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Melanocyte-Directed Enzyme Prodrug Therapy (MDEPT): Development of a Targeted Treatment for Malignant Melanoma

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Abstract—A novel prodrug rationally designed to function as a tyrosinase substrate has been synthesised to allow targeted treatment of malignant melanoma. This agent has been evaluated for tyrosinase-mediated drug release, and has been shown to act in the desired manner. Furthermore, differential cytotoxicity has been demonstrated in cell lines which express tyrosinase and those which do not.

Introduction

Disseminated melanoma is a highly metastatic malignancy which is usually fatal. Systemic chemotherapy is often the only recourse,[1] but to date the results have been very disappointing and the lack of selective cytotoxicity often leads to intolerable side effects. With the increasing occurrence of this disease, there is a clear and urgent need for improved treatments with enhanced specificity.

Improvements in treatment may be attained by the development of a selective prodrug strategy that can specifically target the malignant cells. In an ideal case, the prodrug would be entirely nontoxic, but would be metabolised to liberate a toxic moiety solely at the desired site of action. In this way, not only can more toxic agents be utilised than would otherwise be possible, but systemic toxicity is also greatly reduced. This approach has already been exploited in cancer chemotherapy in the ADEPT (antibody-directed enzyme/prodrug therapy)[2] and VDEPT (virus-directed enzyme/prodrug therapy)[3] protocols, but these have some limitations. For example, in both cases, drug liberation and thus selective toxicity is rarely wholly site-specific, as the targeting vector (the antibody or virus) binds, to some extent, nonselectively.

Our approach differs from these earlier protocols in that our activating mechanism relies upon the enzyme tyrosinase, which is already present in melanoma cells. Indeed, the enzyme is uniquely associated with melanocytes,[4] suggesting a highly focused drug delivery system. Thus, the attachment of a lethal drug to tyrosine (or a tyrosine analogue) would, we hoped, liberate the free drug at the tumour site, via the mechanism depicted in Scheme 1.

For this activation to occur, our proposed tyrosinase substrates have to fulfil certain requirements. Predominantly, the drug must exhibit a catechol or phenolic moiety, to permit tyrosinase oxidation and thus drug release.[5,6] Secondly, the drug linker must be stable until drug release is required, suggesting the use of a carbamate linkage or other such group.

To evaluate the release properties of such prodrugs in biological systems, we required the incorporation of a known cytotoxic agent into our tyrosinase substrates. Nitrogen mustards have been exploited in a number of previous prodrug investigations,[7] and it thus seemed logical to exploit this moiety in our own approach. This led to a target structural motif **2** as shown in Scheme 2.

Key words: Tyrosinase; melanoma; antitumour compound; prodrug.
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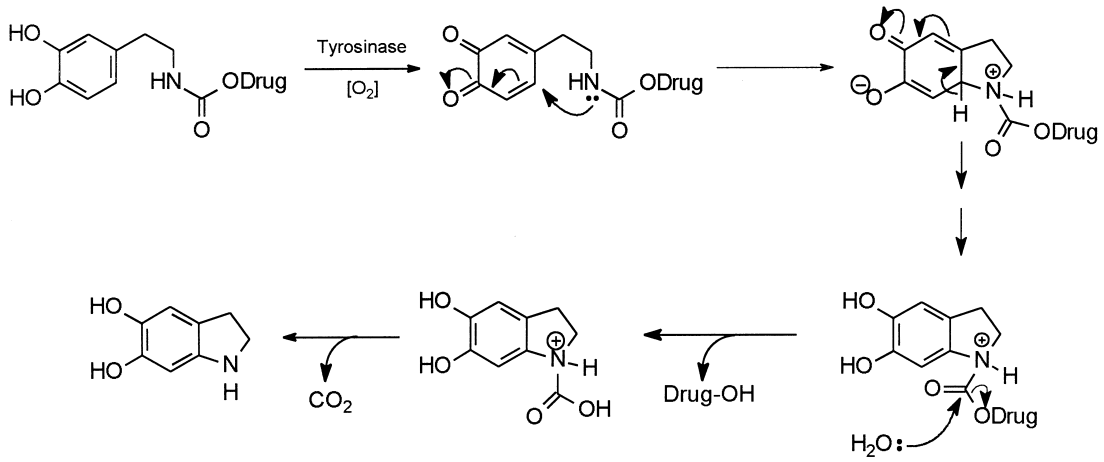
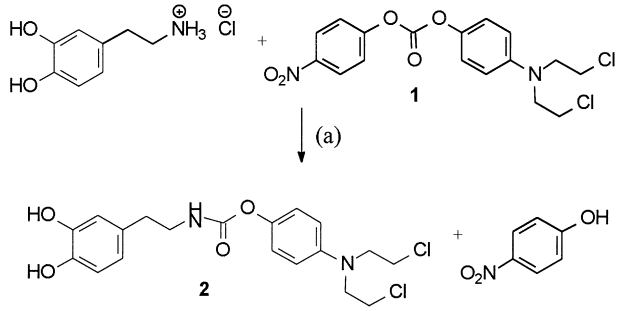
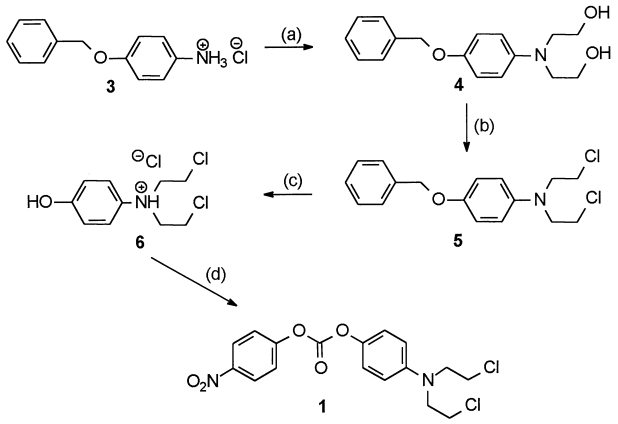


Scheme 1. Proposed liberation of toxic agent by tyrosinase action upon prodrug.



Scheme 2. Synthesis of prodrug **2**. Reagents: (a) dimethylformamide, triethylamine, 73%.



Scheme 3. Synthesis of PNMC. Reagents: (a) triethylamine, ethylene oxide, 88%, (b) mesyl chloride, pyridine, 59%, (c) $HCl_{(g)}$ then $H_{2(g)}$, Pd/C, 51%, (d) *p*-nitrophenylchloroformate, triethylamine, 64%.

Results and Discussion

Synthesis of the prodrug

It was envisaged that our desired prodrug **2** could be formed from the attachment of the tyrosinase substrate to the activated mustard moiety *p*-nitrophenyl mustard carbonate (PNMC) **1**, as shown in Scheme 2.

The PNMC was formed using adaptations of literature protocol. Benzyloxyaniline hydrochloride **3** was first neutralised to liberate the free amine, and treatment with ethylene oxide gave facile access to the di-(hydroxyethyl)amino derivative **4**.[8] Conversion to the di-(chloroethyl)amino functionality to give the desired mustard **5** was accomplished with mesyl chloride in anhydrous pyridine.[9] Treatment with hydrogen chloride generated the hydrochloride salt, hydrogenation of which removed the benzyl ether to give the free *N*-mustard salt **6**. Finally, refluxing with commercially available *p*-nitrophenyl chloroformate and triethylamine gave straightforward access to the desired PNMC **1**,[10] ready for coupling to the proposed tyrosinase substrates (Scheme 3).

Coupling of the PNMC with the 3-hydroxytyramine proceeded smoothly in anhydrous dimethlyformamide to give the prodrug **2** shown in Scheme 2, in 73% yield. With the prodrug in hand, its efficacy to act in our MDEPT protocol was assessed using scanning oximetry, gas chromatography/mass spectrometry (GC–MS) and cytotoxicity screening.

Scanning oximetry

When tyrosinase substrates are processed according to the pathway in Scheme 1, molecular oxygen is absorbed from the surrounding solution in order to effect the enzymatic oxidation. The resultant depletion of dissolved oxygen can be measured by the use of an oxygen sensor, which monitors the relative levels of oxygen in the substrate/tyrosinase solution. This technique is known as scanning oximetry.[11,12] The oximetry cell in which the measurements are conducted is situated in the beam path of a UV spectrophotometer, which is particularly useful for detecting formation of the transient intermediates proposed in Scheme 1. In this way, changes in the UV spectrum can also be monitored as oxygen uptake progresses, and the two sets of data combined to give more meaningful interpretations.

In our initial assay, oxygen uptake was monitored for 15 min. For the prodrug **2**, a rapid oxidation occurred, which we speculate can be attributed to the formation of the *ortho*-quinone product **7** shown in Figure 1. After

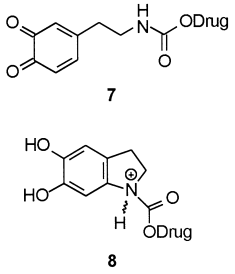


Figure 1. Postulated intermediates in tyrosinase oxidation.

the formation of this *ortho*-quinone product **7**, we hoped to see cyclization of this reactive species to generate the cyclised product and concerted drug release. Indeed, we observed a steady increase in a UV peak at 420 nm. Although we have no direct evidence, we tentatively believe this may result from the desired cyclised product **1**. The initial *ortho*-quinone product should be subject to further oxidation by tyrosinase and in longer assays (over 30 min), gradual additional oxygen uptake was observed. This uptake of oxygen would be expected to be stoichiometric with respect to the prodrug; however, only submaximal oxygen uptake was observed. We postulate that this effect may be due to residence of the oxidised product in the tyrosinase active site, effectively acting as a slow-release enzyme inhibitor. Longer experiment times, or experiments with increased tyrosinase levels should provide more information on this sub-maximal uptake, and we hope to conduct these studies in the near future.

Tyrosinase-dependent drug release: GC–MS evaluation

Before proceeding further with our biological investigations, we wished to confirm that our prodrug would, indeed, release the cytotoxic mustard agent upon treatment with tyrosinase. Although tyrosinase has been shown to process hindered tyrosine residues in proteins,[13,14] we wished to confirm that the steric bulk of our *N*-mustard moiety would not interfere with the enzymatic processing of the prodrug.

In a straightforward assay, the prodrug was dissolved in dimethylsulfoxide and added to a solution of tyrosinase in pH 7.2 phosphate buffer. After gentle agitation, aliquots were injected onto a GC–MS system, and the resultant chromatogram examined for evidence of *N*-mustard liberation. The retention time of any liberated moieties were compared with the free *N*-mustard (prepared by liberation of the free base of **6** with triethylamine). A sample chromatogram is shown in Figure 2. To our satisfaction, the chromatogram for the prodrug showed liberation of the nitrogen mustard, and this suggested that the prodrug was indeed a substrate for tyrosinase. It should be noted that in experiments conducted in buffer solutions without tyrosinase, the *N*-mustard could not be detected by GC–MS, even after agitation for extended periods at elevated temperatures.

Cytotoxicity screening

Having established that our prodrug was a substrate for tyrosinase, we wished to determine its relative toxicity in cell lines that expressed the tyrosinase enzyme and in those which did not. For these purposes we selected five lines, CHO (Chinese hamster ovary), Caki-2 (renal adenocarcinoma), C32 (amelanotic melanoma), G361 (melanoma) and StMI11a (melanoma). Tyrosinase activity in these lines (determined according to literature precedent[15]) is detailed in Figure 3. It can be seen that the three melanoma cell lines show a high level of tyrosinase activity, whilst the Caki-2 line shows only a small, but measurable, level of activity. The CHO cell line, as anticipated, demonstrates little discernible tyrosinase activity and is thus our control cell line. The cytotoxicity studies were conducted using the Sulphorhodamine B assay, according to the literature protocol,[16,17] and the toxicities of both the prodrug **2** and the free *N*-mustard **6** against the various cell lines are shown in Table 1.[18]

As can be seen in Table 1, the cell lines utilised in this study exhibit very similar sensitivities to the free *N*-mustard **6** (i.e. around 1 μM). However, the prodrug **2** shows relatively lower toxicity in the tyrosinase-deficient CHO, Caki-2 and amelanotic C32 cell lines, but demonstrates much greater efficacy in the StMI11a cell line, which shows the highest level of tyrosinase activity. The prodrug thus appears to be functioning as a melanocyte-directed prodrug, liberating the toxic moiety selectively in the desired manner.

Conclusions

The prodrug **2** was rationally designed to act as a substrate for tyrosinase, to allow selective delivery of a toxic agent to those cell lines expressing tyrosinase. The compound was readily prepared from PNMC **1**, and its ability to act as a tyrosinase substrate was assessed by GC-MS studies and oxygen consumption in oximetry experiments. Furthermore, cell line screening showed **2** to demonstrate increased cytotoxicity against tyrosinase-upregulated lines, compared with cell lines displaying little or no tyrosinase activity. Further tyrosinase substrates are currently being prepared in our laboratories, as are a number of novel cytotoxic agents which could ultimately replace the *N*-mustard moiety in the prodrugs.

Experimental

All NMR spectra were recorded on a Bruker WM250 or Jeol AX400 spectrometer, using $CHCl_3$ as an internal standard unless stated otherwise (7.26 ppm for ^1H NMR, 77.0 ppm for ^{13}C NMR). ^{13}C spectra were recorded using Distortionless Enhancement by Polarization Transfer. Mass spectra were recorded on a Fisons VG Autospec. Infra red spectra were recorded on a Perkin–Elmer Paragon 1000 FT-IR spectrometer. Melting points were determined using an Electrothermal digital melting point apparatus, and are uncorrected. GC–MS analyses were performed on a Fisons GC–MS system (GC 8000 gas chromatogram fitted with a Hewlett Packard HP-1 crosslinked methyl

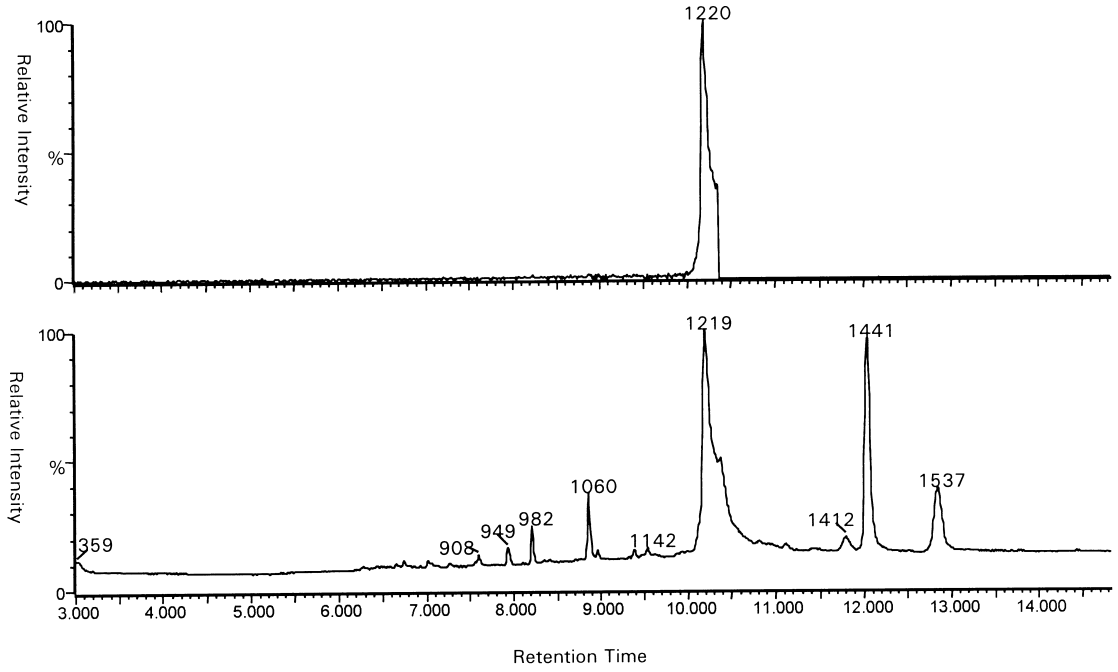


Figure 2. Typical GC–MS output. The upper trace shows the GC output, scanning eluted components selectively for m/z 233 (corresponding to $[M]^+$ for **6**). The lower trace is the GC trace of the crude enzyme reaction mixture. The free *N*-mustard is clearly distinguished at 10.2 min.

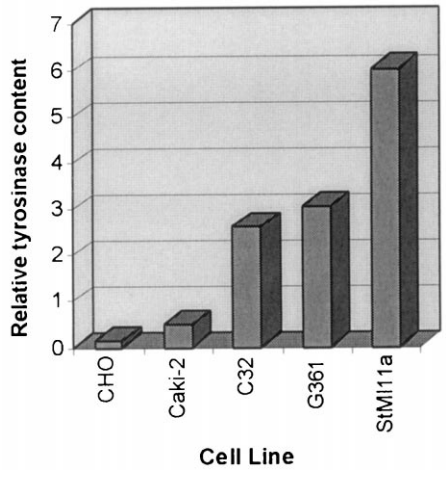


Figure 3. Relative tyrosinase activity of the cell lines.

silicone gum column, 25 m × 0.2 mm × 0.5 μm film thickness, linked to a Trio 1000 EI mass spectrometer) with conditions as stated. Scanning oximetry was conducted in a quartz cell equipped with a YSI 3500 biological oxygen monitor and mechanical stirrer, and the assembly placed in the optical path of a Hewlett-Packard 8452A U.V./Visible spectrometer. Unless stated otherwise, all chemicals and materials were obtained from the Sigma-Aldrich Chemical Company, the B.D.H. Chemical Company or Lancaster Chemicals and were used as received. Silica gel for column chromatography was obtained from Merck, with a mesh size 0.2 mm. Silica thin layer chromatography was performed on precoated aluminium sheets, with a 0.2 mm thickness. Compounds were detected by quenching of fluorescence at 254 nm. Anhydrous solvents were purchased and used as received.

Table 1. Cytotoxicity of prodrug **2** in the five cell lines (IC_{50}/μM)

	CHO	Caki-2	C32	G361	StMI11a
6	1.7	1.1	1.2	0.45	1.6
2	18.9	45.2	13.0	8.2	2.8

Benzyl-*p*-(bis-2-hydroxyethylamino)phenyl ether (4). 4-Benzyloxyaniline hydrochloride (11.8 g, 0.05 mol) was suspended in glacial acetic acid (70 ml) and water (70 ml) and cooled to 0°C. Ethylene oxide (CAUTION: toxic, irritant, mutagen, teratogen, carcinogen) (17.64 g, 20 mL, 0.4 mol) was then added, in 1 mL portions, the solution allowed to warm to room temperature and stirred until no starting material could be detected by TLC. The solution was then concentrated in vacuo ($T < 60$°C) to give a red/brown syrup, which was redissolved in chloroform (100 mL). This solution was washed with water (50 mL) and $NaHCO_3$ solution (50 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. Recrystallization (toluene/hexane) gave the diol as a pale cream powder (12.55 g, 88%); m.p. 96–97°C (lit.[19] 93–94°C); R_f (silica, ethyl acetate) 0.3; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.47 (4H, t, $J = 5.0$ Hz, $2\times NCH_2$), 3.80 (4H, t, $J = 5.0$ Hz, $2\times CH_2Cl$), 5.01 (2H, s, CH_2Ph), 6.74 (2H, d, $J = 9.2$ Hz, Ar), 6.90 (2H, d, $J = 9.2$ Hz, Ar), 7.41 (5H, m, Ar).

Benzyl-*p*-(bis-2-chloroethylamino)phenyl ether (5). The (bis-hydroxyethylamino)phenyl ether **4** (2.0 g, 7.2 mmol) was dissolved in anhydrous pyridine (11 mL) and cooled to 0°C. Mesyl chloride (28.8 mmol, 2.23 mL) was added and the solution stirred at 2–4°C for 20 min, followed by heating at 80°C for 30 min. Ethyl acetate (30 mL) and water (30 mL) were then added, the organic fraction collected, dried ($MgSO_4$), filtered, and concentrated

in vacuo. Column chromatography (silica, dichloromethane) gave the di-chloride (1.38 g, 59%) as a white powder; mp 108–109°C (lit.[18] 105–06°C); R_f (silica, dichloromethane) 0.7; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.62 (8H, m, 4×CH_2), 5.00 (2H, s, PhCH_2), 6.73 (2H, d, J=8.6 Hz, Ar), 6.92 (2H, d, J=8.6 Hz, Ar), 7.29–7.44 (5H, m, Ar).

***p*-(Bis-2-chloroethylamino)phenol hydrochloride (6).** Hydrogen chloride gas was bubbled through a solution of the di-chloride **5** (3.3 g, 0.01 mol) in methanol (35 mL), until complete dissolution occurred. Filtration and concentration in vacuo gave the hydrochloride salt as a white powder (mp 140–141°C (lit.[8] 135–136°C)), which was immediately re-suspended in ethanol (40 mL) containing 10% palladium on carbon (0.17 g). The suspension was stirred under an atmosphere of hydrogen until no starting material was detected by TLC. The suspension was then filtered over Celite® and concentrated in vacuo to give the hydrochloride as a white solid (1.37 g, 51%); mp 176–178°C (lit.[18] 170–173°C); ^{1}H NMR (400 MHz, $CDCl_3$, free amine) δ 3.52 (8H, m, 4×CH_2), 6.57 (2H, d, J=9.0 Hz, Ar), 6.67 (2H, d, J=9.0 Hz, Ar).

Carbonic acid *p*-(bis-2-chloroethylamino)phenol ester-*p*-nitrophenyl ester (PNMC) (1). The bis-chloroethylamine hydrochloride salt **6** (1.35 g, 0.057 mol) and triethylamine (1.17 g, 1.61 mL, 0.114 mol) in toluene (15 mL) were slowly added, over 15 min, to a refluxing solution of *p*-nitrophenyl chloroformate (1 g, 0.05 mol) in toluene (15 mL) and the mixture refluxed for 1 h. The reaction was then cooled, concentrated in vacuo and purified by column chromatography (silica, dichloromethane) to give the diester as a yellow oil (1.27 g, 64%), which solidified upon standing; mp 97–99°C; R_f 0.77 (silica, dichloromethane); ν_{max} (KBr disc) 1767, 1615, 1594, 1512, 1347, 1180, 814 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.61 (4H, m, 2×CH_2), 3.71 (4H, m, 2×CH_2), 6.70 (2H, d, J=9.2 Hz, Ar), 7.16 (2H, d, J=9.2 Hz, Ar), 7.47 (2H, d, J=9.1 Hz, Ar), 8.30 (2H, d, J=9.1 Hz, Ar); ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.2 (CH_2), 53.6 (CH_2), 112.4 (CH), 121.7 (CH), 121.8 (CH), 125.3 (CH), 140.5 (C), 141.3 (C), 142.7 (C), 143.1 (C), 153.6 (C); m/z (CI) 399 ([M+H]+, 75%), 348 (100), 120 (20), 63 (15); (CI: found: [M+H]+, 399.0525. $C_{17}H_{16}Cl_2N_2O_5$ requires [M+H]+, 399.0514).

{2′-(3″,4″-Di-hydroxyphenyl)-ethyl} carbamic acid *p*-(bis-2-chloroethylamino)phenyl ester (2). A solution of the carbonate **1** (0.1 g, 0.26 mmol), 3-hydroxytyramine hydrochloride (0.1 g, 0.53 mmol) and triethylamine (0.05 g, 0.07 mL, 0.5 mmol) in anhydrous DMF (2 mL) was stirred at room temperature for 72 h. The mixture was then concentrated to dryness in vacuo. Column chromatography (silica, dichloromethane:methanol 100:1 →9:1, v/v) gave the carbamate (0.08 g, 73%) as a colourless viscous oil; R_f 0.45 (silica, dichloromethane: methanol, 9/1, v/v); ν_{max} (KBr disc) 3421, 1718, 1653, 1507, 1218 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.60 (2H, br t, J=6.3 Hz, PhCH_2), 3.32 (2H, br q, J=6.3 Hz, CH_2NH), 3.48 (4H, t, J=6.2 Hz, 2×NCH_2), 3.55 (4H, t, J=6.2 Hz, 2×CH_2Cl), 5.17 (1H, br t, J=6.3 Hz, NH), 6.50 (2H, d, J=7.9 Hz, Ar), 6.58 (2H, s, Ar), 6.64 (1H, d, J=7.7 Hz, Ar), 6.88 (2H, d, J=7.9 Hz, Ar); ^{13}C NMR (100 MHz, $CDCl_3$) (35.3 (CH_2), 40.7 (CH_2), 42.7 (CH_2), 53.9 (CH_2), 112.8 (CH), 115.6 (CH), 116.8 (CH), 120.9 (CH), 123.0 (CH), 130.9 (C), 142.5 (C), 143.0 (C), 144.1 (C), 144.4 (C), 156.1(C); m/z (CI) 413 ([M+H]+, 10%), 233 (50), 184 (100), 123 (35); (CI: found: [M+H]+, 413.1045. $C_{19}H_{22}Cl_2N_2O_4$ requires [M+H]+, 413.1034).

Scanning oximetry. Tyrosinase solution (3.65 mL) (phosphate buffer, pH 7.2, 300 units of mushroom tyrosinase (Sigma) per mL) was placed in the scanning oximetry apparatus and to this cell was added 100 mL of a 10 mM solution of the prodrug under investigation. With constant stirring, the U.V. spectrum and oxygen uptake were then measured at 5 s intervals for a period of 15 min. The overall change in the U.V. spectrum could then be calculated by subtracting the spectrum recorded at 5 s from the spectrum recorded at 15 min.

Tyrosinase degradation studies. To a solution of mushroom tyrosinase (Sigma, 3000 units mg^{-1}) in phosphate buffer (pH 7.2, 0.2 mL) was added a solution of the prodrug under investigation (2 mg) in DMSO (0.2 mL). The mixture was then stirred gently for 1 h in a water bath maintained at 25°C. Aliquots (0.5 L) were injected into the GC–MS system with an oven temperature of 150°C for 5 min, warming to 250°C over a period of 5 min and maintaining this final temperature for a further 40 min. The liberated free mustard was detected by scanning the eluted components from the GC, observing m/z 233 ([M]$^+$). The free mustard was found to elute at ca. 10.2 min under these conditions.

Acknowledgements

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