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Biomimetic Oxidation of the Antimelanoma Agent 4-S-Cysteaminylphenol and Related Catechol Thioethers: Isolation and Reaction Behaviour of Novel Dihydrobenzothiazinequinones

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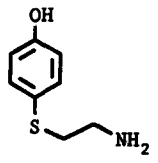
Abstract. Enzymatic and chemical oxidation of 4-S-cysteaminylphenol (**1**) and 4-S-cysteaminylcatechol (**2**) leads to the formation of the hitherto unknown dihydrobenzothiazine-6,7-quinone **4** *via* intramolecular cyclisation of the *o*-quinone **6**. Oxidation of 4-S-cysteinylcatechol (**3**) gives the corresponding dihydrobenzothiazinequinone **9**, which undergoes rearrangement with partial decarboxylation to give 6,7-dihydroxybenzothiazine derivatives, isolated as **8** and **5** after reduction and acetylation of the mixture. Oxidation of **2** and **3** with periodate or iodate gives mainly the iodinated quinones **7** and **13**, respectively.

4-S-Cysteaminylphenol (**1**) and related 4-(2-aminoethylthio)phenols and catechols have been the subject of intensive investigations over the past decade as promising targeting agents for the treatment of disseminated malignant melanoma[1,2]. Following initial reports of depigmenting effects on growing black hair[3], these compounds have been shown to possess potent and selective antitumour effects *in vivo*, as evidenced by the direct growth inhibition of murine melanoma[4] and the increased lifespan of B16 melanoma-bearing mice[5].

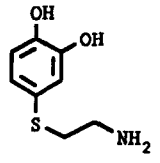
The detailed molecular mechanisms underlying the melanocytotoxic effects of **1** and its congeners have remained so far little understood. There is, however, much circumstantial evidence suggesting that the biological activity of these compounds depends on a metabolic oxidation step promoted by the copper enzyme tyrosinase. This would lead to the

intracellular generation of highly reactive o-quinones capable of targeting crucial sulphydryl-dependent enzymes, thereby interfering with cell growth and proliferation[6-8].

Though widely implicated, this mechanism has never been substantiated by the chemical characterisation of the oxidation products of 1 under biologically relevant conditions[9]. In an attempt to fill this gap, we have carried out a detailed investigation of the tyrosinase catalysed oxidation of 1, and have extended the study to the enzymatic and chemical oxidation of the catechol analogues 2 and 3.



1



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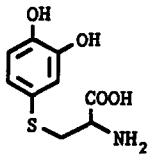
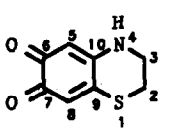


3

Exposure of 1 x 10^{-3}M 1 to mushroom tyrosinase in aqueous phosphate buffer, pH 6.8, resulted in the rapid generation of a violet chromophore (λ_{max}= 565 nm) which persisted for some hours before fading into a featureless absorption throughout the UV-visible spectrum. Chromatographic analysis of the oxidation mixture at the violet pigment stage revealed the presence of a single major product whose absorption properties accounted for the overall spectrophotometric features of the oxidation mixture. This product was isolated by ethyl acetate extraction of the reaction mixture, and was identified as the hitherto unknown dihydro-1,4-benzothiazine-6,7-dione (4). The structural assignment was secured by reductive acetylation with Zn/acetic anhydride, affording the triacetate 5.



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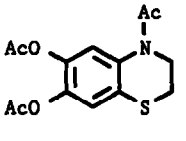

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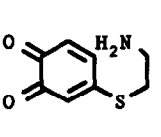


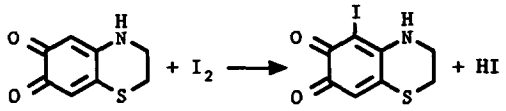
6

Formation of compound 4 is likely to result from intramolecular cyclisation of the o-quinone 6 produced by tyrosinase oxidation of 1. To support this mechanism, the catechol 2 was subjected to chemical and enzymatic oxidation, under which conditions the o-quinone 6 is an obligatory intermediate. With all the systems used (tyrosinase, peroxidase/H_2O_2, $K_3Fe(CN)_6$, $(NH_4)_2S_2O_8$) the reaction led invariably to 4 as the major product.

Oxidation of 2 with sodium periodate (1:1 molar ratio) in aqueous phosphate buffer, pH 7.0, proceeded differently, to yield, besides some

4, a major green product which could be isolated by extraction with CH_2Cl_2. The ^1H-NMR spectrum suggested a C-5 substituted derivative of 4, while the EI/MS spectrum displayed a molecular ion peak at m/z 307 and a base peak at m/z 181. The product was thus formulated as 7. Reductive acetylation of 7 afforded the triacetate 5 with loss of iodine[10].

A plausible mechanism of formation of 7 involves the oxidation of 2 by periodate to give the o-quinone 6, which immediately cyclises to a dihydroxydihydrobenzothiazine intermediate. Under conditions of complete consumption of periodate, iodate would oxidise the dihydrobenzothiazine to 4, being converted to iodide. The latter, in the presence of iodate, would be oxidised to iodine, the actual iodinating agent[11]. In accord with the proposed mechanism, oxidation of 2 with potassium iodate in neutral phosphate buffer afforded 7 in good yield. That the species suffering iodination is indeed 4 was apparent from careful spectrophotometric analysis of the reaction course, as well as from reaction of 4 with alcoholic iodine, affording 7 in quantitative yield (scheme 1). The regiochemistry of the iodination reaction parallels that observed with other aminochrome-type compounds[11].

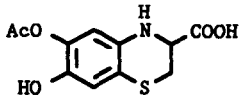


4 7

Scheme 1.

In another series of experiments, the oxidation behaviour of 3 was investigated under the same conditions adopted for 2. With all the oxidising systems used, except hypervalent iodine oxides, a violet chromophore at 560 nm was formed initially, which was rapidly replaced by a new chromophore centred at about 480 nm; eventually, the mixture turned to dark brown. Attempts to isolate the initial intermediate(s) were defeated under a variety of conditions. A procedure was then developed, which involved reduction of the mixture at the 480 nm stage with sodium borohydride, followed by acidification to destroy borate complexes. Acetylation[12] with acetic anhydride/pyridine afforded a complex mixture of products, the major of which could be isolated and characterised as 6-acetoxy-3-carboxy-7-hydroxy-2,3-dihydro-4H-1,4-benzothiazine (8). The compound resisted all attempts at further acetylation, indicating an unexpected instability of the acetyl groups in the carboxylated heterocyclic ring.

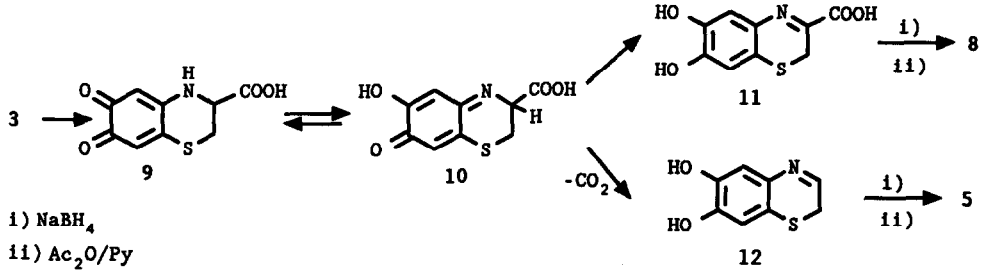
Notably, closer inspection of the reaction mixture revealed also the presence of small but significant amounts of 5, suggesting the occurrence of a decarboxylation step during the oxidation of 3. The possibility that



8

5 was artifactually generated during work-up of the mixture was ruled out in careful control experiments, as well as by the lack of detectable amounts of this compound in the very early stages of the reaction.

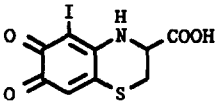
Although the parent compounds of the acetates **8** and **5** eluded direct characterisation, they can be envisaged to arise from the rearrangement of the dihydrobenzothiazine quinone **9** or, more likely, its *p*-quinonimine tautomer **10** (scheme 2). This reaction, which resembles the rearrangement of dopachrome[13], would proceed with partial decarboxylation to give the 6,7-dihydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid **11** and the 6,7-dihydroxy-2*H*-1,4-benzothiazine **12**. Support to this view comes from the spectrophotometric detection, just after the transient violet phase due to **9**, of a strong absorption maximum around 380 nm, suggestive of a benzothiazine-type chromophore[14]. If the proposed mechanism is correct, **8** and **5** would then arise from reduction of **11** and **12**, or some oxidised counterparts, but not from **9** or **4**.



i) $NaBH_4$
ii) Ac_2O/Py

Scheme 2.

As expected, oxidation of **3** with periodate or iodate gave a major green product identified as **13**. This arises evidently by trapping of the dihydrobenzothiazinequinone **9** with iodine. It is not clear at present why substitution with iodine at C-5 would decrease the tendency of the quinone to suffer rearrangement.



13

In conclusion, the results reported in this paper, besides laying the groundwork for studies of the mechanism of action of melanocytotoxic 4-(2-aminoethylthio)phenols, uncover novel aspects of the chemistry of

benzothiazines that are likely to engender further research. Especially noticeable is the characterisation of compounds **4**, **7** and **13**, which provide the first example of isolable quinones in the benzothiazine series[15]. The reaction behaviour and biological activity of the novel compounds are the focus of ongoing work in our laboratory.

EXPERIMENTAL

^{1}H-NMR (270 MHz) and ^{13}C-NMR (67.9 MHz) spectra were performed with a Bruker WH 270 spectrometer. EI-MS and high resolution mass spectra were recorded on a Kratos MS 50 mass spectrometer. Main fragmentation peaks are reported with their relative intensities (percent values are in brackets). Analytical and preparative HPLC was carried out using a 4x250 mm Spherisorb S5-ODS2 column (Phase Separation Ltd.) or a 10x250 mm RP18 Econosil column (Alltech). Mushroom tyrosinase (2200 units/mg) and horseradish peroxidase (220 units/mg) were purchased as lyophilised powders from Sigma (Milan). 3,4-Dihydroxythiophenol was prepared as previously described[16]. 4-S-Cysteinylcatechol (**3**) was prepared as described by Ito *et al*[17].

Preparation of 1-2.

Compounds **1** and **2** were prepared by reacting 4-hydroxythiophenol (3.0 g, 2.4×10^{-2} mol) or 3,4-dihydroxythiophenol (3.4g, 2.4×10^{-2} mol) with bromoethylamine hydrobromide (5.5 g, 2.7×10^{-2}mol) in absolute ethanol (20 ml) in the presence of $NaHCO_3$ (2.0 g, 2.4×10^{-2} mol). The reaction mixture was refluxed under nitrogen for 6 h and evaporated to dryness. The residue was taken up in 1*N* HCl (15 ml) and extracted twice with ethyl ether to remove the unreacted thiol. The aqueous phase, evaporated to dryness, gave a residue which afforded, after further washing with ethyl acetate, 4.7 g (2.28×10^{-2} mol) of **1** as the hydrochloride (yield 95%).

In the case of **2**, the residue from the aqueous phase was taken up with 2*N* HCl (2 ml) and chromatographed on Dowex 50-WX2. Elution with 2*N* HCl gave **2** hydrochloride as white crystals (3.3 g, 63% yield).

All products gave satisfactory analytical and spectral data[17,18].

Tyrosinase-catalysed oxidation of 1: isolation of 4.

To a stirred solution of **1** hydrochloride (100 mg, 4.8×10^{-4} mol) in 0.05 *M* phosphate buffer, pH 6.8 (77 ml), mushroom tyrosinase (10 mg) was added. After 2 hours, when most of **1** had disappeared, the solution was extracted exhaustively with ethyl acetate. The combined organic layers were evaporated to dryness, at 25°C, to give **4** as a violet oil (70 mg,

80% yield). The product was homogeneous on TLC ($CHCl_3/CH_3OH/H_2O$ 80:18:2) and HPLC (eluant: linear gradient of CH_3CN in 0.1 *M* aqueous formic acid (pH 4.0) from 10% to 100% v/v in 40 min, Rt=8.5 min; flow rate 1 ml/min; analytical wavelength = 350 nm). UV (H_2O) λ_{max}: 376, 562 nm; EI-MS: m/z 183 ($M+2^+$,80%), 181 (M^+,100%), 149 (72%); exact mass calcd. for $C_8H_7NO_2S$: 181.0197; found 181.0207. ^{1}H-NMR (CD_3OD), δ: 3.14 (2H, t, J=5.7 Hz, H-2), 3.72 (2H, t, J=5.7 Hz, H-3), 5.62 (1H, s, H-5), 6.41 (1H, s, H-8). ^{13}C-NMR[19] (DMSO-d_6), δ: 24.75 (C-2), 42.58 (C-3), 99.03 (C-5), 124.79 (C-8), 145.78 (C-9), 151.93 (C-10), 179.95 (C-7).

Reductive acetylation of 4.

A suspension of **4** (50 mg) and Zn powder (5 mg) in acetic anhydride (1.5 ml) was refluxed for 40 min. After cooling, the reaction mixture was filtered and the filtrate was evaporated to dryness. The residue was taken up in water and extracted with ethyl acetate. The organic phase was evaporated to dryness and then chromatographed on preparative TLC (eluant: benzene/acetone 8:2) to give **5** as a colourless oil (45 mg). EI-MS m/z: 309 (M^+,62%), 267 (77%), 225 (100%), 183 (90%); exact mass calcd. for $C_{14}H_{15}NO_5S$: 309.0669; found 309.0676. ^{1}H-NMR (CD_3OD), δ: 2.30 (3H, s, CH_3), 2.31 (3H, s, CH_3), 2.34 (3H, s, CH_3), 3.27 (2H, t, J=4.0 Hz, H-2), 4.02 (2H, t, J=4.0 Hz, H-3), 7.16 (1H, s, H-5), 7.33 (1H, s, H-8). ^{13}C-NMR ($CDCl_3$) δ: 20.49 (CH_3), 20.54 (CH_3), 22.50 (CH_3), 28.28 (C-2), 41.20 (C-3), 121.40 (C-5 and C-8, overlapped), 127.58, 135.70, 138.61, 140.22, 167.90 (CO), 168.07 (CO), 169.68 (CO).

Enzymic or chemical oxidation of 2.

Oxidation of **2** ($5x10^{-3}$ *M*) in 0.05 *M* phosphate buffer, pH 6.8, was carried out with a) tyrosinase (244 U/ml); b) peroxidase (24 U/ml) and H_2O_2 ($2x10^{-2}$ *M*); c) potassium ferricyanide ($2x10^{-2}$ *M*); or d) ammonium persulphate ($2x10^{-2}$*M*). Work up of the reaction mixtures as above afforded **4** in comparable yields.

Oxidation of 2 with sodium periodate or potassium iodate.

To a stirred solution of **2** hydrochloride (100 mg, $4.5x10^{-4}$ mol) in 0.1 *M* phosphate buffer, pH 7 (34 ml), a solution of $NaIO_4$(96 mg, $4.5x10^{-4}$ mol) in water (2 ml) was added. After 6 h the mixture was acidified to pH 4 and repeatedly extracted with CH_2Cl_2. The combined organic layers were evaporated to dryness to give **7** as a greenish oil (60 mg, 43%). UV λ_{max}(H_2O): 385, 587 nm; (CH_3OH): 378, 578 nm. EI-MS, m/z: 309 ($M+2^+$,97%), 307 (M^+,59%), 181 (100%), 149 (74%); exact mass calcd. for $C_8H_6NO_2SI$: 306.9162; found 306.9166. ^{1}H-NMR (CD_3OD), δ(ppm): 3.16 (2H, t, J=5.6

Hz,-CH_2), 3.83 (2H, t, J=5.6 Hz,-CH_2), 6.38 (1H, s, H-8). ^{13}C-NMR[19] (DMSO-d_6), δ: 24.85 (C-2), 42.33 (C-3), 123.05 (C-8), 145.11 (C-9), 149.51 (C-10), 170.29 (C-6), 176.22 (C-7).

Oxidation of **2** hydrochloride (100 mg, 4.5×10^{-4} mol) in 0.1 *M* phosphate buffer, pH 7 (34 ml), with $NaIO_4$(193 mg, 9.0×10^{-4} mol) in water (2 ml) and work up as above afforded exclusively **4**.

Oxidation of **2** hydrochloride (30 mg, 1.3×10^{-4} mol) with KIO_3 (58 mg, 2.7×10^{-4} mol) in water (70 ml) led to a greenish precipitate after 30 min. The mixture was centrifuged, the solid residue was washed with water and dried over P_2O_5 to afford pure **7** (34 mg, 85%).

Peroxidase-catalysed oxidation of 3: isolation of 8.

To a stirred solution of **3** hydrochloride (90 mg, 3.4×10^{-4} mol) in 0.1 M phosphate buffer, pH 7 (70 ml), peroxidase (9 mg) and H_2O_2 (1.4×10^{-3} mol) were added. After 15 min, the reaction was stopped by addition of sodium borohydride. The resulting mixture was acidified to pH 2 with 6*N* HCl and evaporated to dryness at 25° C. The residue was repeatedly acetylated with acetic anhydride/pyridine 1:1. After removal of the solvents, the residue was eventually dissolved in methanol (2 ml) and chromatographed on preparative HPLC (eluant: 0.01 M ammonium acetate (pH 4)/CH_3CN 85:15; flow rate 6 ml/min; analytical wavelength λ=280) to give two major peaks eluted after 4 and 30 min. The 4 min peak was re-chromatographed on HPLC (eluant: 0.01 M ammonium acetate (pH 4)/CH_3CN 9:1; flow rate 6 ml/min; analytical wavelength λ=280). The major peak (elution time: 7 min) was collected, extracted with ethyl acetate and evaporated to dryness to give **8** as a colourless oil (20 mg). EI-MS, m/z: 269 (M^+,2%), 227 (6%), 225 (5%), 181 (100%). ^{1}H-NMR (CD_3OD), δ: 2.11 (3H, s, CH_3), 2.88 (1H, dd, J=12.8 and J=9.5 Hz, H-2), 3.44 (1H, dd, J=12.8 and J=7.8 Hz, H-2), 5.43 (1H, dd, J=9.5 and J=7.8 Hz, H-3), 6.78 and 6.83 (1H each, s, H-5 and H-8). The 30 min peak was re-chromatographed on HPLC (eluant: H_2O/CH_3CN 75:25) to give **5** (7 mg).

Oxidation of 3 with sodium periodate or potassium iodate.

To a stirred solution of **3** hydrochloride (26 mg, 9.8×10^{-5} mol) in 0.1 *M* phosphate buffer, pH 7 (40 ml), a solution of $NaIO_4$ (27 mg, 1.25×10^{-4} mol) in water (2 ml) was added. After 10 min the mixture was acidified to pH 3 and extracted with ethyl acetate. The organic phase was evaporated to dryness to give **13** as a green-violet oil (10 mg). EI-MS, m/z: 351 (M^+,5%), 307 (5%), 225 (8%), 181 (100%). UV λ_{max}(CH_3OH): 550, 408 nm. ^{1}H-NMR (CD_3OD), δ: 3.17 (1H, dd, J=13.5 and J=3.7 Hz, H-2), 3.47 (1H, dd, J=13.5 and J=2.2 Hz, H-2), 4.97 (1H, dd, J=2.2 and J=3.7 Hz, H-3), 6.35

(1H, s, H-8). Oxidation of **3** hydrochloride (23 mg, 8.7×10^{-5} mol) with KIO_3 (28 mg, 1.3×10^{-4} mol) in water (43 ml) was carried out as above to give **13** (16 mg).

ACKNOWLEDGMENTS

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19. Some resonances could not be detected owing to the gradual decomposition of the sample during acquisition of the spectrum.

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