# Decomposition of Cinnabarinic Acid by Hydrogen Peroxide



Michael K. Manthey, Stephen G. Pyne* and Roger J. W. Truscott*

Department of Chemistry, University of Wollongong,
P. O. Box 1144, Wollongong, 2500 Australia

Received September 17, 1991

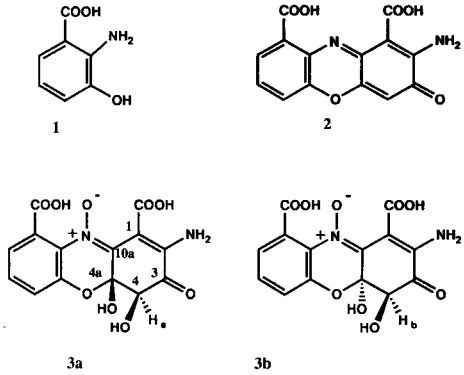
The hydrogen peroxide-induced decomposition of cinnabarinic acid **2** has been examined. Two major compounds were found to arise from the decomposition; 3-hydroxyanthranilic acid **1**, and an isomeric mixture of two novel hemiketals.

*J. Heterocyclic Chem.*, **29**, 263 (1992).

The oxidation of 3-hydroxyanthranilic acid **1** has been implicated in such diverse processes as the pigmentation of certain species of marsupials [1] and fungi [2], and in the sclerotization of silk proteins [3]. The autoxidation of **1** is complex, [4,5,6] but a mechanism has recently been proposed to account for the numerous oxidation products obtained [7]. A central feature of this mechanism is the importance of the reactive oxygen species, hydrogen peroxide and $O_2^{\cdot-}$, which are formed during the oxidation, and both of which act to decompose cinnabarinic acid **2**, one of the major autoxidation products of **1**. The degradative action of hydrogen peroxide upon **2** has been reported by Ogawa *et al.*, [5] who followed the decomposition spectrophotometrically, and isolated **1** from the complex mixture of decomposition products [5]. During our continuing studies upon the autoxidation of **1**, we re-examined the degradation of **2** by hydrogen peroxide since identification of the components present in oxidised solutions of **1** will aid both our understanding of the overall oxidation pathway and the mechanism of reaction of this reactive tryptophan metabolite with biopolymers [8].

Results and Discussion.

Treatment of **2** in an aqueous buffered solution at *p*H 7.0 with hydrogen peroxide (4 molar equivalents), resulted in the gradual discolouration of the original deep orange solution to one light yellow in colour. Preliminary examination of this solution by thin layer chromatography, indicated a number of products, of which two seemed to dominate. Partitioning of the crude acidified (*p*H 3.5) solution with ethyl acetate, afforded separation of the two major products. The organic fraction yielded a considerable quantity of almost pure **1** (36%). The remaining aqueous fraction yielded a highly fluorescent yellow compound, which microanalytical and mass spectral data indicated to have a molecular formula of $C_{14}H_{10}N_2O_9$. This compound was later found to exist as an isomeric mixture for which the structures **3a** and **3b** are proposed. The instability of **3** in organic solvents (for example, complete decomposition of **3** occurred in methanol over a period of 24 hours) and in moderately acidic or basic aqueous conditions, made it necessary to carry out all characterisation procedures in neutral aqueous solution. Even in neutral aqueous media,




significant decomposition of **3** occurred over a period of weeks. Although no chromatographic method could be found which could adequately resolve the isomers, analysis by $^1$H nmr spectroscopy indicated that **3a** and **3b** were always present in a 2:1 ratio respectively.

The high field (400 MHz) $^1$H nmr spectrum of **3** revealed relatively little structural information, although two isolated singlets at $\delta$ 5.14 and $\delta$ 5.63 with relative integral intensities of 2:1 respectively, were indicated. The upper field resonance ($\delta$ 5.14) was assigned to $H_a$ of **3a**, which would be expected to be the thermodynamically favoured isomer due to the equatorial configuration of its secondary hydroxyl group [9]; the large downfield shift of $H_b$ in relation to $H_a$ then being due to the location of $H_b$ within the anisotropic deshielding zones of the skeletal cyclohexenone ring system of **3b** [10].

The $^{13}$C nmr spectral data for **3** (Table 1), is also consistent with an isomeric mixture whose isomerism is centred around C-4a. $^{13}$C nmr spectral assignments for **3** are based (in part) on those reported for actinocin [11] and the $\alpha$ and the $\beta$ forms of D-glucose [12]. From an examination of conformational effects upon $^{13}$C nmr chemical shifts, it would be expected that C-4 of **3a** would experience a significant upfield shift relative to C-4 of **3b** due to the axial configuration of the hydroxyl substituent directly bonded to C-4 [13]. This should also result in an increased shielding of the $^{13}$C nuclei adjacent to C-4; a fact which is borne out by the upfield shifts suffered by C-4a and to a lesser extent C-3 of **3a**. The long wavelength uv absorption maximum of the isomeric mixture ($\lambda$ max 397 nm, log $\epsilon$ 4.30) suggests

Table 1

$^{13}$C NMR Spectral Data and Assignments for **3** (measured in deuterium oxide/DMSO-$d_6$ 1:1 from TMS) [a]

| | C-1 | C-2 [b] | C-3 | C-4 | C4a | C-10a [b] | COOH | COOH |
|---|---|---|---|---|---|---|---|---|
| **3a** [c] | 89.5 | 165.4 | 169.3 | 77.0 | 92.7 | 153.8 | 167.8 | 169.0 |
| **3b** [c] | 90.0 | 165.1 | 168.7 | 80.5 | 94.3 | 154.8 | 167.6 | 169.1 |

[a] See **3a** and **3b** for numbering system employed. [b] May be interchanged. [c] Aromatics **3a** 127.3, 128.6, 143.1, 119.5, 124.4, 124.8; **3b** 127.3, 128.2, 142.8, 118.9, 124.4, 125.7.

that the extended conjugation of **2** (λ max 454 nm, log ε 4.36) [14] has not been disrupted, and that the C=N functionality of **2** is intact. The instability of **3** in organic solvents, acidic, basic and to a lesser extent neutral aqueous solution would be expected to be characteristic features of a nitrone [15] and this was supported by the presence of a strong ir absorption band at 1278 cm$^{-1}$ [15] in **3**, which was absent in **2**. This finding is consistent with a general method of nitrone synthesis which involves treatment of azomethines with hydrogen peroxide [16].

Regarding a possible mechanism to account for the formation of **3** from **2**, one involving an epoxidation of **2** by hydrogen peroxide [18,17] with subsequent hydrolysis and anomerization about the hemiketal centre, accompanied by oxidation of the ring nitrogen of **2** by hydrogen peroxide [15,19] would afford an isomeric mixture of **3**.

## EXPERIMENTAL

The nmr spectra were obtained in deuterium oxide/DMSO-$d_6$ (1:1) as described previously [6]. Infrared spectra were recorded on a Perkin Elmer Infrared Spectrophotometer model 783 as potassium bromide disks. Ultraviolet spectra were recorded on a Shimadzu UV-VIS Recording Spectrophotometer Model UV-265. Microanalyses were performed by the Australian National University Analytical Services Unit, Canberra, Australia. Thin layer chromatography was carried out on aluminium-backed silica gel plates $F_{254}$ (Merck) employing butanol/acetic acid/water (4:2:1) as the developing solvent system. 3-Hydroxyanthranilic acid, Sephadex G-10 and G-25 were obtained from Sigma.

2-Amino-3*H*-phenoxazin-3-one-1,9-dicarboxylic Acid **2**.

Preparation according to the method of Whitfield, [14] employing an extended reaction time of 8 hours afforded **2** as an orange solid of mp >300° (lit mp >300°) [14]; $^1$H nmr (DMSO-$d_6$): δ 6.57 (s, 1H, C = C*H*CO), 7.51-7.93 (m, 3H, 3(Ar*H*)), 8.70 (br s, 1H, N$H_2$), 9.67 (br s, 1H, N$H_2$), 12.03 (br s, 2H, 2(COOH).

Decomposition of **2** by Hydrogen Peroxide.

To a stirred solution of **2** (1 g, 3.3 mmoles) in 0.1*M* sodium phosphate buffer (*p*H 7.0, 200 ml) was added hydrogen peroxide (2.3 ml of a 30% solution). The solution was stirred for 12 hours and the resultant yellow solution acidified to *p*H 3.5-4.0 with 1*M* hydrochloric acid and extracted with ethyl acetate (6 x 150 ml). The organic extracts were combined, dried (magnesium sulfate), filtered, and solvent removed under vacuum to afford **1** as an off-white solid (367 mg, 36%). The isolated compound was identical by $^1$H nmr and ir to authentic material.

The aqueous phase was freeze dried to approximately 20 ml and subjected to column chromatography on Sephadex G-25 employing water as the eluent. The major yellow band was collected, freeze dried and chromatography on Sephadex G-25 was repeated. The yellow fraction was freeze dried, and purified twice more on Sephadex G-10 employing water as the eluent. Lyophilising the yellow fraction afforded 2 amino-4,4a-dihydro-4,4a-dihydroxy-3*H*-phenoxazin-3-one-1,9-dicarboxylic acid 10-oxide **3** (as a mixture of the isomers **3a** and **3b**) as an unstable yellow solid (150 mg, 13%), mp 188-195° dec; $^1$H nmr: δ 5.14 (s, 1H), 5.63 (s, 1H), 6.9-7.50 (m, 6H); $^{13}$C nmr: δ 76.97, 80.49, 89.51, 90.05, 92.65, 94.29, 118.91, 119.52, 124.39 (br), 124.82, 125.72, 127.25 (br), 128.20, 128.60, 142.85, 143.14, 153.83, 154.84, 165.14, 165.39, 167.17, 167.75, 168.80, 169.10, 169.30, 169.77; ir: 3542, 3450, 3212, 3079, 2891, 1775, 1703, 1683, 1618, 1592, 1551, 1497, 1471, 1406, 1384, 1365, 1278, 1156, 1092, 758 cm$^{-1}$; uv (water): λ max 231.4 nm (log ε 4.10), 249.6 nm (log ε 4.01, shoulder), 396.8 nm (log ε 4.30).

*Anal.* Calcd. for $C_{14}H_{10}N_2O_9$: C, 48.01; H, 2.88; N, 8.00. Found: C, 48.56; H, 3.19; N, 7.67.

Acknowledgement.

We wish to thank the National Health and Medical Research Council and the Government Employees Assistance to Medical Research Fund for financial support.

## REFERENCES AND NOTES

[1] E. M. Nicholls, *Int. J. Biochem.*, **2**, 593 (1971).

[2] G. W. K. Cavill, P. S. Clezy and J. R. Tetaz, *J. Chem. Soc.*, 2646 (1957).

[3] P. C. J. Brunet and B. C. Coles, *Proc. Roy. Soc. Biol.*, **187**, 133 (1974).

[4] H. Iwahashi, T. Ishii, R. Sugata and R. Kido, *Biochem. J.*, **251**, 893 (1988).

[5] H. Ogawa, Y. Nagamura and I. Ishigura, *Hoppe-Seylers Z. Physicol. Chem.*, **364**, 1507 (1983).

[6] M. K. Manthey, S. G. Pyne and R. J. W. Truscott, *J. Org. Chem.*, **55**, 4581 (1990).

[7] M. K. Manthey, S. G. Pyne and R. J. W. Truscott, *Biochim. Biophys. Acta*, **207**, 1034 (1990).

[8] R. J. W. Truscott and F. Martin, *Exp. Eye Res.*, **49**, 927 (1989).

[9] Confirmed by inspection of molecular models and an analysis of **3a** employing Alchemy II software, Tripos Associates, St. Louis.

[10] F. A. Bovey, in Nuclear Magnetic Resonance Spectroscopy, 2nd Ed, Academic Press, Inc, San Diego, 1988.

[11] V. Hollstein, E. Breitmaur and A. Jung, *J. Am. Chem. Soc.*, **96**, 8036 (1974).

[12] A. S. Perlin and B. Casu, *Tetrahedron Letters*, 2921 (1969).

[13] A. S. Perlin, B. Casu and H. J. Koch, *Can. J. Chem.*, **48**, 2596 (1970).

[14] F. G. Whitfield, MSc Thesis, The University of New South Wales, N. S. W. Australia, 1959.

[15] J. Hamer and A. Macaluso, *Chem. Rev.*, **64**, 473 (1964).

[16] G. Tennant, in Comprehensive Organic Chemistry, D. Barton and W. Ollis eds, Pergamon Press, Oxford, 1979, p 503.

[17] R. D. Temple, *J. Org. Chem.*, **35**, 1275 (1970).

[18] D. Vargo and M. S. Jorns, *J. Am. Chem. Soc.*, **101**, 7623 (1979).

[19] S. Patai, in The Chemistry of the Amino Group, Interscience Publishers, New York, 1968.