d), 121.3 (d). [c] 137.0 (s), 129.4 (d), 126.5 (d), 124.4 (d). [d] May be interchanged. [e] May be interchanged.

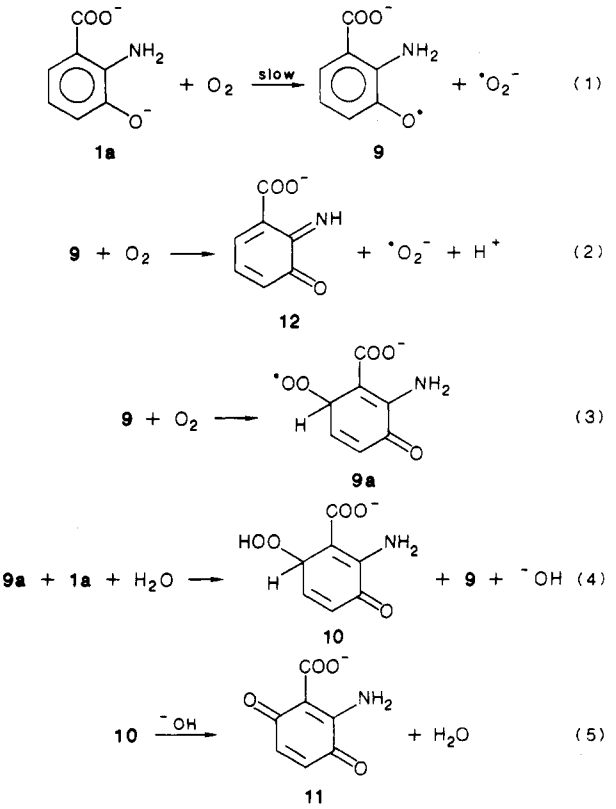
Scheme I



analogous reaction of 1 in the presence of aniline (5 equiv) at pH 7 gave 6 and the dianiline adduct 8 (6:8, 54:46) in about 20% yield. The dianiline adduct 8 could be obtained in moderate yield from the silver oxide oxidation of 1 in the presence of aniline in acetic acid.

Proposed Mechanism for the Autoxidation of 1 at pH 11.7. Any proposed mechanism for the formation of compounds 2, 6, 7, and 8 from 1 must account for the following experimental observations: (1) oxidation of 1 in the presence of aniline at pH 11.7 in 95% $H_2{}^{18}O$ gave 7a in which no incorporation of ^{18}O into the carbonyl oxygens had occurred;[9] (2) upon reexposure to the autoxidation conditions at pH 7 or pH 11.7 compound 8 was not converted to 7a but was recovered essentially unchanged,[10] even in the presence of hydrogen peroxide (2 equiv)[11] at pH 7; (3) compound 7a was not converted to 8 but was returned unchanged upon exposure to aniline (5 equiv) in dimethylformamide even in the presence of an acid catalyst (HCl); (4) cinnabarinic acid (2) and the diadduct 8 are formed at pH 7 but are not detected at pH 11.7. Clearly all the experimental evidence suggests that the C-2 carbonyl oxygen of 6 and 7 arises from molecular oxygen and not via hydrolysis of a labile C-2 imine intermediate. We suggest that 6 and 7 arise from a common precursor, p-quinone 11. Electron transfer from the phenoxide anion of 1 to molecular oxygen generates 9 which gives 10 according to Scheme II. Quinol hydroperoxides have recently been implicated as intermediates in the oxidation of resorcinols,[12] phenols,[13] and catechols.[14] Base-catalyzed elimination of water from 10 gives 11, which then suffers conjugate addition of amine (RNH_2 = 1, aniline or aliphatic amine) and then further oxidation to give 6 or 7. Alternative or competitive mechanisms involving conjugate addition of amine to 9 or reaction of 9 with superoxide to

Scheme II



give 10 could conceivably also be involved.

Proposed Mechanism for the Oxidation of 1 at pH 7. We suggest that at pH 7 a second competitive process leading to the quinone imine 12[16] also operates (Scheme II). Conjugate addition of either one or two molecules of aniline and then further oxidation gives 2 and 8, respectively. To account for the pH dependence of the oxidation of 1 a referee has suggested step (3) (Scheme II) to be reversible. At high pH, 9a is removed by reaction (4) to drive equilibrium (3) to the right and form 11 (via 10). At pH 7 (low concentration of 1a), the rate of reaction (2) becomes competitive with (4); equilibrium (3) is not forced to the right and more 12 is obtained. Whether products similar to 6, 7, and 8 are involved in protein tanning is currently under investigation. Interestingly initial results from our laboratories show that the enzymatic oxidation of 1 with tyrosinase in the presence of aniline gives 8 as the major product.

Experimental Section

Solvents used were of analytical or HPLC grade. Melting points are uncorrected. IR spectra were taken with a Perkin-Elmer infrared spectrophotometer, Model 783. UV spectra were recorded on a Shimadzu UV-vis recording spectrophotometer, Model UV-160. NMR spectra were recorded on a JEOL FX 90Q FT NMR spectrophotometer using TMS as an internal standard unless otherwise indicated. High resolution mass spectra were carried out on an AEI MS-902 using heptaperfluorotributylamine as reference. Thin layer chromatography (TLC) was carried out

(9) Analysis of the $[M - CO_2]^+$ base peak in the EI mass spectrum of 7 showed no ^{18}O incorporation. Analysis of the $[M - CO_2]^+$ peak ensures that any ^{18}O incorporation into the CO_2H group by exchange is excluded from the analysis.

(10) About 5% of decarboxylated 9 was obtained however.

(11) We assume that hydrogen peroxide generated in these reactions may facilitate the hydrolysis of 2 and 8.

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on aluminum-backed silica gel plates F_{254} (Merck) while column chromatography was performed by using silica gel (0.063–0.2 mm (Merck)) as the chromatographic adsorbent. 3-Hydroxyanthranilic acid and *N*-α-*t*-Boc-L-lysine were purchased from Sigma Chemical Company. Aniline was distilled prior to use.

6-Amino-3-[(2-carboxy-6-hydroxyphenyl)amino]-2,5-dioxo-1,3-cyclohexadiene-1-carboxylic Acid (6). CO_2-free air was bubbled through a solution of 3-hydroxyanthranilic acid (200 mg) in a 0.1 M sodium phosphate buffer (50 mL, pH 11.7) for 24 h. The resulted deep red solution was acidified to pH 2.5 with concentrated HCl and the resulting red precipitate of **6** collected by filtration: yield, 208 mg (50%); mp >300 °C (H_2O saturated butanol); ^1H NMR (DMSO-d_6) δ 5.08 (s, 1 H); ^{13}C NMR (DMSO-d_6) δ 94.8, 98.4, 121.3, 122.4, 123.6, 128.5, 129.1, 149.1, 152.3, 157.6, 167.8, 168.3, 175.0 (s), 180.1 (s); IR (Nujol mull) 3360, 3310, 3200, 1680, 1600, 1560, 1305, 1255, 1152, 960, 887, 840, 805 cm^{-1}; UV (EtOH) 262 nm (log ε 4.04), 274 (4.06), 330 (4.20), (3.45); only HRMS (FAB-MS, [M + 2])[17] calcd for $C_{14}H_{10}N_2O_7 + H_2$ 320.0645, found 320.0654.

6-Amino-3-(phenylamino)-2,5-dioxo-1,3-cyclohexadiene-1-carboxylic Acid (7a). CO_2-free air was bubbled through a stirred solution of 3-hydroxyanthranilic acid (200 mg) in a 0.1 M sodium phosphate buffer (50 mL, pH 11.7) containing 5 equiv of aniline for 24 h. The resulting pink precipitate was collected by filtration to give the sodium salt of **7** (238 mg, 65%): IR (Nujol mull) 3546, 3343, 3260, 3160, 1650, 1600, 1560, 1370, 1312, 1240, 850, 817, 745, 693 cm^{-1}; FAB-MS, *m/z* 214 (M − 44, − CO_2), 23 (Na^+).

The salt was dissolved in glacial acetic acid (100 mL) and water (10 mL). The solution was freeze-dried and recrystallized from dioxane to yield **7a** as a brilliant purple solid: mp 248 °C, lit.[7] mp 245 °C; ^1H NMR (DMSO-d_6) 5.79 (s, 1 H), 7.4 (m, 10 H); ^{13}C NMR (DMSO-d_6), δ 94.70, 96.54, 124.40, 126.46, 129.33, 137.01, 148.68, 156.78, 167.76, 174.93, 179.77; IR (Nujol mull) 3285, 3160, 1710, 1603, 1576, 1550, 1250, 1210, 1115, 1080, 868, 738, 700 cm^{-1}; UV (DMSO) 263 nm (log ε 4.22), 345.5 (4.21), 521.5 (3.32).

6-Amino-3-[(phenylmethyl)amino]-2,5-dioxo-1,3-cyclohexadiene-1-carboxylic Acid (7b). The sodium salt of **7b** was prepared analogously to **7a** except benzylamine was substituted for aniline: yield 119.6 mg (51%); IR (Nujol mull) 3540, 3360, 3308, 3140, 1650, 1599, 1560, 1490, 1242, 976 cm^{-1}; FAB-MS, *m/z* 228, 23 (Na^+).

The salt was converted into the free acid in an analogous fashion to that for **7a**: mp 186–187 °C (dioxane/ethanol); ^1H NMR (DMSO-d_6) 4.19 (d (coupled to N–H), *J* = 3.3 Hz, 2 H), 5.19 (s, 1 H), 7.02 (s, 5 H), 8.64 (bt, N–H, 1 H), 9.21 (b, 1 H), 9.72 (b, 1 H), 13.15 (b, 1 H); ^{13}C NMR (DMSO-d_6) δ 44.61 (t), 93.75 (s), 94.37 (d), 126.38 (br d), 127.63 (d), 135.82 (s), 149.98 (s), 156.71 (s), 167.06 (s), 172.48 (s), 178.68 (s); IR (Nujol mull) 3420, 3280, 3140, 1712, 1560, 1440, 1250, 1215, 1063, 875, 780, 740, 725, 692 cm^{-1}; UV (DMSO) 271.5 nm (log ε 3.88), 334.0 (4.21), 497.0 (2.88); HRMS calcd for $C_{14}H_{12}N_2O_4$ 272.0797, found 272.0796.

6-Amino-3-[(2-phenylethyl)amino]-2,5-dioxo-1,3-cyclohexadiene-1-carboxylic Acid (7c). The sodium salt of **7c** was prepared analogously to **7** except that 2-phenylethylamine was substituted for aniline: yield 246 mg (61%); IR (Nujol mull) 3445, 3363, 3308, 3140, 1648, 1598, 1560, 1490, 1338, 1233, 1177, 803, 738, 695 cm^{-1}; FAB-MS, *m/z* 242, 23.

The salt was converted into the free acid in an analogous fashion to that for **7c**: mp 178 °C (dioxane); ^1H NMR (DMSO-d_6) δ 2.87 (t, *J* = 7 Hz, 2 H), 3.48 (t, *J* = 7 Hz, 2 H), 5.59 (s, H) 7.26 (br, 5 H); ^{13}C NMR (DMSO-d_6) δ 33.30 (t), 43.60 (t), 94.45 (s), 94.45 (d), 126.23 (d), 128.33 (d), 128.33 (d), 138.53 (s), 150.63 (s), 157.80 (s), 167.91 (s), 173.22 (s), 179.23 (s); IR (Nujol mull) 3296, 3240, 3190, 1690, 1590, 1540, 1510, 1250, 780, 748, 698 cm^{-1}; UV (DMSO) 271.0 nm (log ε 3.81), 334.0 (4.20), 503 (2.83); HRMS calcd for $C_{15}H_{14}N_2O_4$ 286.0954, found 286.0952.

6-Amino-3-[[5-[[(*tert*-butyloxy)carbonyl]amino]-5-carboxy-1-pentyl]amino]-2,5-dioxo-1,3-cyclohexadiene-1-carboxylic Acid (7d). CO_2-free air was bubbled through a solution of 3-hydroxyanthranilic acid (200 mg) in a 0.1 M sodium phosphate buffer (50 mL, pH 11.7) containing 5 equiv of *N*-α-*t*-Boc-lysine for 24 h. The resulting red solution was acidified to pH 2.5 with concentrated HCl and extracted with ethyl acetate (2 × 50 mL washes). The organic extract was dried ($MgSO_4$), filtered, and rotary evaporated to yield a red solid which was chromatographed, using ethyl acetate/methanol gradient elution on silica gel. The resulting red band was collected. Removal of solvent gave **7d**: yield 200 mg (37.0%); mp 138–140 °C (methanol/water); ^1H NMR (DMSO-d_6) δ 1.37 (m, 13 H), 3.1 (bt, 2 H), 3.99 (bt, 1 H), 5.56 (s, 1 H), 7.00 (bd, 1 H), 8.41 (bt, 1 H), 9.53 (b, 1 H), 10.04 (b, 1 H), 13.12 (b, 2 H); ^{13}C (DMSO-d_6) 23.053, 26.86, 28.13, 30.42, 42.1 (t), 53.31 (d), 77.96 (s), 94.21, 94.27, 150.83, 155.51, 157.95, 167.95, 172.94, 174.11, 179.53; IR (Nujol mull) 3340, 3300, 3185, 1700, 1690, 1530, 1250, 1165, 770 cm^{-1}; UV (MeOH) 267.0 nm (log ε 4.14), 326.0 (4.55), 499.5 (3.07); HRMS calcd for $C_{18}H_{25}N_3O_8$ 411.1642, found 411.1635.

6-Amino-3-(phenylamino)-2-(phenylimino)-5-oxo-1,3-cyclohexadiene-1-carboxylic Acid (8). To a suspension of Ag_2O (1.7 g) in 80 mL of glacial acetic acid was added aniline (5.2 mmol) and 3-hydroxyanthranilic acid (1.3 mmol) in small portions. Vigorous shaking for 1 h gave a deep red solution which was filtered free of Ag_2O and Ag. Addition of water (500 mL) to the filtrate resulted in a black precipitate which was collected by filtration and dried over P_2O_5. The filtrate was extracted with 3 × 100 mL washes of CH_2Cl_2 and the combined extracts were washed with 2 × 50 mL portions of water. The organic fraction was dried with $MgSO_4$ and filtered and the solvent was removed to yield a black oily residue. This residue was combined with the previous precipitate and chromatographed on silica gel. The following solvent gradient was used: CH_2Cl, CH_2Cl_2/EtOAc (1:1), EtOAc, EtOAc/MeOH (9:1). The various fractions are collected and spotted onto TLC plates. The red fraction with an R_f value of 0.38 (EtOAc/hexane, 1:1) was rotary evaporated to leave **8** as a red solid (104 mg, 24%). Instability of **8** prevented further purification. For example, attempted recrystallization of **8** from ethanol/water produced **7a** quantitatively via intramolecular acid-catalyzed imine hydrolysis: ^1H NMR ($CDCl_3$) 5.93 (s, 1 H), 6.8–7.3 (m, 10 H); ^{13}C NMR ($CDCl_3$) 95.1, 100.5, 120.1, 123.8, 126.3, 127.1, 129.8, 130.6, 136.0, 145.1, 148.8, 152.0, 155.0, 170.3, 177.6; IR ($CHCl_3$) 3420, 3380, 3260, 1730, 1665, 1646, 1585, 1540, 1496, 1370, 1350, 960 cm^{-1}; MS (CI), *m/z* 333 (M^+), 289 (M − CO_2).

Autoxidation of 3-Hydroxyanthranilic Acid in $H_2{}^{18}O$. 3-Hydroxyanthranilic acid (5 mg) and Na_3PO_4 (25 mg) were dissolved in $H_2{}^{18}O$ (0.6 mL). Small amounts of sodium were added until the pH was approximately 11.0. Air was blown through the stirred solution for 4 h. The resulting red solution was acidified with glacial acetic acid and the precipitate collected by filtration. Mass spectral data indicates that virtually pure **6** results with no incorporation of ^{18}O into the quinoid system [refer to ref 9].

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Registry No. 1, 548-93-6; 2, 606-59-7; 3, 71745-80-7; 4, 63040-26-6; 6, 112817-49-9; **7a**, 68054-47-7; **7a**·Na, 112817-50-2; **7b**, 112817-51-3; **7b**·Na, 112817-52-4; **7c**, 112817-53-5; **7c**·Na, 112817-56-8; **7d**, 112817-54-6; 8, 112817-55-7; *N*-α-*t*-BOC-L-lysine, 13734-28-6; aniline, 62-53-3; benzylamine, 100-46-9; 2-phenylethylamine, 64-04-0.

(17) A [M + 2] peak in the mass spectrum is typical of quinones, see: Zeller, K.-P. In *The Chemistry of the Quinoid Compounds*; Patai, S., Ed.; Wiley-Interscience: New York, 1974; Part I, Chapter 5.