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Original article

Dopamine- and tyramine-based derivatives of triazenes: Activation by tyrosinase and implications for prodrug design

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ABSTRACT

A range of triazene derivatives were synthesized and investigated as prodrug candidates for melanocyte-directed enzyme prodrug therapy (MDEPT). The prodrugs contained a tyramine or dopamine promoiety required for tyrosinase activation and this was joined via a urea functional group to the cytotoxic triazene. The stability of each of the prodrugs in phosphate buffer, human plasma and in mushroom tyrosinase is discussed. The identification of the main peak formed after the tyrosinase reaction was attempted by LC–MS and the conversion of prodrug to the quinone was confirmed.

1. Introduction

Disseminated melanoma represents one of the most treatment-resistant of all human malignancies. Drug resistance is not only acquired following drug therapy, but may be also present in untreated lesions. Consequently, adjuvant therapy, immunotherapy or chemotherapy, provide only modest improvements in outcome. Among new cancer cases, melanoma is continuing to rise in incidence at a rate exceeding all other cancers [1–3].

Melanocyte-directed enzyme prodrug therapy (MDEPT) has been suggested as a possible selective strategy towards the treatment of malignant melanoma [4]. MDEPT offers a highly selective triggering mechanism for drug delivery because of the unique occurrence of tyrosinase enzyme expression in melanocytes and melanoma cells. When melanocytes become malignant, the genes expressing tyrosinase become up-regulated, resulting in a marked increase in the tyrosinase levels within the cancerous cells [5]. A number of tyrosinase-dependent prodrug strategies have been investigated for the treatment of melanoma, in which tyrosinase mediates the release of an anticancer agent from prodrugs *via* a cyclization-drug release mechanism [6]. Jordan and co-workers have synthesized several prodrugs **1** of nitrogen mustards as the cytotoxic agent connected via a carbamate, urea or thiourea group to the tyrosinase sensitive unit [4,7,8]. These prodrugs act as tyrosinase substrates and release the mustard drug after enzymatic oxidation [7–9].

Dacarbazine (DTIC), **2**, remains the single FDA-approved chemotherapeutic agent in common use for metastatic melanoma. Among all the chemotherapeutic agents tested against this malignancy, DTIC is the most active single agent and thus considered the reference drug [10,11]. Related 1-aryl-3,3-dimethyltriazenes, **3** (see Scheme 1), are also documented as anticancer drugs, but with reduced selectivity for tumour cells [12,13]. The biological action of DTIC and the dimethyltriazenes, **3**, is a consequence of their capacity to alkylate DNA and RNA via the corresponding monomethyltriazene **4** following metabolic oxidation by cytochrome P450 enzymes (Scheme 1) [12,14,15]. Thus, improvements in the treatment of melanoma may be attained by the development of a prodrug strategy that can specifically deliver the ultimate methylating agent to malignant cells. Since monomethyltriazenes are as good leaving groups as alcohols [16], we envisaged that coupling them to tyrosinase substrates would identify suitable prodrugs **5** capable of regenerating the alkylating metabolite **4** via the pathway depicted in Scheme 2. We now report the synthesis of triazene

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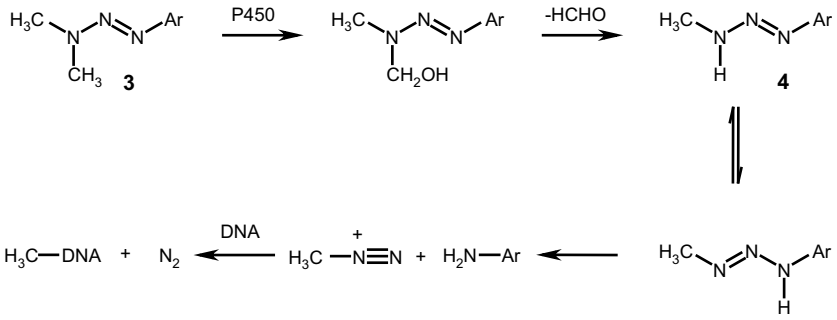
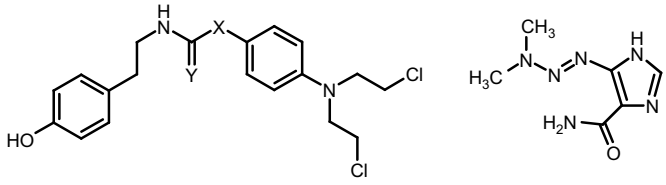


Scheme 1. Metabolic pathway for dimethyltriazenes.

derivatives **5a**–**g** (Scheme 3), and their evaluation as substrates for tyrosinase.



1 X = N, O; Y = O, S

2

2. Results and discussion

2.1. Synthesis

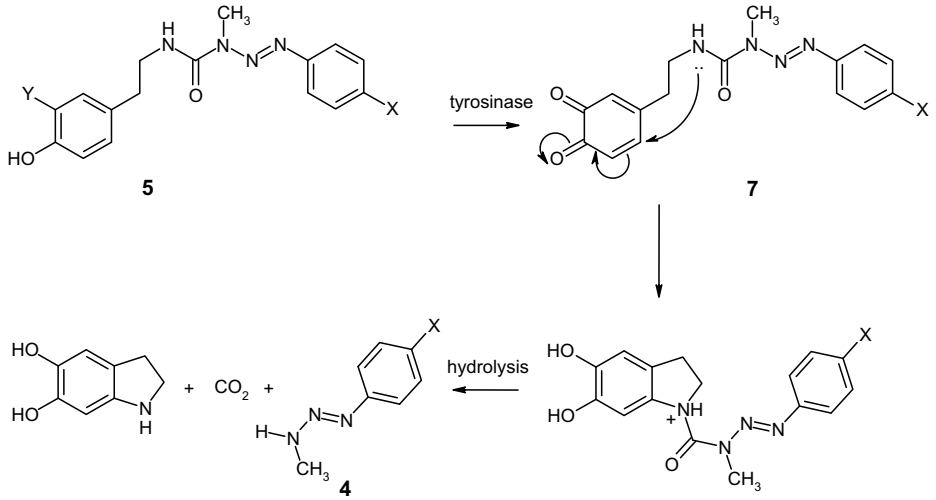
Initial attempts to synthesize compounds **5** involved the preparation of ethyl carbamates, **6a**, from the corresponding monomethyltriazenes **4**, according to the published procedure (Scheme 3) [17]. However, carbamates **6a** proved to be unreactive towards tyramine and dopamine in the presence of a wide range of bases. Consequently, we synthesized the corresponding 4-nitrophenyl carbamate derivatives **6b** and gratifyingly reaction of these with tyramine and dopamine in the presence of triethylamine gave the desired compounds **5a**–**g** in good yields.

2.2. Stability in phosphate buffer and human plasma

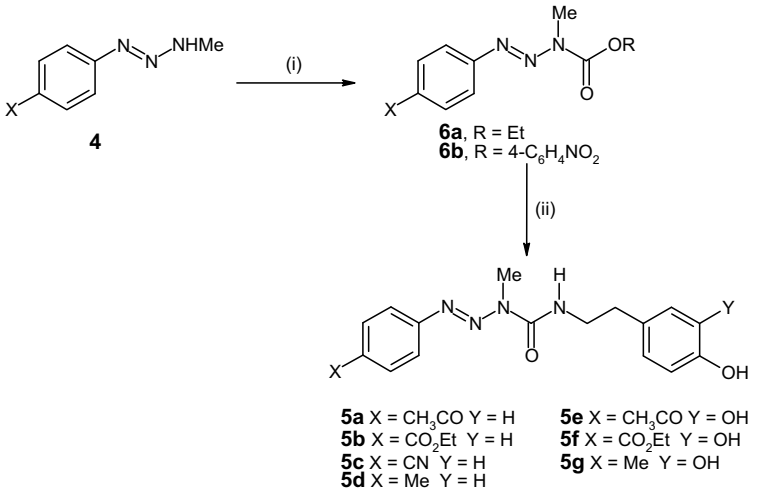
The hydrolysis of triazene derivatives, **5a**–**g**, to the corresponding 1-aryl-3-methyltriazenes, **4**, was studied both in isotonic phosphate buffer (PBS, containing 20% of dimethylsulfoxide) and in 80% human plasma, at 37 °C. These reactions were followed by HPLC by monitoring both the loss of starting material and the formation of products. The kinetics of the hydrolysis of each prodrug in both media followed pseudo-first-order kinetics over four to five half-lives.

Half-lives for the hydrolysis in PBS are presented in Table 1. Inspection of Table 1 shows that these compounds are generally very stable in this medium; no measurable decomposition of the tyramine-based derivatives **5a**–**c** over 15 days or of the dopamine derivative **5g** over 2 days could be detected, while the tyramine derivative **5d** (half-life 15 days) and the dopamine derivatives **5e**,**f** (half-lives 15 h and 20 h, respectively) hydrolyzed only very slowly. It seems, however, that the prodrugs containing dopamine are the more reactive.

We were interested in examining the stability of the prodrugs in human plasma because it contains a range of enzymes that could potentially catalyze the hydrolysis of such urea derivatives. The dopamine-based triazene derivatives **5e**–**g** are hydrolyzed to the corresponding 1-aryl-3-methyltriazene **4** with half-lives ranging from 6 h to 15 h, implying that they are hydrolyzed in human plasma only ca. 1.5 times faster than in pH 7.4 buffer. In contrast, the tyramine-based derivatives **5a**,**c**,**d** are stable in human plasma for 2 days, while **5b** is hydrolyzed with a half-life of 71 h. These results



Scheme 2. Activation pathway for tyramine-based triazene prodrugs triggered by tyrosinase.



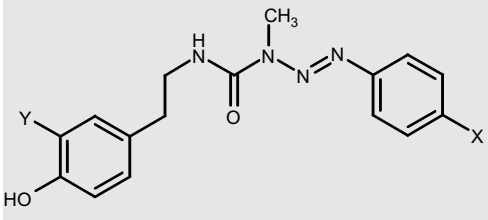
Scheme 3. Synthesis of triazene prodrugs **5**. (i) Ethyl or 4-nitrophenyl chloroformate, pyridine, CH_2Cl_2; (ii) tyramine or dopamine, triethylamine, THF.

indicate that tyramine substrates are less prone to plasma hydrolysis when compared with their dopamine counterparts.

2.3. Triazene derivatives as a substrate for tyrosinase

The ability of triazene derivatives **5** to act as substrates of tyrosinase was studied by HPLC by determining the half-lives for activation for each compound in the presence of the enzyme (Table 1). HPLC analysis of reaction mixtures showed that both tyramine, **5a–d**, and dopamine derivatives, **5e–g**, are rapidly consumed with half-lives ranging from 6 to 18 min, thus indicating that they are excellent tyrosinase substrates. These triazene prodrugs have shorter half-lives in the presence of tyrosinase than the urea-linked *N*-mustard prodrugs described by Knaggs et al. [9]. For compound **5c** $K_m = 0.31 \pm 0.08$ mM and $V_{max} = 0.030 \pm 0.005$ mM/min, and for compound **5f** $K_m = 0.71 \pm 0.4$ mM and $V_{max} 0.31 \pm 0.14$ mM/min.

Table 1
Triazene prodrug **5** half-lives, $t_{1/2}$, in both PBS buffer (20% DMSO) and 80% human plasma at 37 °C, their log *P* values, and their ability to act as tyrosinase substrates (100 U/mL).



5			log P_{exp}	$t_{1/2}$		
Compound	X	Y		pH 7.4 buffer[a] (h)	80% human plasma[a] (h)	Tyrosinase[b] (min)
5a	Ac	H	3.25 ± 0.07	[c]	[d]	13.0
5b	CO_2Et	H	3.96 ± 0.09	[c]	71.5	12.9
5c	CN	H	2.89 ± 0.06	[c]	[d]	11.8
5d	Me	H	3.90 ± 0.06	364	[d]	18.2
5e	Ac	OH	3.24 ± 0.13	15.1	9.30	9.9
5f	CO_2Et	OH	3.78 ± 0.09	20.8	14.7	6.1
5g	Me	OH	4.17 ± 0.03	[d]	5.66	8.4

[a] Substrate concentration 10^{-5} M.
[b] Substrate concentration 5×10^{-5} M.
[c] Stable for 15 days.
[d] Stable for 2 days.

These K_m values compare favourably with those reported for tyramine (0.51 mM) and dopamine (2.2 mM). The V_{max} values correspond to turnover numbers of 0.6 s^{-1} and 6 s^{-1} for **5c** and **5f**, respectively, which are rather smaller than the 25.9 s^{-1} and 439 s^{-1} observed for tyramine and dopamine substrates [18], though the relative sizes between the tyramine and dopamine derivatives are similar. This observation is consistent with the report that tyrosinase accepts mono-, di- and trihydroxyphenols as substrates but displays the highest turnover rates for dihydroxyphenols [19,20]. The rates of decomposition of tyramine derivatives **5e–g** are not significantly affected by the substituents in the triazene moiety, presumably reflecting the significant distance between these substituents and the catechol moiety. Interestingly, although the half-lives of the tyramine derivatives **5a–d** are little affected by the substituent in the triazene moiety also, there is a correlation between log $t_{1/2}$ values and the Hammett σ_p values yielding a ρ value of −0.2 ($r^2 = 0.99$, Fig. 1). The origin of this rather small effect is not clear.

Inspection of the HPLC traces of the reaction mixtures of the tyrosinase-catalysed reactions revealed that triazenes **5** were

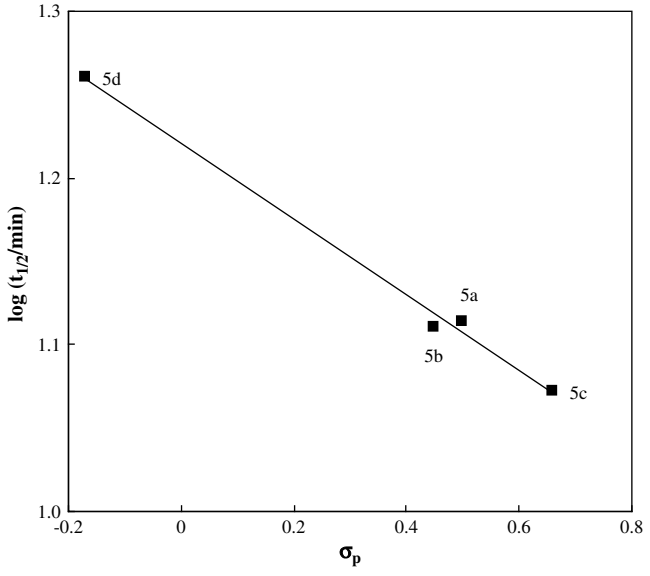


Fig. 1. Hammett plot of the half-lives for the decomposition of tyramine-based triazenes **5a–d** against σ_p values for the triazene substituents.

Table 2
APCI MS of the triazene substrates **5b**,**c**,**e** and their corresponding metabolites produced by tyrosinase activation.

Compound	5b	5c	5e
Substrate molecular ion		322.7[a]	355.0[a]
	415.7[b]	368.4[b]	400.6[b]
Metabolite molecular ion	383.6[a]	336.6[a]	
	429.3[b]	382.2[b]	398.6[b]

[a] Molecular ion.
[b] Formic acid adduct.

rapidly converted to a metabolite that did not correspond either to the appropriate monomethyltriazene **4** or its aniline decomposition product [12]. To gain some insight to the structure of this metabolite, an LC–MS study of the reaction mixtures was performed. It was not possible to obtain efficient ionization of the substrates or products by electrospray ionization (positive or negative modes) using the chromatographic conditions used for kinetic experiments. However, using the APCI source we were able to detect formic acid adducts of the substrates **5** and their corresponding metabolites; in some cases we could also detect the molecular ions (Table 2). For this reason ammonium formate was added to the aqueous eluent. To achieve adequate HPLC separations, we also required the pH of the eluent to be kept low; therefore the aqueous reaction solutions were acidified to pH 3.5 using formic acid.

We were able to identify the main HPLC peak formed through activation of triazenes **5b**,**c** and **e** by tyrosinase. In the case of the tyramine derivatives, **5b**,**c**, the mass of the metabolite was 14 units higher than the mass of the substrate (as exemplified for **5c** in Fig. 2). This mass difference can be ascribed to the conversion of **5b**, and **5c** to the corresponding *ortho*-quinone **7** (Scheme 2). In the case of the dopamine derivative, **5e**, chromatographic separation proved more difficult since the product peak overlapped with the substrate, as expected for a quinone product with similar structure and polarity to the substrate. Separation proved possible, however, using a gradient program and lower formate concentration. Formation of the *ortho*-quinone **7** corresponding to **5e** was confirmed by LC–MS since the metabolite peak, which eluted 0.5 min earlier than the substrate, gave a formic acid adduct ion of 2 mass units less than the substrate (Table 2). Overall, these results indicate that although the prodrugs are substrates for tyrosinase, the release of the cytotoxic monomethyltriazene **4** does not occur

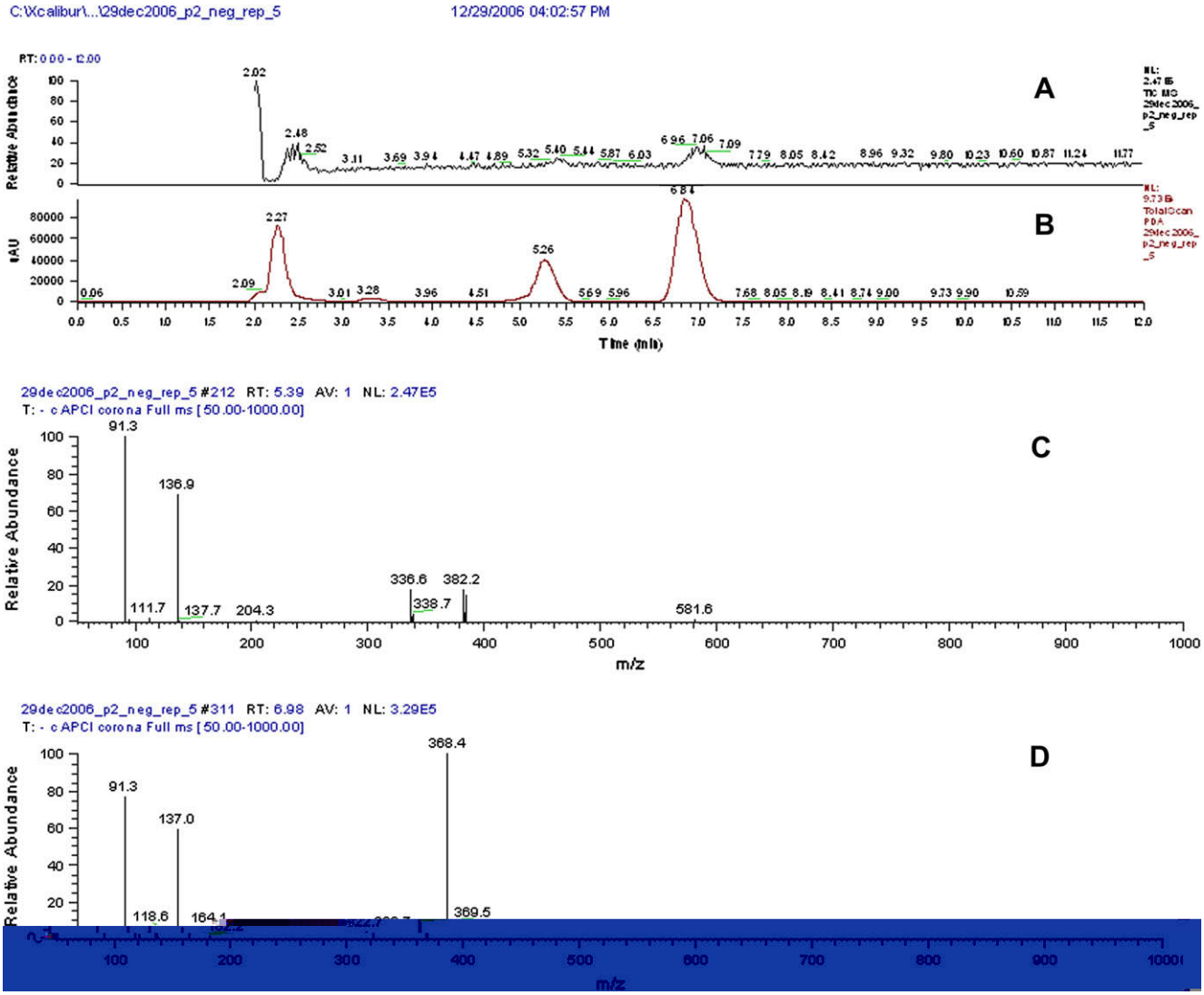


Fig. 2. TIC (A) and DAD (B) chromatograms of a solution of triazene **5c** after enzymatic reaction with tyrosinase; the substrate is detected at 6.8 min and the product at 5.3 min. Mass spectra for the product (C) and the triazene substrate (D) peaks.

under the conditions used. This suggests that subsequent cyclization activation of the *ortho*-quinone **7** (Scheme 2) is not an efficient process. Our results are consistent with recent studies that show that in urea or carbamate derivatives of dopamine the nitrogen atom is not sufficiently nucleophilic to effect the necessary cyclization that results in release of the antitumour agent [21,22].

3. Conclusions

In conclusion, the synthesis and analysis of a range of prodrugs derived from tyramine and dopamine, of potential use within MDEPT, have been evaluated. These compounds display excellent stability both in pH 7.4 buffer and in human plasma and simultaneously are good substrates for tyrosinase. The tyrosinase-catalysed reaction is very efficient in liberating an oxidized metabolite, but our HPLC and LC–MS data demonstrate that release of a cytotoxic monomethyltriazene does not occur for the urea-linked derivatives **5**. The present work demonstrates that drug release, which requires an activation process involving an intramolecular Michael addition reaction, requires a much better nucleophile than the urea nitrogen atom. Future work will therefore focus on the design of derivatives that have improved cyclization capacity while retaining excellent stability in aqueous solution. Such an approach shall also take account of the fact that dopamine derivatives are more rapidly metabolized by tyrosinase.

4. Experimental

4.1. General information

Melting points were determined using a Kofler camera Bock Monoscop M and are uncorrected. Elemental analyses were performed by Instituto Tecnológico e Nuclear, Portugal. FTIR spectra were recorded as KBr discs using a Nicolet Impact 400 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$ or DMSO solutions using a Bruker AM 400 WB. Coupling constants, *J*, are expressed in Hz. Chemicals were purchased from Sigma–Aldrich Chemical Company (U.K.) and B.D.H. Merck Chemical Company (U.K.). All chemicals and solvents were of reagent grade, except buffer substances and HPLC solvents, which were analytical grade and LiChrosolv® grade, respectively. Column chromatography was performed using silica gel 60 mesh 70–230 (Merck).

4.2. Synthesis

4.2.1. General procedures for the synthesis of prodrugs **5a–g**

A dichloromethane solution (5 mL) containing the appropriate monomethyltriazene (1.31 mmol) was added to a solution of 4-nitrophenyl chloroformate (1.58 mmol) and pyridine (1.31 mmol, 106 μL). After stirring for 24 h at room temperature, the reaction mixture was filtered and concentrated in vacuo. The crude product so-obtained was purified by column chromatography and used immediately. The pure carbamate (0.425 mmol) was dissolved in THF (10 mL) and tyramine (or dopamine) (0.863 mmol) and triethylamine (1.3 mmol, 180 μL) were added. After 48 h at room temperature, the solution was concentrated in vacuo and the crude product purified by column chromatography (ethylic ether-petroleum ether, 7:3).

4.2.2. 3-[2-(4-Hydroxyphenyl)ethylaminocarbonyl]-3-methyl-1-(4-acetylphenyl)triazene (**5a**)

Yield 50%; m.p. 178–180 °C; ν_{max}/cm^{-1} 3409, 3362, 1748, 1672; ^{1}H NMR ($CDCl_3$): δ 2.67 (3H, s, C*H*$_3$CO), 2.87 (2H, t, *J* = 8.0 Hz, C*H*$_2$Ar), 3.48 (3H, s, NC*H*$_3$), 3.67 (2H, q, *J* = 8.0 Hz, NHC*H*$_2$CH$_2$), 5.47 (1H, s, O*H*), 6.85–7.13 (4H, AA′BB′, *J* = 8.0 Hz, OH*Ar*), 7.44–8.03 (4H, AA′BB′, *J* = 8.0 Hz, CO*Ar*); ^{13}C NMR (100 MHz, $CDCl_3$): δ 27.29 (*C*H$_3$CO), 29.18 (NCH$_3$), 35.21 (*C*H$_2$Ar), 42.56 (NCH$_2$), 115.66–136.41 (*C*Ar), 152.37 (*C*NN), 154.16 (*C*OH), 156.18 (*C*O), 197.67 (CH$_3$*C*O). Anal. Calcd for $C_{18}H_{20}N_4O_3$: C, 63.5; H, 5.9; N, 16.5. Found: C, 63.5; H, 5.9; N, 16.4.

4.2.3. 3-[2-(4-Hydroxyphenyl)ethylaminocarbonyl]-3-methyl-1-(4-ethoxycarbonylphenyl)triazene (**5b**)

Yield 68%; m.p. 158–160 °C; ν_{max}/cm^{-1} 3415, 3280, 2962, 1708, 1676; ^{1}H NMR ($CDCl_3$): δ 1.42 (3H, t, *J* = 7.0 Hz, C*H*$_3$CH$_2$), 2.84 (2H, t, *J* = 6.8 Hz, C*H*$_2$Ar), 3.46 (3H, s, NC*H*$_3$), 3.65 (2H, q, *J* = 6.4 Hz, NHC*H*$_2$CH$_2$Ar), 4.40 (2H, q, *J* = 7.0 Hz, CH$_3$C*H*$_2$), 6.14 (1H, s, O*H*), 6.52 (1H, br t, *J* = 5.2 Hz, N*H*), 6.85–7.10 (4H, AA′BB′, *J* = 8.4 Hz, HO*Ar*), 7.41–8.09 (4H, AA′BB′, *J* = 8.8 Hz, N*Ar*CO); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.33 (*C*H$_3$CH$_2$), 28.61 (NCH$_3$), 34.98 (*C*H$_2$Ar), 41.68 (NCH$_2$), 61.33 (CH$_3$*C*H$_2$), 115.69–130.65 (*C*Ar), 152.36 (*C*NN), 154.36 (*C*OH), 154.87 (*C*O), 166.48 (*C*OCH$_2$CH$_3$). Anal. Calcd for $C_{19}H_{22}N_4O_4$: C, 61.6; H, 6.0; N, 15.1. Found: C, 61.5; H, 5.8; N, 14.9.

4.2.4. 3-[2-(4-Hydroxyphenyl)ethylaminocarbonyl]-3-methyl-1-(4-cyanophenyl)triazene (**5c**)

Yield 55%; m.p. 156–158 °C; ν_{max}/cm^{-1} 3221, 3411, 3018, 2966, 2224, 1678, 1613; ^{1}H NMR ($CDCl_3$): δ 2.83 (2H, t, *J* = 6.8 Hz, C*H*$_2$Ar), 3.45 (3H, s, NC*H*$_3$), 3.59–3.36 (2H, q, *J* = 4.0 Hz, C*H*$_2$CH$_2$Ar), 6.56 (1H, br t, N*H*CH$_2$), 6.72 (1H, s, ArO*H*), 6.79–7.08 (4H, AA′BB′, *J* = 4.8 Hz, Ar*H*), 7.47–7.74 (4H, AA′BB′, *J* = 8.4 Hz, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$): δ 27.38 (NCH$_3$), 33.49 (*C*H$_2$Ar), 40.29 (*C*H$_2$CH$_2$Ar), 109.80 (*C*CN), 114.24–117.46 (*C*Ar), 121.14 (*C*N), 128.46 (*C*Ar), 131.94 (*C*Ar), 150.73 (*C*NN); 152.61 (*C*OH); 154.13 (*C*O). Anal. Calcd for $C_{17}H_{17}N_5O_2$: C, 63.2; H, 5.3; N, 21.7. Found: C, 63.2; H, 5.5; N, 21.6.

4.2.5. 3-[2-(4-Hydroxyphenyl)ethylaminocarbonyl]-3-methyl-1-(4-tolyl)triazene (**5d**)

Yield 53%; m.p. 196–197 °C; ν_{max}/cm^{-1} 3363, 3027, 2923, 1678; ^{1}H NMR ($CDCl_3$): δ 2.41 (3H, s, C*H*$_3$Ar), 2.85 (2H, t, *J* = 6.8 Hz, C*H*$_2$Ar), 3.45 (3H, s, NC*H*$_3$), 3.64 (2H, q, *J* = 6.2 Hz, C*H*$_2$CH$_2$Ar), 5.64 (1H, s, O*H*), 6.55 (1H, br t, N*H*), 6.85–7.12 (4H, AA′BB′, *J* = 7.6 Hz, *Ar*Me), 7.24–7.34 (4H, AA′BB′, *J* = 7.8 Hz, *Ar*CN); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.23 (*C*H$_3$Ar), 28.65 (NCH$_3$), 35.30 (*C*H$_2$Ar), 42.46 (NCH$_2$), 115.66–138.39 (*C*Ar), 146.91 (*C*NN), 154.54 (*C*OH), 156.17 (*C*O). Anal. $C_{17}H_{20}N_4O_2$: C, 65.4; H, 6.5; N, 17.9. Found: C, 65.3; H, 6.3; N, 17.7.

4.2.6. 3-[2-(3,4-Dihydroxyphenyl)ethylaminocarbonyl]-3-methyl-1-(4-acetylphenyl)triazene (**5e**)

Yield 45%; m.p. 158–160 °C; ν_{max}/cm^{-1} 3560, 3500, 3400, 1720, 1650; ^{1}H NMR (DMSO-d_6): δ 2.65 (3H, s, C*H*$_3$CO), 2.77 (2H, t, *J* = 7.2 Hz, C*H*$_2$Ar), 3.45 (3H, s, NC*H*$_3$), 3.56 (2H, t, *J* = 7 Hz, NHC*H*$_2$), 6.58–6.74 (3H, m, *Ar*), 7.65–8.09 (4H, AA′BB′, *J* = 7.0 Hz, *Ar*Ac); ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.29 (*C*H$_3$), 29.18 (NCH$_3$), 35.49 (*C*H$_2$Ar), 42.61 (NCH$_2$), 116.02–136.41 (*C*Ar), 144.09 (*C*OH), 145.64 (*C*OH), 152.37 (*C*NN), 154.14 (*C*O), 197.68(*C*O). Anal. Calcd for $C_{18}H_{20}N_4O_4$: C, 60.7; H, 5.7; N, 15.7. Found: C, 60.5; H, 5.3; N, 15.6.

4.2.7. 3-[2-(3,4-Dihydroxyphenyl)ethylaminocarbonyl]-3-methyl-1-(4-ethoxycarbonylphenyl)triazene (**5f**)

Yield 56%; m.p. 173–174 °C; ν_{max}/cm^{-1} 3410, 1708, 1602; ^{1}H NMR (DMSO-d_6): δ 1.35 (3H, t, *J* = 7.2 Hz, C*H*$_3$CH$_2$O), 2.66 (2H, t, *J* = 8.0 Hz, C*H*$_2$Ar), 3.35 (5H, br s, NC*H*$_3$ and NC*H*$_2$), 4.34 (2H, q, *J* = 7.2 Hz, OC*H*$_2$), 6.48–6.65 (3H, m, *Ar*), 7.82–8.04 (4H, AA′BB′, *J* = 8.0 Hz, *Ar*CO$_2$Et), 7.91 (1H, br t, *J* = 8.0 Hz, N*H*), 8.70 (1H, s, O*H*), 8.81 (1H, s, O*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.65 (*C*H$_3$), 29.18 (NCH$_3$), 35.50 (*C*H$_2$Ar), 42.64 (NCH$_2$), 61.33 (OCH$_2$), 116.01–130.69 (*C*Ar), 144.09 (*C*OH), 145.63 (*C*OH), 152.48 (*C*NN), 154.12 (CO), 165.76 (CO). Anal. Calcd for $C_{17}H_{20}N_4O_3$: C, 62.2; H, 6.1; N, 15.2. Found: C, 62.2; H, 5.9; N, 15.1.

4.2.8. 3-[2-(3,4-Dihydroxyphenyl)ethylaminocarbonyl]-3-methyl-1-(4-tolyl)triazene (**5g**)

Yield 26%; m.p. 151–153 °C; ν_{max}/cm^{-1} 3500, 3317, 3408, 1683; ^{1}H NMR (DMSO-d_6): δ 2.35 (3H, s, CH_3Ar), 2.65 (2H, t, $J = 8.0$ Hz, NCH$_2$CH_2Ar), 3.32 (3H, s, NCH_3), 3.37 (2H, m, NCH_2CH$_2$Ar), 6.48–6.67 (3H, m, *Ar*), 7.28–7.61 (4H, AA′BB′, $J = 8.0$ Hz, Ar-triazene), 7.73 (1H, br t, $J = 5.6$ Hz, N*H*), 8.71 (1H, O*H*), 8.82 (1H, O*H*); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.25 (*C*H$_3$), 28.66 (NCH$_3$), 35.59 (*C*H$_2$Ar), 42.49 (NCH$_2$), 116.01–138.39 (*C*Ar), 144.07 (*C*OH), 145.64 (*C*OH), 146.90 (*C*NN), 154.51 (*C*O). Anal. Calcd for $C_{17}H_{20}N_4O_3$: C, 62.2; H, 6.1; N, 17.1. Found: C, 62.5; H, 6.0; N, 17.1.

4.3. HPLC analysis

The analytical high-performance liquid chromatography (HPLC) system comprised a Merck Hitachi L-7110 pump with an L-7400 UV detector, a manual sample injection module equipped with a 20 μL loop, a Merck Lichrospher® 100 RP$_{18}$ 125 mm × 4.6 mm (5 μm) column equipped with a Merck Lichrocart pre-column (Merck, Germany). For compounds **5a**–**d** an acetonitrile/water (45:55) isocratic solvent system was used. For compounds **5e**–**g** a gradient elution beginning with a mobile phase of 95% water/5% acetonitrile ramping to 95% acetonitrile/5% water over 30 min was used. The column effluent was monitored at 300 nm for all compounds (**5a**–**g**).

4.4. Apparent partition coefficients

The apparent partition coefficients (log P_{exp}) were determined at room temperature using 1-octanol–pH 7.4 phosphate buffer. Each phase was mutually saturated before the experiment. The volumes of each phase were chosen so that solute concentrations in the aqueous phase after distribution were readily measurable. The compounds were dissolved in 1-octanol and the 1-octanol–phosphate buffer mixtures were shaken for 30 min to reach an equilibrium distribution. Each phase was analyzed separately by HPLC, but the organic phase was diluted in acetonitrile (1:10) before HPLC analysis. Partition coefficients, P_{exp}, were calculated from the ratio of the peak area in octanol to the peak area in buffer.

4.5. Hydrolysis in buffer solution

Reaction mixtures were analyzed using HPLC. Usually, a 10 μL aliquot of a 10^{-2} M stock solution of prodrug **5** in acetonitrile was added to 10 mL of the appropriate thermostated buffer solution (8 mL PBS and 2 mL DMSO). At regular intervals, samples of the reaction mixture were analyzed by HPLC using the following conditions: mobile phase, acetonitrile/water (the composition of which depended on the compound); flow rate 1.0 mL/min.

4.6. Hydrolysis in human plasma

Human plasma was obtained from the pooled, heparinised blood of healthy donors, and was frozen and stored at −70 °C prior to use. For the hydrolysis experiments, the substrates were incubated at 37 °C in human plasma that had been diluted to 80% (v/v) with pH 7.4 isotonic phosphate buffer. At appropriate intervals, aliquots of 200 μL were added to 400 μL of acetonitrile to both quench the reaction and precipitate plasma proteins. These samples were centrifuged and the supernatant analyzed by HPLC for the presence of substrate and products.

4.7. Tyrosinase degradation studies

Mushroom tyrosinase (Sigma) was used at a concentration of 100 U/mL in a solution comprising phosphate buffer (pH 7.4, 2.4 mL) and DMSO (0.6 mL) at 37 °C. A solution of the prodrug under investigation (15 μL of 10^{-2} M solution) was added and the mixture incubated with gentle stirring at 37 °C. Aliquots were collected at selected time intervals and quenched by addition of an organic solvent (acetonitrile). The disappearance of the prodrug, with concomitant appearance of metabolites, was followed by HPLC for evaluation of the hydrolysis kinetics.

4.8. LC–MS assays

Analysis was performed on a Surveyor HPLC from Thermo Finnegan, San Jose, C.A., USA equipped with an autosampler and a diode array detector. The mass spectrometry system was an LCQ ion trap mass spectrometer also from Thermo Finnegan, equipped with an APCI source. The LC–MS system was controlled by Excalibur version 1.3 software (Thermo Finnegan – Surveyor, San Jose, USA). The analytical column was a Lichrospher RP-8 (125 × 4 mm, 5 μm) from Merck with a guard column of the same material. Separations were carried out with a flow rate of 0.7 mL/min at 50 °C. Injections were made with 20–100 μL of the 10^{-4} to 10^{-5} M solutions of the substrates in acetonitrile or the corresponding solutions after enzymatic reaction.

In the case of the tyrosine derivatives, an isocratic system comprising 45% ammonium formate (p.a. Fluka, 20 mM, pH 3.5 with formic acid), 45% acetonitrile (LC–MS grade, Reidel-de Haën) and 10% methanol (LC–MS grade, Reidel-de Haën) was used. For the dopamine derivatives, separation was carried out using a gradient program, starting with 95% ammonium formate (2 mM, pH 3.5)/5% acetonitrile for 7 min ramping to 35% acetonitrile over the next 10 min. Detection by UV absorption was in the range 200–400 nm.

The following conditions were used for the APCI source in negative mode: vaporizer temperature, 400 °C; discharge current, 5 μA; temperature of the heated capillary 150 °C. Nitrogen was used as sheath gas and auxiliary gas with a gas flow rate of 80 and 40 arbitrary units, respectively. The flow was diverted from the mass detector in the first 2 min of each run.

LC–MS was performed in total ion content mode (TIC) from m/z 50 to 1000.

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