Tetrahedron Vol. 48, No. 39, pp. 8515-8522, 1992
Printed in Great Britain

0040-4020/92 $5.00+.00

A New Oxidation Pathway of the Neurotoxin 6-Aminodopamine. Isolation and Characterisation of a Dimer with a Tetrahydro[3,4a]iminoethanophenoxazine Ring System.

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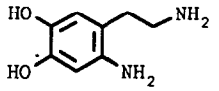
(*Received in UK* 7 *July* 1992)

Abstract: Oxidation of the neurotoxin 6-aminodopamine (**1**) is known to proceed through the *o*-quinone **3**, which undergoes intramolecular cyclisation to give 5,6-dihydroxyindole (**6**). In a re-examination of the reaction, we have found that at concentrations of **1** higher than 5 x 10^{-3} M a quite different course prevails, leading to the formation of the novel 7-amino-8-(2-aminoethyl)-3-hydroxy-2-oxo-2,3,4,10-tetrahydro[3,4a]iminoethanophenoxazine (**7**). Product **7** was formed by aerobic, chemical (persulphate, periodate) or enzymatic (tyrosinase, peroxidase/H_2O_2) oxidation of **1**. On acetylation, **7** afforded the tetraacetate **8**. Oxidation of the model compound 5-amino-4-methylcatechol (**9**) proceeded similarly as that of **1**, to give the tetrahydrophenoxazinedione **11**.

6-Aminodopamine (**1**) and its congener 6-hydroxydopamine (**2**) have been the subject of keen interest in neurobiological circles owing to their ability to bring about selective degeneration of catecholaminergic neurons.[1] While it seems clear now that the selectivity of these dopamine analogues is derived from their high affinity uptake by the neuron membrane pump,[2] there is still much uncertainty about the molecular mechanisms underlying the neurotoxic effects.



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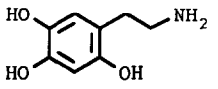


2

Common theories revolve around the marked facility of these catecholamines to oxidation, leading to the intracellular formation of electrophilic quinones[3] and highly reactive oxygen species, such as

superoxide ions, hydrogen peroxide and hydroxyl radicals.[4] Neuronal destruction would then result from the targeting of critical functionalities within vital biomolecules, including enzymes[5] and lipids.

Direct investigation of the metabolic fate of **1** and **2** in nerve cells has so far been unrewarding, and information has mainly been sought through studies of the oxidative behaviour of these neurotoxins under physiologically relevant conditions. Chemical[6] and electrochemical[7] experiments have shown that the oxidation of both catecholamines involves the initial generation of the corresponding *o*-quinones **3** and **4**. These undergo rapid intramolecular cyclization to give a common aminochrome intermediate (**5**), which smoothly rearranges to 5,6-dihydroxyindole (**6**), thereby leading the oxidation pathway to merge into the final stages of the biosynthesis of melanins, the principal pigments of mammals[8] (Figure 1).

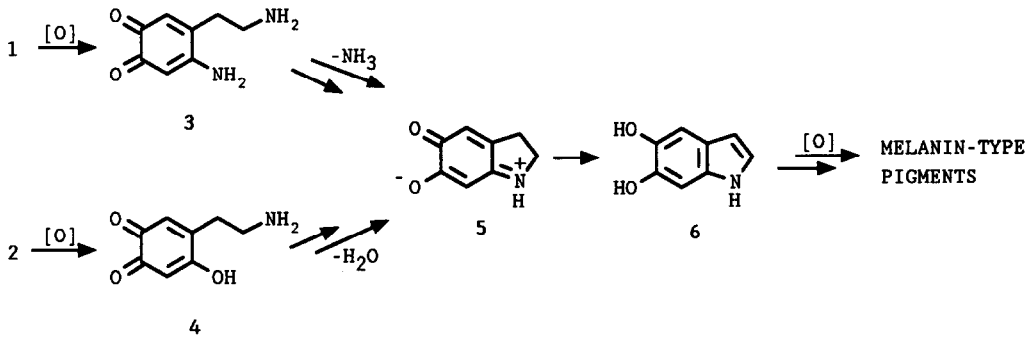


Figure 1. Commonly accepted oxidation pathways of catecholamines 1 and 2.

In his synthetic approach to melanin precursors, Harley-Mason[9] in 1953 proposed the oxidation of **1** and **2** as a most convenient route to the indole **6**. Recently, however, in the course of our continuing studies of the oxidation chemistry of catecholamines,[10] we repeated the reaction under the reported conditions[9] and found that, in the case of **1**, the major product was not the indole **6** but a new compound whose features did not match those of any of the known oxidation products of **1**. This finding prompted us to re-investigate the oxidation chemistry of this neurotoxic catecholamine.

Preliminarily, an improved two-step procedure for the preparation of **1** was developed, which afforded the product on a gram-scale in good yield. This involves nitration of dopamine with sodium nitrite in aqueous sulphuric acid, followed by $SnCl_2$/HCl reduction of the resulting 2-nitrodopamine.

When a dilute solution of **1** (e.g. 1 x 10^{-3} M) in 0.08 M sodium

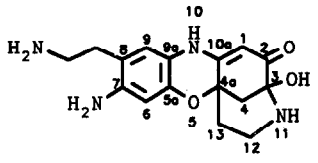
bicarbonate was oxidised in air, a 20% yield of **6** was obtained (HPLC evidence) along with copious brownish-black materials, resembling melanin pigments. However, at concentrations higher than 5 x 10^{-3} M, up to 6.9 x 10^{-2} M, i.e. under the conditions used by Harley-Mason,[9] the autoxidation of **1** led to a major yellow product (λ_{max} 368 nm), but little or no **6**.

Formation of the compound was also observed when **1** was oxidised with enzymatic systems, *e.g.* peroxidase/H_2O_2 and tyrosinase/O_2, or chemical oxidants, such as sodium periodate and ammonium persulphate. This latter reagent was preferable for preparative scale oxidation of **1**, which afforded the product in about 40% yield as a yellow glassy oil. The positive FAB-MS spectrum exhibited a pseudomolecular ion peak $(M+H)^+$ at m/z 317. The ^1H NMR spectrum (D_2O) showed three 1H singlets at δ 5.33, 6.35 and 6.60, and a complex pattern of signals in the aliphatic region. In particular, two distinct sets of CH_2CH_2 resonances could be discerned, one consisting of two 2H triplets at δ 2.62 and 2.90, and the other showing as four 1H signals at δ 1.64 (ddd), 1.95 (dd), 2.80 (ddd), 3.27 (dd), due apparently to a proximal asymmetric center. An additional feature of the high field region was the presence of a D_2O-exchangeable AB system which could be detected soon after dissolution of the sample, or using DMSO-d_6 as the solvent (doublets at δ 2.76 and 2.86, J= 14 Hz).

The ^{13}C NMR spectrum displayed in the aliphatic region the resonances of the four methylenes of the aminoethyl chains plus a signal (δ 41.00) for the exchangeable methylene group. Other prominent features of the spectrum were two signals at δ 73.46 and 84.46, ascribable to deshielded sp^3 quaternary carbons, and a pattern of three resonances at δ 97.55, 156.90, and 184.51, suggestive of a β-aminoenone system.[11] The remaining signals present in the sp^2 region accounted for a tetrasubstituted phenyl ring.

On the basis of these data, the compound was formulated as 7-amino-8-(2-aminoethyl)-3-hydroxy-2-oxo-2,3,4,10-tetrahydro[3,4a]imino-ethanophenoxazine (**7**), characterised by the presence of a cyclic hemiaminal function. The same structural feature is shared by the tetrahydromethanobenzofuroazocine adducts recently described in the oxidative coupling of dopa with resorcinols.[12] The stability of the cyclic hemiaminal function can be ascribed on one side to the favourable equilibrium of formation of the 6-membered ring, and on the other side to the considerable strain which would be introduced in the rigid tetracyclic skeleton upon dehydration to an imine derivative.

Consistent with the structural assignment, compound **7** was smoothly reduced by sodium borohydride and was oxidised by $NaIO_4$ to give a labile



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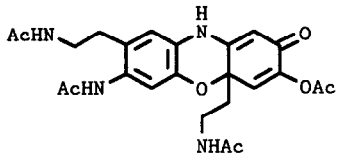


8

yellow species with an absorption maximum at 420 nm. Acetylation of **7** with Ac_2O/pyridine afforded a tetraacetyl derivative with an absorption maximum at 395 nm. Spectral analysis revealed the lack of the cyclic methylene group and the quaternary carbon at δ 84.46, and their replacement by a trisubstituted double bond (doublet at δ 128.13 and singlet at δ 132.71 in the ^{13}C-NMR spectrum, singlet in the proton spectrum at δ 6.74). On these grounds, the compound was formulated as **8**, arising evidently by opening of the cyclic carbinolamine system of **7** induced by N-acetylation, with concomitant conversion of the carbonyl group thus generated into the corresponding enolacetate.

Mechanistically, formation of compound **7** is likely to result from condensation of one molecule of **1** with the quinone **3**, as depicted in figure 2.

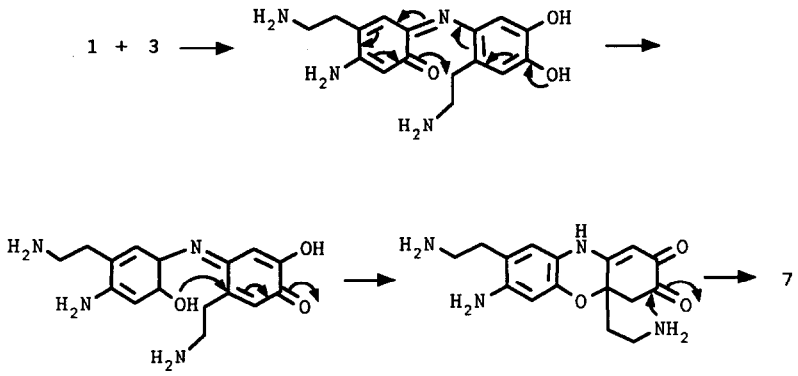
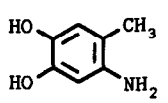


Figure 2. Proposed mechanism of formation of compound 7.

Indirect support for the above scheme was obtained in a separate investigation of the oxidative behaviour of the model compound 5-amino-4-methylcatechol (**9**). Periodate oxidation of **9** afforded in good yield the corresponding *o*-quinone **10** (λ_{max} 500 nm). Reaction of equimolar amounts of **9** and the quinone **10** in acetate buffer at pH 6.5 afforded a major orange-red product (λ_{max} 425 nm) which was assigned the structure of the tetrahydrophenoxazinedione **11** by straightforward spectral



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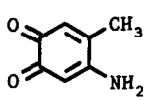

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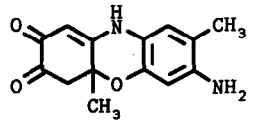

11

analysis.

Interestingly enough, the dimer **11**, unlike **7**, proved rather unstable and underwent rapid decomposition in solution giving rise to orange-red insoluble materials. Such a behaviour is conceivably due to the presence in **11** of an amino group and a carbonyl function, which may undergo repeated intermolecular condensations leading to polymeric species, whereas in **7** a similar process would be prevented by the cyclic hemiaminal function protecting the carbonyl group.

In conclusion, the results of this study provide evidence for the existence of a new oxidation pathway of the neurotoxin **1** which may compete with the more general route to indole intermediates. Apart from the chemical interest connected with the reactivity of 4-aminocatechol systems, the observed dichotomy in the oxidation behaviour of **1** may provide a new lead to the understanding at the molecular level of the mechanism of action of this catecholamine.

EXPERIMENTAL

Melting points are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 270 or 200 MHz spectrometer using tetramethylsilane or dioxane (taken at δ 3.55) as internal standards. Electron impact mass spectra (EIMS) were obtained at 70 eV. Elemental analyses were performed by Analytische Laboratorien, Gummerbach 1 Elbach, F.R.G.. Analytical and preparative HPLC was carried out using a 4 x 250 mm RP 18 Lichrochart and a 21.4 x 250 mm C 18 Rainin Dynamax column, respectively. The flow rate was maintained at 1 mL/min with the analytical column and at 13 mL/min with the preparative column. Analytical wavelength was 280 nm.

Dopamine hydrochloride and 4-methylcatechol were from Aldrich Chemie. 5,6-Dihydroxyindole (**6**) was prepared according to a standard synthetic procedure.[13] All other chemicals were of the highest purity available.

6-Aminodopamine (**1**).

A modification of the general procedures reported for nitration of catecholic compounds[14] was utilised. In brief, 20% sulphuric acid (25 mL) was cautiously added to a solution of dopamine hydrochloride (5 g) and sodium nitrite (6.30 g) in water (150 mL) cooled in an ice bath. A yellow solid separated from the mixture, which was collected by filtration and was washed with water and methanol to afford 2-nitrodopamine hydrogensulphate (6.40 g, 82% yield): m.p. 165-167 °C, UV λ_{max}(DMSO) 287, 363, 452 (log ε 3.02, 3.48, 3.82); ^{1}H-NMR (DMSO-d_6) δ (ppm): 3.04 (4H, s, $-CH_2CH_2NH_2$), 6.82 (1H, s, H-6), 7.47 (1H, s, H-3). 2-Nitrodopamine hydrogensulphate (3 g) was added under stirring to a solution of stannous chloride dihydrate (6.87 g) in conc. HCl (24 mL) and the resulting mixture was allowed to stand at 80 °C for 30 min. After cooling, the solid which separated was filtered and washed repeatedly with cold conc. HCl. Crystallisation from ethanol-ether afforded

6-aminodopamine dihydrochloride (1.86 g, 76% yield) as white plates: m.p. 240-242 °C; UV λ_{max} (EtOH) 285 nm (log ϵ 3.39); ^{1}H-NMR (D_2O, dioxane internal standard) δ (ppm): 2.84 (2H, t, J=7.8 Hz, -$\underline{CH_2}CH_2NH_2$), 3.18 (2H, t, J= 7.8 Hz, -$CH_2\underline{CH_2}NH_2$), 6.74, 6.79 (1H each, s, s, H-2, H-5); ^{13}C-NMR (D_2O, dioxane internal standard) δ (ppm): 26.98 (t), 39.41 (t), 111.91 (d), 117.54 (d), 120.50 (s), 122.20 (s), 144.11 (s), 145.13 (s). The identity of the compound was secured by comparison of its chromatographic and spectral properties with those of an authentic sample prepared according to a standard procedure.[9]

Oxidation of **1**.

a) Aerobic oxidation. Aerobic oxidation of **1** under the conditions reported by Harley-Mason[9] afforded a yellowish-brown mixture which, on HPLC (eluent 0.1 M sodium acetate buffer, pH 4.0-methanol 75:25) did not show appreciable amounts of **6**. Spectrophotometric analysis of the reaction mixture showed a well defined absorption maximum at 368 nm.

In subsequent experiments, formation of **6** in the aerobic oxidation of **1** at various concentrations was investigated. Solutions of the catecholamine ranging from 1.0 to 40 x 10^{-3} M in 0.08 M sodium bicarbonate were vigorously stirred in air, and the oxidation course was monitored spectrophotometrically. After 60 minutes, when nearly all **1** had disappeared, 10 mL aliquots of the reaction mixtures were withdrawn, acidified to pH 3.0, filtered through a 45 μm Millipore filter to remove the brownish precipitate formed and injected on HPLC for analysis of **6**. The yields of the indole, as estimated from comparison of peak areas with external calibration curves, were found to vary from 20% in the experiment with 1.0 x 10^{-3} M **1** to less than 1% in that with 40 x 10^{-3} M **1**.

b) Oxidation with ammonium persulphate. Isolation of 7-amino--8-(2-aminoethyl)-3-hydroxy-2-oxo-2,3,4,10-tetrahydro[3,4a]iminoethano-phenoxazine (7). To a solution of **1** dihydrochloride (200 mg, 0.84 x 10^{-3} mol) in 0.2 M sodium acetate buffer, pH 6.5 (15 mL), was added under stirring a solution of ammonium persulphate (95 mg, 0.42 x 10^{-3} mol) in water (5 mL). After 90 min, the resulting yellowish brown mixture was evaporated to dryness *in vacuo* at 30 °C and the residue, taken up in water, was chromatographed on a Biogel P-2 (200-400 mesh) column (95 x 1.7 cm) using water as the eluent. Seven mL fractions were collected and monitored by UV. Fractions 22-28 which showed absorption maxima at 275 and 368 nm were collected and evaporated to dryness *in vacuo* under reduced pressure. The residue (123 mg) thus obtained was further purified by preparative HPLC chromatography (eluent water-methanol 70:30) to give 7-amino-8-(2-aminoethyl)-3-hydroxy-2-oxo-2,3,4,10-tetrahydro[3,4a]imino-ethanophenoxazine (**7**) (100 mg, 38% yield) as a glassy oil. UV λ_{max}(H_2O) 272, 368 nm; (0.1 M HCl) 260, 346 nm; FABMS (thioglycerol matrix): m/z 317 ($M+H^+$); ^{1}H-NMR (D_2O, internal reference dioxane) δ 1.64 (1H, ddd, J=14.2, 14.2, 5.8 Hz), 1.95 (1H, dd, J=14.2, 4.4 Hz), 2.20 (1H, d, J=13.0 Hz, exchangeable) 2.32 (1H, d, J=13.0 Hz, exchangeable), 2.62 (2H, t, J=8.8 Hz), 2.80 (1H, ddd, J=14.2, 14.2, 4.4 Hz), 2.90 (2H, t, J=8.8 Hz), 3.27 (1H, dd, J=14.2, 5.8 Hz), 5.33 (1H, s), 6.35 (1H, s, shifts to 6.95 in DCl), 6.60 (1H, s, shifts to 7.05 in DCl); ^{13}C-NMR (D_2O, internal reference dioxane) δ 27.36 (t), 28.74 (t), 39.23 (t), 40.05 (t), 41.00 (m, appearing as t in non-protic solvents), 73.46 (s), 84.46 (s), 97.55 (d), 106.76 (d), 118.12 (s), 118.19 (d), 119.08 (s), 142.60 (s), 143.33 (s), 156.90 (s), 184.51 (s). Anal. calcd. for $C_{16}H_{20}N_4O_3$: C, 60.73; H, 6.38; N, 17.72. Found: C, 60.82; H, 6.30; N, 17.70.

Acetylation of **7**.

Dimer **7** (100 mg) was treated with acetic anhydride (2 mL)-pyridine (200 μl) overnight at room temperature. After removal of the volatile

components, the resulting yellow-orange mixture was purified by chromatography on polyamide (70 x 2.0 cm column) using benzene-methanol 90:10 as the eluent to afford the tetracetate **8** (50 mg, 33% yield) as a glassy yellow-orange oil. UV λ_{max}(EtOH) 270, 395 nm; FABMS (glycerol + thioglycerol matrix): m/z 485 $(M+H)^+$; ^{1}H-NMR (CD_3OD) δ 1.89 (3H, s), 1.93 (3H, s), 2.02 (2H, m), 2.19 (3H, s), 2.25 (3H, s), 2.69 (2H, t, J=7.9 Hz), 3.26 (4H, m), 5.48 (1H, s), 6.62 (1H, s), 6.74 (1H, s), 7.13 (1H, s); ^{13}C-NMR (CD_3OD) δ 20.39 (q), 22.58 (q), 22.59 (q), 23.28 (q), 31.71 (t), 35.29 (t), 37.37 (t), 40.95 (t), 75.23 (s), 98.64 (d), 116.35 (d), 117.67 (d), 126.12 (s), 128.13 (d), 129.70 (s), 132.71 (s), 139.57 (s), 146.63 (s), 156.68 (s), 170.56 (s), 172.78 (s), 172.93 (s), 173.44 (s), 179.49 (s).

*4-Amino-5-methylcatechol hydrochloride (**9**).*

The catechol **9** was prepared by a two-step procedure identical to that described for **1**. The crude precipitate obtained from nitration of 4-methylcatechol with sodium nitrite/20% sulphuric acid was subjected to stannous chloride/hydrogen chloride reduction affording, after washing with cold conc. HCl and crystallisation from ethanol/ether, a 60% overall yield of **9** as colourless prisms, m.p. 218-220°C; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.12 (3H, s, $-CH_3$), 6.66 (1H,s, H-3), 6.92 (1H,s, H-6), 9.70 (bs, 2H, D_2O exchangeable, $-NH_2$), 9.93 (bs, 2H, D_2O exchangeable, -OH); ^{13}C-NMR (DMSO-d_6) δ (ppm): 16.20 (q), 111.09 (d), 111.78 (d), 120.47 (s), 121.90 (s), 143.44 (s), 144.90 (s). Calcd. for $C_7H_{10}NO_2Cl$: C, 47.85; H, 5.74; N, 7.98. Found: C, 48.00; H, 5.71; N, 8.01.

*4-Amino-5-methyl-1,2-benzoquinone (**10**).*

To a solution of **9** (300 mg) in 0.2 M sodium acetate buffer, pH 6.5 (12 mL) was added under vigorous stirring a solution of sodium periodate (370 mg) in water (2 mL). The brownish red solid which separated was rapidly filtered, washed with cold water and dried *in vacuo* to give **10** (210 mg, 90% yield). UV λ_{max}(EtOH) 280, 500 nm; ^{1}H-NMR (DMSO-d_6) δ (ppm): 2.12 (3H, s, $-CH_3$), 5.45 (1H,s, H-3), 6.25 (1H,s, H-6), 7.85 (bs, 2H, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6) δ (ppm): 17.68 (q), 97.59 (d), 129.68 (d), 146.50 (s), 158.68 (s), 174.49 (s), 183.59 (s).

*Reaction of **9** with the quinone **10**. Isolation of the tetrahydrophenoxazinedione **11**.*

To a solution of **9** (175 mg, 1.0×10^{-3} mol) in 0.2 M sodium acetate buffer, pH 6.5 (15 mL) a solution of quinone **10** (137 mg, 1.0×10^{-3} mol) in methanol (10 mL) was added dropwise under vigorous stirring. The resulting mixture was kept under nitrogen atmosphere under stirring. After 12 h, TLC analysis (cellulose, eluent water) revealed the presence of a major orange-red product (Rf 0.5) along with small amounts of quinone **10** (Rf 0.8). The mixture was carefully concentrated *in vacuo* at 30°C and then rapidly chromatographed on a 3 x 35 cm cellulose column using water as the eluent. The orange red band eluted (200 mg) was freed from more polar reddish brown materials by chromatography on a 0.5x25 cm Sephadex LH-20 column (eluent: 95% ethanol-water 80:20) affording pure **11** (77 mg, 30% yield) as an orange-red oil. UV λ_{max} (EtOH) 425 nm; EIMS m/z 258 (M^+), 256, 243, 230, 228, 215; ^{1}H-NMR[15] (DMSO-d_6) δ 1.21 (3H, s), 1.99 (3H, s), 3.04 (1H, d, J=15.0 Hz), 3.30 (1H, d, J=15.0 Hz), 5.68 (1H, s), 6.26 (1H, s), 6.67 (1H, s); ^{13}C-NMR[15] (DMSO-d_6) δ 16.77 (q), 23.44 (q), 51.62 (t), 71.83 (s), 92.91 (d), 101.40 (d), 115.84 (d), 116.77 (s), 138.62 (s), 140. 80 (s), 144.46 (s), 156.39 (s), 178.11 (s), 194.05 (s). HRMS m/z calcd. for $C_{14}H_{14}N_2O_3$ 258.1004, found 258.1010. Compound **11** was also formed by reaction of **9** with half-molar equivalent of ammonium persulphate in 0.2 M acetate buffer, pH 6.5 or by incubation of **9** with mushroom tyrosinase in 0.1 M phosphate buffer, pH 7.0 (TLC and UV evidence).

ACKNOWLEDGMENTS

This work was carried out in the frame of the EC project "An integrated approach to the structure of melanins" and was supported in part by C.N.R. (P. F. Chimica Fine II), Rome, Italy.

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15. Owing to the marked instability of the compound, particular attention was paid to record NMR spectra just after dissolution of the sample. Over even short periods of time, the quality of the spectra significantly deteriorated.