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Tyrosinase-Catalysed Oxidation of the New Melanocytotoxic Agent N-4-Hydroxyphenylglycine: Unusual Cleavage of the 4-Aminophenol Ring to Muconic Semialdehyde Derivatives.

Donatella Mascagna, Claudio Costantini, Marco d'Ischia and Giuseppe Prota*

**Department of Organic and Biological Chemistry,
University of Naples
Via Mezzocannone 16, I-80134 Naples, Italy.**

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Abstract. Oxidation of *N*-4-hydroxyphenylglycine (**1**) with tyrosinase in phosphate buffer at pH 6.8 results in two major products, which have been isolated and identified as the novel carboxymethylaminomuconic semialdehyde **3** and the related Schiff base **2**. Tyrosinase-catalysed oxidation of 4-aminophenol under similar conditions affords the analogous ring-opened products **6** and **7**. Formation of these compounds can be envisaged as proceeding through an unusual cleavage of the 4-aminophenol ring subsequent to its enzymatic conversion to the corresponding *o*-quinone. Besides disclosing new aspects of the oxidative reactivity of the 4-aminophenol system, the results of this study provide a chemical background to look into the mechanism of the cytotoxic effects of **1** towards human melanoma cells.

Oxidation of phenolic compounds by the copper-containing enzyme tyrosinase[1,2] has traditionally been a very active issue in studies of the chemistry of biological processes. Reasons for this interest are multiple and include, notably, the involvement of tyrosinase-promoted reactions in the biosynthesis of melanins, the characteristic pigments of mammals[3,4]; in certain sclerotisation processes of natural composite materials[5]; in the oxidation pathway of γ-glutaminyl-4-hydroxybenzene in the mushroom *Agaricus bisporus*[6]; and in the browning phenomena that occur when certain plants are cut and the resultant surface is exposed to air[7]. Additional emphasis has come, during the last two decades, from the promising application of phenolic drugs in the targeted chemotherapy of malignant melanoma[8], an aggressive and increasingly prevalent form of skin cancer that shows marked resistance to virtually all standard

chemotherapeutic agents, such as DTIC (decarbazine), CCNU (lomustine) and the newer cis-platinum. The underlying rationale is that, within aberrant melanocytes, phenols would be metabolically activated by tyrosinase, with consequent formation of highly cytotoxic *o*-quinones capable of interfering with cell growth[9].

Among the various phenolic substrates of tyrosinase that have so far been investigated for the treatment of melanoma, 4-hydroxyanisole[10] and a number of 4-*S*-cysteaminylphenol derivatives[11] have attracted most of the attention. However, their clinical use in chemotherapeutic regimens has been obstacled by their unfavourable pharmacokinetics and significant collateral effects. Recently, in the course of a screening program aimed at developing new prototype antitumour molecules, we have found that *N*-4-hydroxyphenylglycine (1) and some related 4-aminophenol compounds exert remarkable inhibitory effects towards a number of melanotic and amelanotic melanoma cell lines growing *in vitro*[12]. This finding prompted us to investigate in detail the tyrosinase-catalysed oxidation of 1 as a model reaction to gain some insight into the mechanism of the melanocytotoxic effects.

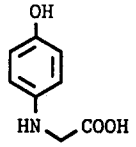
The enzymic oxidation of 1 was carried out in the presence of catalytic amounts of dopa serving as a cofactor. Spectrophotometric monitoring of the reaction progress in phosphate buffer at pH 6.8 showed the formation during the early stages of a yellow chromophore centered at about 345 nm, which persisted without significant modification over a prolonged period of time. A qualitatively similar course was observed when the enzymic oxidation of 1 was carried out in the absence of added dopa, although the kinetics of product formation were significantly slower.

HPLC analysis of the oxidation mixture after about 1 h, when almost all 1 had disappeared, revealed the presence of two detectable products, the most polar of which proved so unstable to escape all attempts at isolation. Preparative HPLC afforded the other product as a yellow oil (λ_{max} 345 nm) soluble in water, slightly soluble in methanol but almost insoluble in other common organic solvents. The ^{1}H-NMR spectrum (D_2O) exhibited a very complex pattern of resonances which, on careful analysis, turned out to be due to an equilibrium mixture of two closely related compounds in the approximate ratio of 2.5:1. Discrimination of the signals belonging to the major component, aided by proton decoupling experiments, allowed to discern the presence of a 4-aminophenol moiety (2H doublets at δ 6.72 and 7.04), a glycine residue (2H singlet at δ 4.21), and two distinct pairs of sp^2 protons. These latter were accounted for by an almost coalesced AB quartet centred at δ 6.30 (J=12.5 Hz) and a

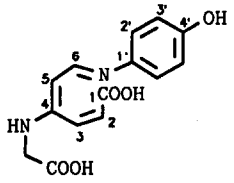
characteristic, widely splitted AX system consisting of two doublets at δ 5.34 and 7.75.

Attempts to run the ^{13}C-NMR spectrum of the compound(s) were invariably defeated, owing to the slow conversion to a more stable mixture of two related products in the same approximate ratio of 2.5:1. These coeluted on HPLC and exhibited longer retention times than the first formed of products. The ^{1}H-NMR spectrum of the major component exhibited features that were strikingly similar to those of the parent compound, with the noticeable exception of two doublets at δ 6.57 and 7.44 (J=16.0 Hz) in place of the AB quartet. The ^{13}C-NMR spectrum displayed likewise a distinct set of signals for the major component of the mixture, along with some partially obscured signals ascribable to the minor isomer. Salient resonances, confirmed by DEPT multiplicity discrimination[13], included those of a 4-aminophenol unit, two carboxyl groups, two sp^2 CH carbons, one low-field at δ 158.44 and the other high-field at δ 95.20, and a glycine-type methylene at δ 56.22. The UV spectrum of the new products showed an absorption maximum at 363 nm. Positive FAB mass spectral analysis revealed a pseudomolecular ion peak at m/z 291 $(M+H)^+$.

From the above spectral data, we argued that we were dealing with two mixtures of stereoisomers in a mutual stereoisomeric relationship. Of the several structures that were considered, one which was compatible with all available structural elements was that of the novel 4-aminophenol-muconic semialdehyde Schiff base **2**.

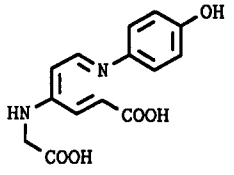


1



2

(Z isomer at C2,C3 double bond)



2

(E isomer at C2,C3 double bond)

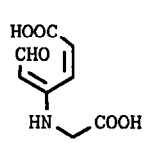
Apparently, the major first-formed product possesses the Z configuration at the carboxyl-bearing double bond, while the principal isomer of the aged mixture corresponds to the thermodynamically more stable E form, as indicated by the conversion in the proton spectrum of the olefinic AB quartet into the trans-coupled pair of doublets. The presence of an aldimine proton vicinal to a β-enamine proton well accounts for the widely splitted AX pairs of doublets typical of both isomers. The minor components of the mixtures could not be separated

chromatographically, nor could be characterised spectrally; probably, they arise from E,Z isomerization at the enamine double bond.

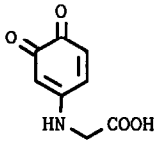
Consistent with the proposed structural assignment is also the significant low-field shift (up to 0.5 ppm) of the signals of the olefinic H-3 and H-5 protons of **2**, Z and E isomers, in dilute DCl as the solvent. This should conceivably be induced by the suppressed ionization of the carboxyl group and the partial protonation of the glycine nitrogen.

When the oxidation mixture of **1** was allowed to stand for 24 h or more, HPLC analysis showed the formation, besides the isomeric mixtures of **2**, of a new, relatively more polar product with absorption maxima at 280 and 332 nm. Positive FAB mass spectral analysis revealed a pseudomolecular ion peak at m/z 200 $(M+H)^+$. The ^{1}H-NMR spectrum displayed, in addition to a 2H singlet at δ 4.25, a rather simple pattern of resonances in the olefinic region, consisting of a pair of doublets at δ 6.46 and 7.84, and, as a most significant feature, an aldehydic signal at δ 9.78 coupled to a signal at δ 5.78. A long-range coupling between the signals at δ 5.78 and 6.46 was also apparent. These data were strongly suggestive of a conjugated dienal fragment bearing a glycine residue on the β carbon linked through the amino group. Support to this view came from the ^{13}C-NMR spectrum, in which signals ascribable to an aldehyde carbonyl, two double bonds and two carboxyl groups were clearly detectable. On these grounds, the compound was unambiguously formulated as the carboxymethylaminomuconic semialdehyde **3**.

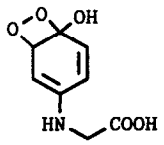
The origin of the products **2** and **3** is intriguing. Their formation appears to be typical of the tyrosinase-catalysed reaction, since they were not produced during aerial or chemical oxidation of **1**, e.g. with $NaIO_4$, and could not be detected in the enzymic oxidation with the peroxidase/H_2O_2 system. Considering the specific ability of tyrosinase to catalyse the oxidation of phenols to o-quinones[1,2], there seems a convincing justification for postulating the intervention of quinone **4** in the title reaction. Alternatively, oxygenated intermediates of the type **5** could be invoked. Unfortunately, all attempts to directly demonstrate the generation of **4** or **5** in the early stages of the oxidation of **1** have been unsuccessful.



3

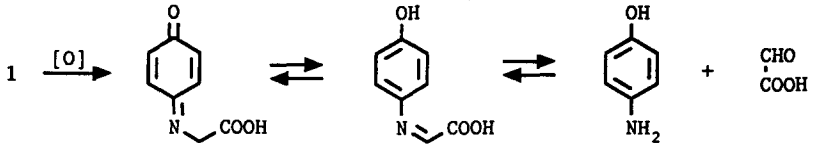


4



5

The presence of a 4-aminophenol moiety in **2** and the observed liberation of small amounts of 4-aminophenol during the very early phases of the reaction (HPLC, TLC) suggest, on the other hand, that the more general oxidative route of **1** to the corresponding *p*-quinone imine is also operative. Loss of the CH_2COOH grouping would then be easily explained as the result of sequential tautomerisation and hydrolysis processes, as depicted in the Scheme.

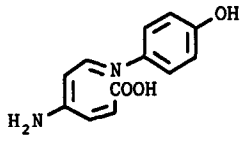


Scheme

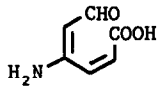
The opening of the 4-aminophenol ring of **1** remains a major mechanistic dilemma. Since addition of catalase to the incubation mixture does not affect the reaction course nor the product pattern, the involvement of some hydrogen peroxide generated during the oxidation would be ruled out. One could therefore argue that the critical event is a non-oxidative, non-enzymatic cleavage suffered by a transient intermediate formed subsequent to the initial enzymatic generation of the quinone **4**. That the postulated precursor is the quinone **4** itself seems unlikely, since the aldehyde **3**, which would derive from hydrolytic fission of this putative intermediate, becomes detectable only in the later stages of the oxidation reaction, probably by degradation of a more complex labile compound. Relevant to this discussion are the observations that under the experimental conditions **2** is fairly stable and is by no means hydrolysed to **3**, and that the reaction of **3** with 4-aminophenol does not lead to significant amounts of **2**, which would rule out any direct interrelationship between the two products. In the light of the foregoing, compound **5** would seem a more plausible intermediate; however, it was difficult to test the validity of any hypothesis in view of the marked complexity of the chemistry involved.

Mechanistic insight into the oxidation of **1** was sought through separate experiments, in which 4-aminophenol was oxidised with tyrosinase under the same conditions used for **1**. Careful analysis of the reaction mixture during the early stages, to detect quinone intermediates or derivatives, did not provide conclusive evidence. It is worthwhile noting, however, that an oxidation product of 4-aminophenol closely related to **2** could be identified when the reaction was approaching completion. This compound, which proved difficult to separate from a mixture of intimately related minor species, showed an absorption maximum

centred at 366 nm and a pattern of resonances in the ^1H NMR spectrum virtually identical to those of the first formed isomer of **2**, with the obvious lack of the glycine methylene protons. It was thus assigned the structure of **6**, Z isomer at the C-2,C-3 double bond. Z/E isomerization was not apparent under the reaction conditions, though it might have escaped our analysis. In analogy with the oxidation of **1**, prolonging the reaction time allowed the formation of significant amounts of 4-amino-5-formyl-pentadienoic acid (**7**) which was isolated and characterised by straightforward analysis of its spectral data. The acid **7** was also formed, together with 4-aminophenol, upon mild hydrolysis of **6** with 0.1 M acetic acid at 50 °C for 2 h.



6



7

While the chemical or enzymatic oxidative cleavage of *o*-quinones to give muconic acids is well documented in the literature[14-17], the generation of muconic semialdehyde derivatives under mild biomimetic conditions is, to the best of our knowledge, unprecedented; the main relevant reports deal solely with the enzymatic extradiol cleavage of catechol, by the action of metapyrocatechase[18], and of 3-hydroxyanthranilic acid[19]. Work is in progress in our laboratory to ascertain whether and to what extent the observed formation of muconic semialdehyde derivatives may account for the cytotoxic effects of **1** towards human melanoma cells.

EXPERIMENTAL.

UV spectra were determined with a Perkin Elmer Lambda 7 spectrophotometer. ^1H-NMR (270 MHz) and ^{13}C-NMR (67.9 MHz) spectra were performed with a Bruker AC 270 spectrometer, using D_2O as the solvent and dioxane as the internal standard. Fast atom bombardment mass spectra (FAB-MS) were determined with a Kratos MS 50 spectrometer. Analytical and preparative HPLC were carried out using a Gilson mod. 305 pump, a Gilson 316 UV detector and a 4 x 250 mm Spherisorb S5-ODS2 column (Phase Separation Ltd.) or a 10 x 250 mm RP18 Econosil column (Alltech). L-Dopa and mushroom tyrosinase (*o*-diphenol: O_2 oxidoreductase, EC 1.14.18.1, 2480 units/mg) were purchased from Sigma Chemical Co. *N*-4-hydroxyphenylglycine (**1**) was prepared by a standard synthetic procedure[20]. 4-Aminophenol was from Carlo Erba. All other chemicals and solvents were of the highest quality available and were used without further purification.

Tyrosinase-catalysed oxidation of 1.

To a solution of **1** (500 mg) in phosphate buffer 0.025 M pH 6.8 (500 ml), mushroom tyrosinase (50 mg) and L-dopa (35 mg) were added, and the

mixture was vigorously stirred at room temperature under oxygen-bubbling. After 1 hour, when most of **1** had disappeared, HPLC analysis (0.1 M acetic acid - MeOH 85:15 v/v, mobile phase; flow rate 1 ml/min; analytical wavelength λ=280 nm) revealed two major peaks eluting at 9.5 and 16 min. The latter was collected by preparative HPLC (NH_4HCO_3 0.1 M - MeOH, 95:5 v/v, mobile phase; flow rate 4 ml/min) to give **2** (Z form) as a yellow oil (83 mg), $\lambda_{max}(H_2O)$ 345 nm; ^{1}H-NMR[21] (D_2O), δ (ppm) 4.21 (2H, s, CH_2), 5.34 (1H, d, J=12 Hz, H-5), 6.28 (1H, d, J=12.5 Hz, H-2 or H-3) 6.32 (1H, d, J=12.5 Hz, H-3 or H-2), 6.72 (2H, d, J=8.6 Hz, H-3′), 7.04 (2H, d, J=8.6 Hz, H-2′), 7.75 (1H, d, J=12 Hz, H-6).

On standing at room temperature, **2** was converted into a more stable isomer eluting on HPLC at 35 min, $\lambda_{max}(H_2O)$ 363 nm; FAB-MS (matrix: nitrobenzyl alcohol): m/z 291 $(M+H)^+$; ^{1}H-NMR[21] (D_2O), δ (ppm) 4.35 (2H, s, CH_2), 5.58 (1H, d, J=13.0 Hz, H-5), 6.57 (1H, d, J=16.0 Hz, H-2), 6.87 (2H, d, J=9.0 Hz, H-3′), 7.13 (2H, d, J=9.0 Hz, H-2′), 7.44 (1H, d, J=16.0 Hz, H-3), 8.04 (1H, d, J=13.0 Hz, H-6); ^{13}C-NMR (D_2O), δ (ppm) 56.22 (t, CH_2), 95.20 (d, C-5), 117.32 (d, 2 x C-3′), 124.87 (d, 2 x C-2′), 130.25 (d, C-2), 138.58 (d, C-3), 139.81 (s, C-4′), 156.45, 157.38 (s,s, C-4, C-1′), 158.44 (d, C-6), 170.65 (COOH), 173.88 (COOH, C-1).

When the oxidation mixture was allowed to stand for 24 hours at room temperature, HPLC analysis showed the disappearance of the peak eluting at 9.5 min and the presence, besides both isomers of **2**, of a new peak eluting at 5.7 min. This was collected by preparative HPLC (eluant as above) and afforded **3** (80 mg) as a courless oil which could not be crystallised, $\lambda_{max}(H_2O)$ 280, 332 nm; FAB-MS (matrix: glycerol/thioglycerol): m/z 200 $(M+H)^+$; ^{1}H-NMR (D_2O), δ (ppm) 4.25 (2H, s, CH_2), 5.78 (1H, dd, J=8.1, 1.0 Hz, H-5), 6.46 (1H, dd, J=5.9, 1.0 Hz, H-2), 7.84 (1H, d, J=5.9 Hz, H-3), 9.78 (1H, d, J=8.1 Hz, H-6); ^{13}C-NMR (D_2O), δ (ppm) 42.37 (t, CH_2), 111.03 (d, C-5), 128.43 (d, C-2), 136.05 (d, C-3), 156.58 (s, C-4), 172.00, 173.42 (s,s, C-1 and COOH), 193.20 (d, C-6).

The remainder of the oxidation mixture was accounted for by polymeric materials which could not be analysed.

Tyrosinase-catalysed oxidation of 4-aminophenol.

The oxidation of 4-aminophenol was carried out under conditions similar to those used for **1**. The reaction mixture consisted of a solution of the phenol (100 mg) in 0.05 M phosphate buffer, pH 6.8 (100 ml), to which L-dopa (10 mg dissolved in 50 ml of water) and then mushroom tyrosinase (10 mg dissolved in 10 ml of water) were added. After 2 hours, HPLC analysis (0.05 M acetate buffer, pH 5.5-methanol 85:15 v/v, mobile phase; flow rate 1 ml/min) revealed the presence, besides highly polar materials, of a broad major peak eluting at 17.4 min. This was collected by preparative HPLC (H_2O-MeOH 80:20 v/v, mobile phase; flow rate 4 ml/min; analytical wavelength λ=280 nm) and was shown to contain compound **6** (C-2,C-3 Z isomer[22]), yellow oil, $\lambda_{max}(H_2O)$ 366 nm; ^{1}H-NMR (D_2O), δ (ppm) 5.50 (1H, d, J=6.7, H-5), 6.73 (1H,1H, collapsed AB q, H-2, H-3), 6.89 (2H, d, J=9.1 Hz, H-3′), 7.17 (2H, d, J=9.1 Hz, H-2′), 7.80 (1H, d, J=6.7 Hz, H-6).

When the oxidation mixture was allowed to stand at room temperature, HPLC analysis showed the slow formation of 4-amino-5-formyl-pentadienoic acid (**7**) (eluting at 6.1 min, 0.1 M acetic acid - methanol 85:15 v/v mobile phase; flow rate 0.7 ml/min). This compound was obtained by preparative HPLC (flow rate 6 ml/min; eluant as above) as a colourless oil (7 mg) $\lambda_{max}(H_2O)$ 280, 315 nm; FAB-MS (matrix: nitrobenzyl alcohol) m/z 233 $(M+H)^+$; ^{1}H-NMR (D_2O), δ (ppm) 5.83 (1H, dd, J=8.5, 1.6 Hz, H-5), 6.38 (1H, dd, J=5.9, 1.6 Hz, H-2), 7.74 (1H, d, J=5.9 Hz, H-3), 9.73 (1H, d, J=8.5 Hz, H-6). ^{13}C-NMR (D_2O), δ (ppm) 111.97 (d, C-5), 129.81 (d, C-2), 135.90 (d, C-3), 156.72 (s, C-4), 172.20, 173.84 (s,s, C-1 and COOH), 193.72 (d, C-6).

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21. Signals due to minor accompanying isomers have not been included. Wherever resolved from the signals of the major product, their integrated areas accounted for variable fractions of a proton.
22. The ^{13}C-NMR spectrum of **6** was very complicated to interpret, due in part to the mixture of products and to the presence of isomeric species. Attempts to enrich the sample in the major species by careful HPLC peak shaving were not successful.