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A novel hydrogen peroxide-dependent oxidation pathway of dopamine via 6-hydroxydopamine

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Dedicated in the memory of Professor Giuseppe Prota, an extraordinary teacher and scientist

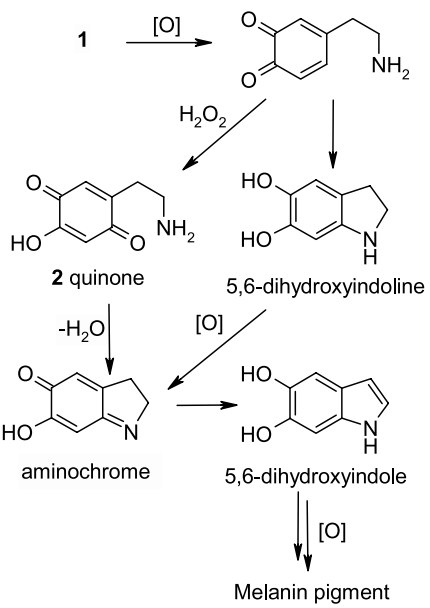
Abstract—In the presence of excess H_2O_2, oxidation of dopamine was diverted from the usual pigment-forming pathway to afford 6-hydroxydopamine and then a colorless reaction mixture comprising a polar non-extractable product. The latter was obtained in 20% yield by oxidation of 6-hydroxydopamine and was tentatively formulated as the novel 5-(2-aminoethyl)-2-hydroxy-5-(3-hydroxy-2-oxotetrahydro-1a*H*-oxireno[2,3]cyclopenta[1,2-*b*]pyrrol-3a(4*H*)-yl)cyclohex-2-ene-1,4-dione by extensive spectral analysis and conversion to a tetraacetyl derivative. Mechanistic experiments suggested that formation of the product proceeds via 6-hydroxydopamine by H_2O_2-dependent epoxidation and cyclization steps followed by dimerization and ring contraction with decarboxylation.

1. Introduction

The oxidation chemistry of dopamine (**1**) and related catecholamines has been an active research subject over the past decades because of its central relevance to nigrostriatal neuronal degeneration in Parkinson's disease,[1–5] and the etiopathogenesis of vitiligo.[6–8] Further incentives for these studies have derived from chemical issues relating to the biomimetic synthesis of indole systems[9,10] and the control of oxidative browning processes in food technology.

The first insight into dopamine oxidation was provided by the elucidation of the classic indoline-aminochrome-5,6-dihydroxyindole pathway depicted in Scheme 1.[11–13] In the presence of hydroperoxides,[4,5] 6-hydroxydopamine† (**2**) quinone may be formed, which affords likewise 5,6-dihydroxyindole, the ultimate monomer precursor of the dark polymeric pigments that constitute the characteristic visible outcome of dopamine oxidation.[2,9] Little is currently known about alternative oxidation pathways of **1**, nor has any intermediate product between the monomer and pigment stage ever been isolated and structurally characterized.

Recently, in a study of the effects of nitrite ions on the hydrogen peroxide-promoted oxidation of **1**,[14] as a possible route to the putative dopamine metabolite 6-nitrodopamine,[15] we noticed the generation at high substrate concentrations of a product whose chromatographic and spectral features did not match with those of the known oxidation products of **1**.[4,5,16,17] We report herein the isolation and spectral characterization of this new product,



Scheme 1.

Keywords: dopamine; 6-hydroxydopamine; oxidation; hydrogen peroxide; dimerization.

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† The common catecholamine nomenclature system was adopted which assigns numbers 3, 4 and 6 to the OH bearing carbons.

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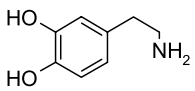
which features an unusual heterocyclic ring system arising by a remarkable H_2O_2-dependent oxidative dimerization route of **2**.



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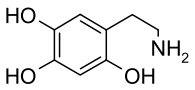

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2. Results and discussion

Oxidation of **1** at 1 mM concentration with horseradish peroxidase (HRP)/H_2O_2 in 0.1 M phosphate buffer, pH 7.4, proceeded smoothly to give in the early stages a main transient species coinciding with **2** quinone,[18,19] which gradually changed into 5,6-dihydroxyindole and then into a dark brown pigment. A quite different pattern of reactivity was observed when a large excess of H_2O_2 and/or higher concentrations of dopamine were used. Under these latter conditions, pigment formation was inhibited and a pale yellow, clear solution developed. HPLC analysis showed small amounts of a species eluted just before **2** quinone and with an absorption maximum centred at 293 nm in acidic media. The product resisted treatment with dithionite or ascorbate or with acids (HPLC evidence), but was smoothly converted by $NaBH_4$ to a slower eluting species with similar absorption spectrum. It could not be recovered on standing at pH>10.

The same product was formed in much higher yields by oxidation of **2** under similar conditions, suggesting that this catecholamine was the actual precursor. Accordingly, in subsequent experiments **2** was preferably used as substrate for preparative purposes. After several attempts, the product was eventually isolated by ion exchange chromatography of the reaction mixture followed by preparative HPLC fractionation. The glassy oil thus obtained contained a single product homogeneous on HPLC and sufficiently, though not indefinitely, stable to be subjected to extensive NMR analysis.

A noticeable feature of the ^1H NMR spectrum in D_2O/DCl was the lack of signals in the aromatic region. The most deshielded resonance was a doublet (J=1.8 Hz) at δ 4.99, coupled to a similar doublet at δ 3.36. In addition, a series of multiplets (8H overall) were apparent in the region between δ 2.0–4.5, suggesting two aminoethyl chains. A singlet (1H) at δ 3.32 was also observed. In DMSO-d_6 the two doublets were shifted upfield at δ 4.53 and 3.00, and additional signals at δ 5.00 (1H, singlet), 3.18 and 2.68 (doublets, J=16.8 Hz) were present, the latter indicating diastereotopic CH_2 protons. Addition of D_2O resulted in the partial disappearance of the doublet at δ 2.68, suggesting different rates of D-exchange of the CH_2 protons. COSY analysis of the spin systems revealed moreover, marked differences in both chemical shifts and coupling constants of the protons of the two ethylamine chains.

Main features of the ^{13}C NMR spectrum (DMSO-d_6) were four quaternary carbons at δ 211.5, 189.6, 187.3, 170.9, three CH signals at δ 88.9 (showing C–D coupling following D_2O exchange), 80.4 and 60.4, three signals for quaternary carbons at δ 75.6, 69.3 and 48.8, and five CH_2 resonances, one of which (δ 34.4) proved sensitive to D_2O exchange.

Overall, these data suggested that: (a) the number of carbons was that expected for two molecules of starting material, minus one carbon; (b) an additional CH_2 was formed; (c) the aromatic units were profoundly modified; (d) the two aminoethyl chains experienced different chemical environments, reflecting adjacent stereocenters and presumably, in one case, cyclization; (e) three carbonyl groups and a deshielded sp^2 carbon were present; (f) an epoxide ring was

Table 1. Spectral data of compound **3** (DMSO-d_6)

	δ ^{13}C	δ ^1H (mult, J Hz)	^1H,^1H COSY	^1H,^{13}C HMBC
C-1	187.3	–	–	–
C-2	170.9	–	–	–
C-3	88.9	5.00 (s)	–	48.8, 75.6
C-4	189.6	–	–	–
C-5	48.8	–	–	–
C-6	34.4	2.68 (d, 16.8)	3.18	–
		3.18 (d, 16.8)	2.68	60.4, 69.3, 170.9, 189.6
CH$_2$CH$_2$N	32.5	2.17 (m)	2.25, 3.47	34.4, 48.8, 170.9
		2.25 (m)	2.17, 3.54	–
CH$_2$N	45.2	3.47 (m)	3.54, 2.17	–
		3.54 (m)	3.47, 2.25	–
C-1′	60.4	3.00	4.53	34.4, 48.8, 69.3, 75.6, 80.4, 211.5
C-1a′	69.3	–	–	–
C-2′	211.5	–	–	–
C-3′	80.4	4.53	3.00	21.0, 69.3, 189.6, 211.5
C-3a′	75.6	–	–	–
C-5′	53.3	3.85 (m)	4.07, 2.72, 2.32	–
		4.07 (m)	3.85, 2.72, 2.32	–
C-6′	21.0	2.32 (m)	2.72, 4.07	–
		2.72 (m)	2.32, 3.85	–

present (1H signal at δ 3.00 correlating with carbon resonances at δ 60.4 (HMQC) and 69.3 (HMBC)).

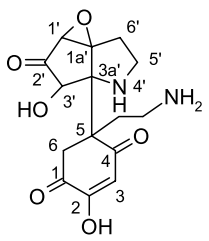
Based on correlation data, a most likely structure for the compound was **3** in which a unit of **2** quinone was linked through the quaternary C-1 carbon to a tetrahydrooxirenocyclopenta[1,2-*b*]pyrrole ring system. Resonances were assigned on the basis of the $^{1}H,^{1}H$ COSY, $^{1}H,^{13}C$ HMQC and HMBC spectra (Table 1). Long range correlations between high field CH_2 protons at δ 2.17/2.25, and the carbon resonances at δ 34.4, 48.8 and 170.9 indicated that the uncyclized ethylamine chain was linked to the cyclohexenedione ring. Distinguishable correlations between proton signal at δ 4.53 (C-3′) and the carbon resonance at δ 21.0 (C-6′), the quaternary epoxide carbon (δ 69.3) and the ketone carbon (δ 211.5) resonances; and between the epoxide proton signal at δ 3.00 (C-1′) with the carbon C-3a′ (δ 75.6), the adjacent epoxide carbon at δ 69.3, and the carbon resonances of the hydroxy ketone moiety (δ 80.4, 211.5) were supportive of the tetrahydrooxirenocyclopentapyrrole ring system. The high field chemical shift of the C-3 carbon was consistent with a vinylogous α-oxocarboxylic acid moiety which is present in a substantially ionized form in neutral aqueous medium.[20–22]

The FAB-MS spectrum showed pseudomolecular M+H (*m/z* 323) and M+Na (*m/z* 345) ion peaks. Intense fragmentation peaks at *m/z* 306 and 289 were also apparent due to consecutive losses of two OH groups.

Structure **3** can exist in eight diastereoisomeric pairs of enantiomers. Careful inspection of the NMR spectra, however, did not provide evidence for a mixture of stereoisomers. Moreover, the product should be formed in different stereoisomers, at least some of these would conceivably elute on HPLC at similar, but not identical, retention times, giving rise to a group or a cluster of peaks, at variance with experimental evidence.

Brief inspection of geometry optimized structures for all diastereomers of **3** predicted lower energy values for those structures featuring a *cis*-fused [3.3.0]azabicyclooctane system. Unfortunately, NOESY and ROESY experiments did not provide conclusive evidence to settle the stereochemical issues.

Attempts to grow crystals suitable for X-ray diffraction analysis either from **3** or its bromide, hydrogensulfate and perchlorate salts, using a variety of solvents (THF, acetic acid, acetone, alcohols) or mixture of solvents and temperature conditions proved unsuccessful, due to partial degradation of the sample or tendency to separate as oil or amorphous solid.



3

Reaction of **3** with acetic anhydride/pyridine at room temperature for 16 h resulted in the quantitative conversion to a main acetylated derivative, which could be purified to give eventually a pale yellow oil homogeneous on TLC and HPLC analysis.

Both the ^{1}H and ^{13}C NMR spectra indicated a tetraacetyl derivative of **3** in which the methylene group and one of the carbonyl groups on the cyclohexenedione ring of **3** were missing and were replaced by two CH groups at δ 6.51/106.2 and 6.68/108.2. Three quaternary carbons resonating at δ 52.3, 138.6 and 166.2, and a carbonyl resonance at δ 190.2 were also apparent. The epoxide ring carbons on the bicyclic moiety resonated markedly up-field at δ 55.2 and 58.1, whereas the epoxide proton was deshielded (δ 3.53), possibly due to the acetylation of the OH group at the C-3′ position, affecting also the geminal proton (δ 5.98). $^{1}H,^{13}C$ HMBC experiments showed correlations between the proton at δ 6.51 and resonances at δ 52.3 and 80.5, assigned to the carbons at the junction of the two ring systems, and the adjacent β-carbonyl carbon (δ 166.2) bearing an acetoxy group. Intense cross peaks in the HMBC spectrum were observed between the β-CH_2 proton signal of the uncyclized ethylamine chain (δ 3.84), the carbon resonance of the adjacent CH_2 (δ 49.2) and a quaternary carbon at δ 171.5, due to the acetamido group. In addition, the signal at δ 5.98 correlated with a carbonyl carbon at δ 204.9, and with the carbon resonances (δ 169.5 and 24.9) of the acetoxy function. These data, coupled with the correlations between the epoxide proton signal (δ 3.53) and the carbon resonances at δ 204.9, 80.5, and 55.2 (quaternary epoxide carbon), corroborated the bicyclic tetrahydrooxirenocyclopentapyrrole ring system. A summary of spectral data with correlations is given in Table 2.

The EI-MS spectrum of the acetylated derivative displayed a weak molecular ion peak at *m/z* 490 and significant peaks

Table 2. Spectral data of compound **4** (DMSO-d_6)

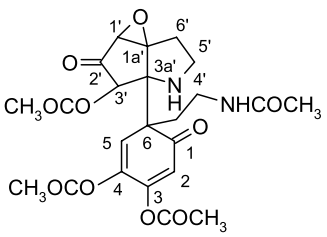
	δ ^{13}C	δ ^{1}H (mult, *J* (Hz))[a]	$^{1}H,^{13}C$ HMBC
C-1	190.2	–	–
C-2	106.2	6.51 (s)	52.3, 80.5, 166.2
C-3	166.2	–	–
C-4	138.6	–	–
C-5	108.2	6.68 (s)	–
C-6	52.3	–	–
CH_2CH_2N	30.7	3.84 (m)	49.2, 171.5
CH_2N	49.2	4.16 (m)	–
C-1′	58.1	3.53 (d, *J*=1.3 Hz)	55.2, 80.5, 204.9
C-1a′	55.2	–	–
C-2′	204.9	–	–
C-3′	84.6	5.98 (d, *J*=1.3 Hz)	24.9, 169.5, 204.9
C-3a′	80.5	–	–
C-5′	48.0	3.59 (m), 4.06 (m)	–
C-6′	24.2	2.28 (m), 2.52 (m)	–
$OCOCH_3$	169.2, 169.5, 169.7	–	–
$OCOCH_3$	20.5, 20.9, 24.9	2.11–2.30 (s)	–
$NHCOCH_3$	171.5	–	–
$NHCOCH_3$	24.3	2.11–2.30 (s)	–

[a] Chemical shift values of the spectrum run in CD_3OD.

at *m/z* 456, 414, 372, 330, denoting formation of a primary fragment (M−2O−2H), as observed in the case of **3**, from which three ketene molecules are sequentially lost.

Overall, these data allowed straightforward formulation of the tetraacetyl derivative as **4**. In this structure, the acetylated OH group at C-3 exerted a lower shielding effect on the adjacent CH carbon compared to the free ionized group in **3**.[20–22]

Unfortunately, compound **4** resisted all attempts at crystallization, affording invariably oily or amorphous materials. Efforts to obtain other derivatives of **3** amenable to crystallization for X-ray analysis proved unrewarding.



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To get some insight into the mechanism of formation of **3** the oxidation of **2** was carried out under a variety of experimental conditions summarized in Table 3. Formation of **3** occurs exclusively in the presence of H_2O_2 or under conditions where H_2O_2 could be generated, e.g. by autoxidation of **2**. Conversely, in the absence of oxygen or in the presence of catalase as H_2O_2 scavenger, formation of **3** was clearly inhibited, **2** quinone being the only product in the early phases of the oxidation. Consistent with the dimeric nature of **3**, a high concentration of the catecholamine also proved an important requisite for its formation.

Kinetic analysis revealed a relatively slow rate of formation of **3**, compared to the decay of **2**, in line with a complex mechanism in which several intermediates are produced.

Table 3. Product formation from **2** under different oxidation conditions and at varying substrate concentrations

[**2**] (mM)	Reaction conditions	Product(s)[a]
10	50 μM Fe^{2+}	**3**[b]
0.5	50 μM Fe^{2+}	**2** quinone[b]
10	50 μM Fe^{2+}, 150 U/mL catalase	**2** quinone
10	3.0 mM Fe^{2+}/EDTA,10 mM H_2O_2	**2** quinone
0.5	3.0 mM Fe^{2+}/EDTA,10 mM H_2O_2	**2** quinone
10	5 mM $NaIO_4$	**3**
10	10 mM	**3** (traces)
0.5	0.5 mM $NaIO_4$	**2** quinone
10	10 mM $K_3Fe(CN)_6$	**3**
10	10 mM $K_3Fe(CN)_6$, O_2 excluded	**2** quinone
10	10 mM $K_3Fe(CN)_6$, 10 mM H_2O_2, O_2 excluded	**3**
10	Tyrosinase[c]	**3**
0.5	Tyrosinase	**2** quinone
10	Peroxidase[d], 10 mM H_2O_2	**3**
0.5	Peroxidase[d], 0.5 mM H_2O_2	**2** quinone
0.5	Peroxidase[d], 5 mM H_2O_2	**3** (traces)

[a] Product analysis was carried out at 3 h reaction time unless otherwise stated.
[b] At 12 h reaction time.
[c] 72 U/μmol of **2**.
[d] 1.5 U/μmol of **2**.

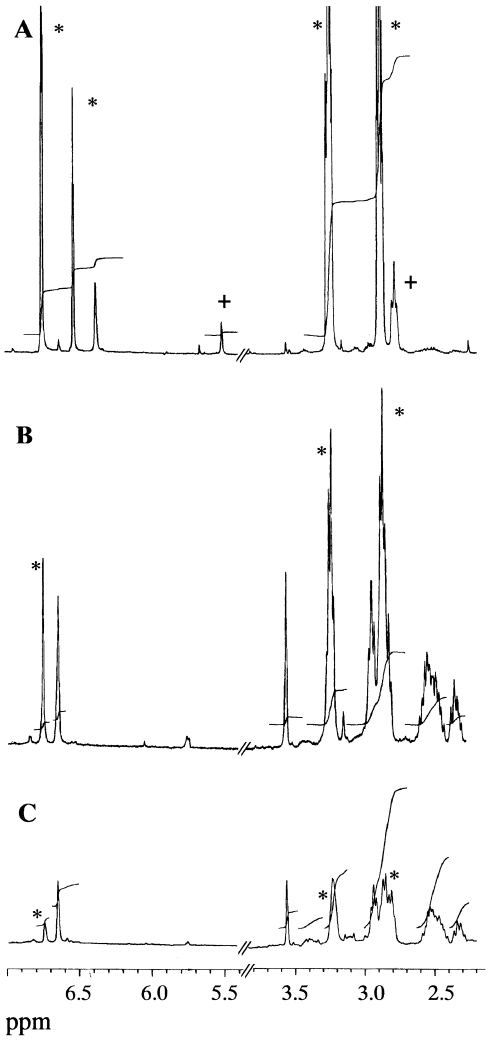
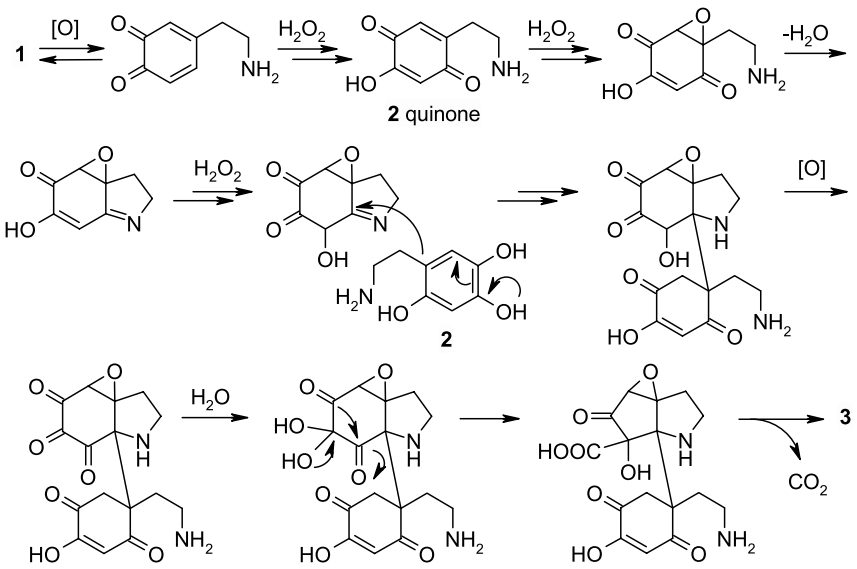


Figure 1. ^{1}H NMR spectrum of the oxidation mixture of **2** in deuterated 0.1 M phosphate buffer (pH 7.4) at the addition of ferricyanide (0.5 mol equiv.) (plot A), at 5 min (plot B), and at 15 min (plot C) reaction time. Symbols mark resonances due to the same species: (*): **2**; (+): **2** quinone.

Structure **3** arises apparently by reaction of one unit of **2** with a highly oxygenated intermediate derived from **2**. This species could be latter conceivably derived by H_2O_2-induced epoxidation of **2** quinone. To probe this route, oxidation of **2** with ferricyanide was run in phosphate buffered D_2O at neutral pH and was monitored by ^{1}H NMR at 400 MHz. (Fig. 1). When the oxidant was added, the resonances of **2** were gradually replaced by a new set of signals including two singlets at δ 6.39 and 5.53 and two 2H triplets (one of which partially overlapped to that of **2**) which were attributed to **2** quinone (plot A).[19] Proton–deuterium exchange accounted for the low integrated area of the high field sp^2 proton signal. The identity of the species as **2** quinone was confirmed by HPLC analysis of the mixture showing the presence of a main component at R_t=20 min which was converted to **2** (R_t=16 min) on treatment with $NaBH_4$. At 5 min reaction time, the resonances of **2** quinone disappeared, whereas those of **2** persisted (plot B). The aliphatic region of the spectrum displayed a series of multiplets and spread over the region between δ 2.1–3.4 ppm which were attributed to the spin systems of two aminoethyl chains (COSY analysis). In



Scheme 2.

addition, singlets at δ 6.65 and 3.54, with comparable integrated areas became apparent (plot B). HPLC analysis of the mixture confirmed the decay of the quinone with formation of a species eluting at around 7 min. After 10 min, following nearly complete decay of **2**, the pattern of aliphatic resonances was simplified, disclosing the presence of an additional singlet at δ 3.22 (plot C) ascribable to an epoxide species. In the later phases of the reaction (2 h) the smooth formation of **3** became apparent with concomitant decay of the less retained components (HPLC analysis).

To determine whether epoxidation occurred prior to or subsequent to cyclization, in another experiment the aminochrome was generated in the NMR tube by ferricyanide oxidation of 5,6-dihydroxindoline (see Scheme 1) in deuterated phosphate buffer at pH 7.4. Formation of the aminochrome was evidenced by complete loss of the indoline resonances and the appearance of two singlets at δ 6.48 and 5.78, the latter showing a lower integrated area with respect to the former due to rapid D-exchange, as well as of two broad 2H signals at δ 3.94 and 3.11. Addition of H_2O_2 resulted in partial conversion of the red aminochrome to dark materials without detectable epoxidic proton signals in the range of δ 3–3.5.

Although the inherent complexity of the chemistry precluded detailed characterization of the transient intermediates, some conclusion can be safely drawn based on the NMR monitoring experiments. First, aminochrome species derived by cyclization of **2** quinone[18] or oxidation of indoline[4] do not seem to undergo facile epoxidation. Second, the spreading of the proton resonances of the ethanamine unit observed in the early stages of the reaction is indicative of a stereogenic center adjacent to the ethylamine chain, e.g. due to epoxidation at the C-1 carbon of the catecholamine.[23,24]

Table 4. HOMO coefficients for **2** as determined by semiempirical methods

Carbon[a]	AM1	PM3
1	0.43	0.44
2	0.11	0.11
3	0.45	0.45
4	0.38	0.39
5	0.02	0.02
6	0.41	0.41

[a] Numbering refers to catecholamine nomenclature (see footnote †).

Overall, these pieces of information allowed to propose a simplified mechanistic scheme for the H_2O_2-promoted oxidation of dopamine via **2**, which is outlined in Scheme 2.

This scheme involves the H_2O_2-promoted conversion of dopamine to **2** quinone,[4] followed by epoxidation and cyclization leading to an α-hydroxyimine intermediate. This would undergo nucleophilic attack by **2** through the quaternary C-1 carbon. Brief inspection of the HOMO of **2** by semiempirical methods (AM1 and PM3) (Table 4) consistently indicated a relatively large coefficient at the C-1 carbon.

The peculiar pattern of oxygenation of the adduct would ensure rapid equilibration with its tautomeric forms as well as a marked facility to oxidation to give a 1,2,3-trioxo species. This latter, in the hydrated form, would undergo a decarboxylative ring contraction in which the driving force would be provided by relief of the steric tension contributed, e.g. by the epoxide ring and the encumbering substituent. This rearrangement would share several features in common with other known rearrangements, such as the benzil–benzilic acid rearrangement.[25] It is known that **2** quinone cyclizes quite slowly compared to dopamine or other related catecholamines,[18,26] so it can well partition among different competing routes depending on concentration and H_2O_2 levels.

3. Experimental

3.1. General methods

For (HR) EI-MS spectra samples were ionized with a 70 eV

beam and the source was taken at 230°C. Main fragmentation peaks are reported with their relative intensities (percent values are in brackets). (HR) FAB-MS spectra were obtained using glycerol as the matrix. ^{1}H spectra were recorded at 400 MHz using a Bruker WM 400 spectrometer; *t*-butyl alcohol (δ 1.23) was added as internal standard. ^{13}C NMR spectra were recorded at 100 MHz. ^{1}H,^{1}H COSY, ^{1}H,^{13}C HMQC, ^{1}H,^{13}C HMBC, NOESY and ROESY experiments were run at 400 MHz using standard pulse programs from the Bruker library.

UV spectra were performed with a Beckmann DU 640 spectrophotometer. Analytical and preparative HPLC was carried out on a Gilson apparatus equipped with a UV detector set at 280 nm using a Sphereclone ODS (5μ, 4.6×250 mm^2) or Econosil (10μ, 22×250 mm^2) column, respectively. For analytical runs, citrate (0.04 M), phosphate (0.04 M), EDTA (0.8 mM), octane-1-sulfonic acid (7.5 mM)-pH 3.1 containing 11% methanol and 5% acetonitrile was used as the eluant, at a flow rate of 1 mL/min. In preparative runs elution conditions were: 0.1 M HCOOH-acetonitrile 98:2 (v/v), flow rate 15 mL/min.

TLC and PLC was carried out on silica gel plates (0.25 and 0.50 mm, respectively) from Merck. Dopamine, 6-hydroxydopamine hydrochloride, hydrogen peroxide (30% solution in water), $NaIO_4$ and $K_3Fe(CN)_6$, were from Aldrich Chemie; mushroom tyrosinase (EC 1.14.18.1), horseradish peroxidase type II (HRP) (EC 1.11.1.7) and catalase from bovine liver (EC 1.11.1.6) were from Sigma. 5,6 dihydroxyindole,[27] and 5,6-dihydroxyindoline hydrobromide[4] were prepared as reported. Molecular mechanics and semiempirical (AM1/PM3) calculations were carried out with Hyperchem 5.0 package produced by Hypercube, Inc. (Waterloo, Ontario, Canada) 1997.

3.2. Oxidation of dopamine (1) and 6-hydroxydopamine (2)

A solution of **1** or **2** hydrochloride (0.5–10 mM) in 0.1 M phosphate buffer, pH 7.4, was treated with HRP (1.5 U/μmol of substrate) and hydrogen peroxide (10 mol equiv.). In the case of **2**, different reaction conditions, oxidants and additives were used as detailed in Table 2. When required, the solution of **2** was purged with a stream of nitrogen for at least 30 min prior to addition of the solution of the oxidant thoroughly purged with nitrogen. The reaction course was followed by periodical HPLC analysis of aliquots of the mixture.

3.3. NMR analyses

A solution of **2** (73 mM) in deuterated 0.1 M phosphate buffer pH 7.4 was treated with $K_3Fe(CN)_6$ (0.5 mol equiv.) and spectra were run soon after addition and at 5 min time intervals. The mixture was eventually analyzed at 2 h reaction time.

In other experiments, a solution of 5,6-dihydroxyindoline hydrobromide (73 mM) in deuterated 0.1 M phosphate buffer pH 7.4 was treated with $K_3Fe(CN)_6$ (2.0 mol equiv.) and with H_2O_2 portionwise up to 2 mol equiv. Spectra were run after each addition and at 10 min intervals over 30 min reaction time.

3.3.1. Isolation of 5-(2-aminoethyl)-2-hydroxy-5-(3-hydroxy-2-oxotetrahydro-1a*H*-oxireno[2,3]cyclopenta-[1,2-*b*]pyrrol-3a(4*H*)-yl)cyclohex-2-ene-1,4-dione (3). A solution of **2** hydrochloride (100 mg, 0.49 mmol) in 0.1 M phosphate buffer, pH 7.4 (32 mL) was treated with $K_3Fe(CN)_6$ (104 mg, 0.32 mmol) under vigorous stirring and after 5 min fluxed with a stream of oxygen. After 3 h the reaction was stopped by addition of 6 M HCl (70 μL) and the mixture fractionated by ion-exchange chromatography (Dowex 50W-X2(H^+) 2×40 cm column) using water (1.4 L), 0.1 M HCl (340 mL), 0.5 M HCl (1 L), 1 M HCl (400 mL), 2 M HCl (100 mL) as the eluants. Fractions eluted with 0.5 M HCl (λ_{max} 293 nm) were collected, evaporated to dryness under reduced pressure and subjected to preparative HPLC to afford **3** (31 mg, 20% yield) as a glassy oil.

Compound **3**. λ_{max} 293 nm (pH 1); δ_H (DMSO-d_6), see Table 1; δ_H (D_2O/DCl) 2.28 (1H, m), 2.41–2.57 (2H, m), 2.91 (1H, m), 3.32 (1H, s), 3.36 (1H, d, J=1.8 Hz), 3.82 (2H, m), 4.12 (1H, m), 4.26 (1H, m), 4.99 (1H, d, J=1.8 Hz); δ_C (DMSO-d_6), see Table 1; δ_C (D_2O/DCl) 20.7 (CH_2), 31.6 (CH_2), 47.4 (CH_2), 50.2 (C), 53.7 (CH_2), 59.7 (CH), 68.6 (C), 75.3 (C), 80.4 (CH), 174.9 (C), 188.7 (C), 211.1 (C); FAB-MS: *m/z* 345 $[M+Na]^+$, 323 $[M+H]^+$. HRFABMS calcd for $C_{15}H_{19}N_2O_6$ $[M+H]^+$ 323.1243, found *m/z* 323.1239.

3.3.2. Preparation of 6-(3-acetoxy-2-oxotetrahydro-1a*H*-oxireno[2,3]cyclopenta[1,2-*b*]pyrrol-3a(4*H*)-yl)-6-(2-acetylamino)-3,4-diacetoxycyclohexa-2,4-dien-1-one (4). Compound **3** (50 mg) was treated with acetic anhydride (2 mL) and pyridine (50 μL) at room temperature for 16 h. The mixture was taken to dryness and fractionated by TLC (benzene–acetone 1:1) to give **4** (15 mg, 20% yield) as a pale yellow oil.

Compound **4**. λ_{max} 318 nm (ethanol); FT-IR ($CHCl_3$) ν_{max} 3689, 3652, 1769, 1752, 1701, 1680, 1674, 1656, 1636, 1602, 1399, 1383, 1307, 1132; δ_H (CD_3OD), see Table 2; δ_C (DMSO-d_6), see Table 2; EI MS *m/z* 490 (5), 456 (48), 414 (72), 372 (76), 370 (100), 330 (79). HREIMS calcd for $C_{23}H_{26}N_2O_{10}$ 490.1587, found *m/z* 490.1582.

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