

Original article

# Towards an efficient prodrug of the alkylating metabolite monomethyltriazene: Synthesis and stability of *N*-acylamino acid derivatives of triazenes

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## Abstract

A series of 3-[ $\alpha$ -(acylamino)acyl]-1-aryl-3-methyltriazenes **6a–l**, potential cytotoxic triazene prodrugs, were synthesised by coupling 1-aryl-3-methyltriazenes to *N*-acylamino acids. Their hydrolysis was studied in isotonic pH 7.4 phosphate buffer and in human plasma, while hydrolysis of the derivative **6a** was studied in more depth across a range of pH values. Prodrugs **6a–l** hydrolyse by cleavage of the triazene acyl group to afford the corresponding monomethyltriazenes. Studies in human plasma demonstrate that acylation of the  $\alpha$ -amino group of the amino acid carrier is an effective means of reducing the chemical reactivity of the  $\alpha$ -aminoacyl derivatives while retaining a rapid rate of enzymatic hydrolysis. These derivatives displayed log *P* values that suggest they should be well absorbed through biological membranes.

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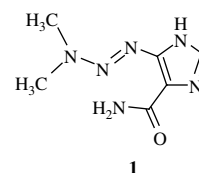
**Keywords:** Triazene; Prodrug; Aminoacyl; Antitumour drugs; Kinetics; Enzymes and enzyme reactions

## 1. Introduction

Prodrug formation is an important means of enhancing drug efficiency. A major requirement of the prodrug derivative is that it must be converted rapidly, or at a controlled rate, to the active therapeutic agent *in vivo* while at the same time be sufficiently stable *in vitro* such that a stable pharmaceutical product may be developed. This is often achieved using the double prodrug concept via molecules that make use of an enzymatic release mechanism followed by a spontaneous chemical reaction [1]. Another important aspect of prodrug design is the need for the carrier moiety to be non-toxic [2]. To this end, biologically compatible compounds, particularly  $\alpha$ -amino acids, have been used as carriers for drugs that contain either the hydroxyl functional group (forming esters) or a carboxyl or amine group (forming amides) [3]. Esters of  $\alpha$ -amino acids are chemically very unstable, the ester group being activated

towards hydrolysis in acidic and neutral solutions by the strong electron-withdrawing inductive effect of the protonated amino group [4].

For many years dacarbazine **1** has been one of the most widely used drugs to treat malignant melanoma [5].



The biological action of dacarbazine and, in general, the anticancer 1-aryl-3,3-dimethyltriazenes (**2**, in Fig. 1) is a consequence of their capacity to alkylate DNA [6].

These compounds suffer metabolic oxidation by cytochrome P450 enzymes to give hydroxymethyltriazenes **3**, which, by loss of formaldehyde, generate the cytotoxic monomethyltriazenes **4** (Fig. 1) [7–9]. These are known alkylating agents, capable of methylating DNA and RNA [10,11]. However, dimethyltriazenes are poorly metabolised by humans

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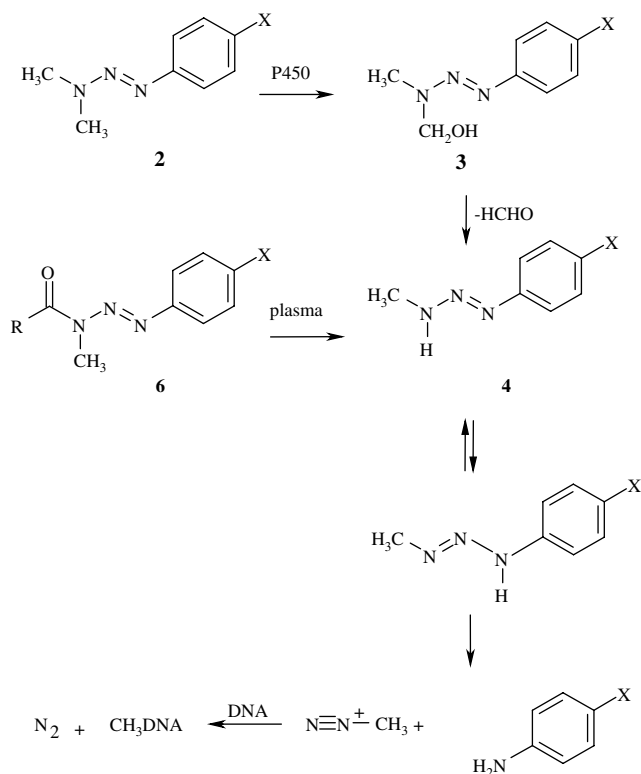
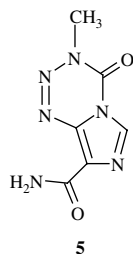


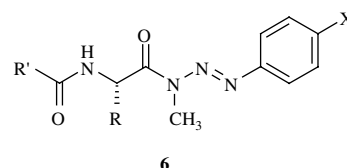
Fig. 1. Metabolism pathways for 1-aryl-3,3-dimethyltriazenes and 3-aminoacyl-1-aryl-3-methyltriazenes.

[12]. Consequently, with the aim of finding suitable prodrugs that by-pass the need for this oxidative metabolism, alongside hydroxymethyltriazenes **3** themselves [13], a range of derivatives of **3** and **4**, including alkyl ether, aryl ether, alkane-thioether, and acyl ester derivatives of **3** and acyl, acyloxymethylcarbamate and ureido derivatives of **4**, have been synthesised by several groups, including ourselves [14–25]. Indeed, one such compound, imidazo[3,4-*e*]tetrazin-5-one temozolomide **5** [18], has received FDA approval for the treatment of a specific type of brain cancer, anaplastic astrocytoma (<http://www.fda.gov/cder/da/da0899.htm>).



Given that the incidence of malignant melanoma is on the increase and is expected to continue to rise [5] there is a need to develop improved pharmacological entities. This, together with the need to employ carrier moieties that are non-toxic, has directed our interests towards triazenes linked to amino acids via the triazene *N*-3 nitrogen atom and the acyl group of the amino acid [26,27]. These triazene amide derivatives were found to have reactivities very similar to those of amino acid esters. However, an *N*-acetylated alanyl derivative

displayed significant chemical stability in isotonic phosphate buffer yet rapid hydrolysis to monomethyltriazene in plasma [27]. To demonstrate the potential of this type of compound as possible bioreversible prodrugs of the anticancer monomethyltriazene system, we here report the synthesis of a series of 3-[ $\alpha$ -(acylamino)acyl]-1-aryl-3-methyltriazenes, **6a–l**, a determination of their log *P* values and an examination of their stability both in isotonic pH 7.4 phosphate buffer and in plasma.



<b>6a</b>	R = CH <sub>3</sub>	R' = CH <sub>3</sub>	X = CN
<b>6b</b>	R = H	R' = CH <sub>3</sub>	X = CN
<b>6c</b>	R = CH <sub>2</sub> Ph	R' = CH <sub>3</sub>	X = CN
<b>6d</b>	R = CH(CH <sub>3</sub> ) <sub>2</sub>	R' = CH <sub>3</sub>	X = CN
<b>6e</b>	R = CH <sub>3</sub>	R' = CH <sub>2</sub> CH <sub>3</sub>	X = CN
<b>6f</b>	R = CH <sub>3</sub>	R' = CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	X = CN
<b>6g</b>	R = CH <sub>3</sub>	R' = Ph	X = CN
<b>6h</b>	R = CH <sub>3</sub>	R' = CH <sub>3</sub>	X = COCH <sub>3</sub>
<b>6i</b>	R = CH <sub>3</sub>	R' = CH <sub>3</sub>	X = COOEt
<b>6j</b>	R = CH <sub>3</sub>	R' = CH <sub>3</sub>	X = CONH <sub>2</sub>
<b>6k</b>	R = CH <sub>3</sub>	R' = CH <sub>3</sub>	X = Br
<b>6l</b>	R = CH <sub>3</sub>	R' = CH <sub>3</sub>	X = CH <sub>3</sub>

## 2. Results and discussion

### 2.1. Synthesis

The  $\alpha$ -(acylamino)acyl derivatives, **6a–l**, were readily synthesised by the conventional coupling reaction of the appropriate 1-aryl-3-methyltriazene **4** with an (*S*)-*N*-acylamino acid using dicyclohexylcarbodiimide activation. The <sup>1</sup>H NMR spectra of **6a–l** display a typical triazene *N*-methyl singlet in the range  $\delta$  3.40–3.53 ppm. For **6d** the two methyl groups of the isopropyl side-chain are diastereotopic and appear as two doublets.

### 2.2. Kinetic studies

The hydrolysis of the  $\alpha$ -(acylamino)acyltriazenes **6a–l** to give the corresponding 3-methyltriazenes **4** (Fig. 1) was studied in isotonic phosphate buffer and in 80% human plasma containing 20% isotonic phosphate buffer. The hydrolysis of **6a** was also studied in more detail across the pH range 1–11 in aqueous buffers. Under the reaction conditions the monomethyltriazenes are unstable, hydrolysing further to the corresponding anilines. These reactions were easily followed either by HPLC (monitoring both the loss of starting material and the formation of products) or by UV spectroscopy (monitoring the loss of starting material). For compound **6g** the

concomitant quantitative formation of *N*-benzoyl-(*S*)-alanine **7** (Scheme 1) was also observed (Fig. 2).

### 2.2.1. Hydrolysis in aqueous buffers

Half-lives for the hydrolysis of triazenes **6a–l** in isotonic phosphate buffer are given in Table 1. Inspection of Table 1 shows that these compounds decompose at physiological pH with half-lives ranging from 7.6 to 84 h.

To better understand the chemistry of these potential pro-drug derivatives, compound **6a** was studied in more detail. Pseudo-first-order rate constants,  $k_{\text{obs}}$ , for its hydrolysis were determined in aqueous buffers (using several buffer concentrations at each pH), in deuterated buffers, and in NaOH and H<sub>2</sub>SO<sub>4</sub> solutions (Table 2). The values of  $k_{\text{obs}}$  depend upon both the pH of the solution and the buffer concentration. Using the intercepts of plots of  $k_{\text{obs}}$  versus buffer concentration, together with  $k_{\text{obs}}$  values determined in NaOH and H<sub>2</sub>SO<sub>4</sub> solutions, a pH-rate profile can be constructed (Fig. 3), which shows that there are regions of acid- and base-catalysis, as well as a pH-independent process. The compound is most stable between pH 3 and 5.5. At pH 7.4 the most important reaction is that catalysed by hydroxide ion (by about a factor of 20). Further, comparison of the second-order rate constant for hydroxide-catalysed reaction,  $5.7 \text{ M}^{-1} \text{ s}^{-1}$ , with that for the corresponding deuterioxide-catalysed reaction,  $7.8 \text{ M}^{-1} \text{ s}^{-1}$ , affords a solvent deuterium isotope effect of 0.73. This value identifies hydrolysis proceeding by hydroxide ion acting as a nucleophilic entity rather than as a general base [28].

The rate constants for catalysis of the hydrolysis reaction by the buffer species,  $k_{\text{B}}$ , were determined from the slopes of plots of  $k_{\text{obs}}$  versus buffer concentration. Fig. 4 shows that the correlation between  $k_{\text{B}}$  and the buffer  $\text{p}K_{\text{a}}'$  (i.e. the  $\text{p}K_{\text{a}}$  corrected for ionic strength [29]) is linear, giving a Brønsted  $\beta$  value of 0.7. Significantly, the sterically hindered base 2,2,4,4-tetramethylpiperidine,  $\text{p}K_{\text{a}}'$  11.21, exhibits a catalytic rate constant  $k_{\text{B}}$  of  $0.41 \text{ M}^{-1} \text{ s}^{-1}$ , essentially not different from that of the non-sterically hindered parent piperidine,  $k_{\text{B}}$   $0.42 \text{ M}^{-1} \text{ s}^{-1}$ , which has an almost identical  $\text{p}K_{\text{a}}'$  of 11.26. Together with the Brønsted plot, these data imply that, in contrast to hydroxide ion, the buffer materials exert catalysis by acting as general bases rather than nucleophiles. This was corroborated by the deuterium isotope effect for the buffer catalysed process, which was determined for pyridine, a relatively weak catalyst, and piperidine, a relatively efficient catalyst. The data are shown in Table 3; the rates are more rapid in H<sub>2</sub>O compared to D<sub>2</sub>O, which identifies proton transfer from

water to the base catalyst as being integral to the rate-limiting hydrolytic step.

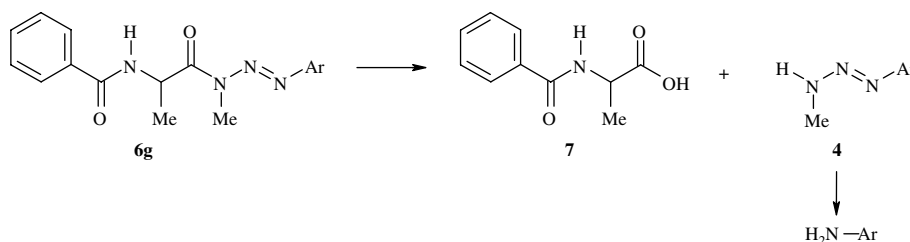
Thus, taken together these studies reveal that in solutions at a physiological pH of 7.4 the hydrolysis of aminoacyltriazenes comprises three components: pH-independent, base-catalysed and buffer-catalysed reactions. Consequently, general conclusions about the factors affecting the relative magnitudes of the  $t_{1/2}$  values for the compounds in Table 1 ought to be made with caution, although in pH 7.4 phosphate buffer the major reaction is the hydroxide ion catalysed process. Nevertheless, certain features are apparent. First, compounds **6a,h–l** reveal that the reaction is influenced by the substituent, -X, present in the aryl group, electron-attracting substituents increasing the rate of hydrolysis (these compounds afford a Hammett correlation with a  $\rho$  value of +0.81;  $r^2 = 0.99$ ;  $n = 6$ ). Second, the structure of the  $\alpha$ -amino acid unit is also important, the glycine derivative **6b** being the most reactive and the sterically hindered valine derivative **6d** the most stable. Third, the structure of the *N*-acyl group (compounds **6a,e–g**) has only a small effect on the rate of hydrolysis.

### 2.2.2. Hydrolysis in human plasma

Blood serum and plasma are known to contain a range of enzymes that catalyse the hydrolysis of esters and amides [30]. Consequently, we were particularly interested in examining the hydrolysis of the  $\alpha$ -(acylamino)acyltriazenes **6a–l** in human plasma. The results in Table 1 clearly show that, in plasma, all the compounds studied liberate the corresponding monomethyltriazenes; that they do so at rates that are 3–15 times faster than in phosphate buffer also implies that the  $\alpha$ -(acylamino)acyltriazenes are substrates for plasma enzymes. This is in contradistinction to the more reactive parent aminoacyltriazenes, which were found not to be substrates for plasma enzymes [26].

Although the prodrugs liberate the cytotoxic monomethyltriazene more rapidly in plasma than in phosphate buffer, Fig. 5 reveals a correlation (albeit with an  $r^2$  correlation coefficient of 0.67) between the half-lives for the two processes. The implication is that similar electronic and steric effects operate in both cases.

We observed previously that, despite formally being amide derivatives, 3-acyltriazenes have a reactivity similar to that of esters [15,21]. Since substrate lipophilicity is one of the main features that influence esterase activity, lower enzyme reactivity being associated with increasing lipophilicity [31], we examined the effect of substrate lipophilicity on the rate of



Scheme 1.

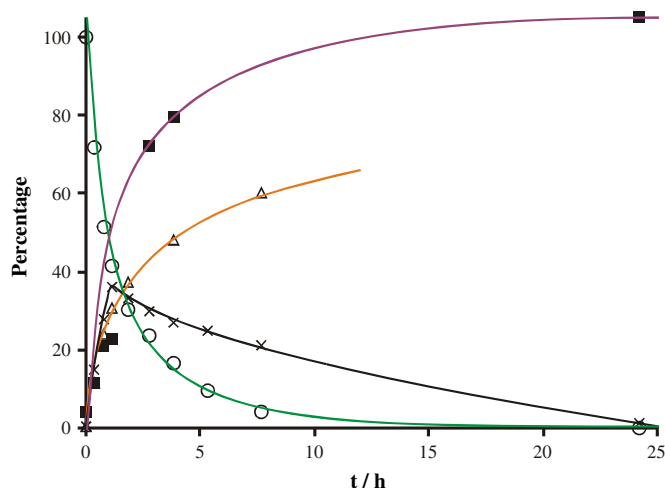


Fig. 2. Time course for the hydrolysis of **6g** in 80% human plasma at 37 °C: ○, **6g**; ■, *N*-benzoyl-(*S*)-alanine; ×, **4**; △, corresponding aniline.

monomethyltriazene liberation from the prodrugs **6a–l**. The partition coefficients between octanol and pH 7.4 phosphate buffer at 25 °C for compounds **6a–l** (Table 1) give values of log *P* that cover a wide range of lipophilicities (0.69–3.04). However, Fig. 6 reveals no correlation between lipophilicity and plasma stability and we infer that prodrug stability depends only on those factors that govern the chemical reactivity of the aminoacyltriazene unit.

### 3. Conclusions

$\alpha$ -(Acylamino)acyltriazenes **6a–l** have two amide functional groups susceptible to hydrolysis, the *N*-acyl group and the triazene amide. Our results – dependence on the triazene aryl substituent, reduced reactivity with bulky substituents at the  $\alpha$ -carbon of the amino acid, minimal effect of the *N*-acyl group, and isolation of the *N*-acylamino acid hydrolysis co-product – point to reaction proceeding via hydrolysis of the triazene amide (Fig. 7) and therefore that the  $\alpha$ -(acylamino)acyl derivatives provide a potential prodrug system for

Table 1  
Half-lives,  $t_{1/2}$ , for the triazenes **6a–l** in both pH 7.7 isotonic phosphate buffer and 80% human plasma at 37 °C, together with log *P* values

	$t_{1/2}$ (h)		log $P_{\text{exp}}^b$	log $P_{\text{calc}}$
	pH 7.7 buffer <sup>a</sup>	Plasma <sup>b</sup>		
<b>6a</b>	9.8 ± 0.4	1.0 ± 0.1	1.41 ± 0.01	1.93 ± 0.51
<b>6b</b>	7.6 ± 0.1	1.2 ± 0.2	1.1 ± 0.03	1.58 ± 0.50
<b>6c</b>	13.9 ± 0.0	2.4 ± 0.3	2.76 ± 0.05	3.86 ± 0.52
<b>6d</b>	84.3 ± 1.8	5.7 ± 1.1	2.18 ± 0.04	2.81 ± 0.51
<b>6e</b>	10.1 ± 0.3	1.3 ± 0.2	1.87 ± 0.06	2.46 ± 0.51
<b>6f</b>	9.1 ± 0.2	1.1 ± 0.2	2.28 ± 0.02	2.99 ± 0.51
<b>6g</b>	9.2 ± 0.0	1.6 ± 0.3	3.04 ± 0.03	3.84 ± 0.51
<b>6h</b>	13.9 ± 0.4	2.3 ± 0.3	1.51 ± 0.09	1.81 ± 0.49
<b>6i</b>	15.7 ± 0.6	4.6 ± 0.5	2.52 ± 0.04	2.87 ± 0.48
<b>6j</b>	18.4 ± 0.2	7.6 ± 0.6	0.69 ± 0.06	0.89 ± 0.48
<b>6k</b>	25.5 ± 0.2	5.7 ± 0.9	2.8 ± 0.04	3.01 ± 0.54
<b>6l</b>	46.5 ± 1.5	15.1 ± 1.2	2.45 ± 0.02	2.45 ± 0.46

<sup>a</sup> Values represent the means of at least 2 determinations ± SD.

<sup>b</sup> Values represent the means of at least 3 determinations ± SD.

the anticancer monomethyltriazenes. The compounds are suitably stable in phosphate buffer ( $t_{1/2}$ ; ca. 8–84 h) while, in plasma, those containing an electron withdrawing group in the triazene moiety together with a non-bulky group (acylamino)acyl moiety decompose over 1–2 h. By way of comparison, temozolomide, the recently introduced clinically approved triazene, is likewise a chemically-activated prodrug that has a half-life in both pH 7.4 phosphate buffer and plasma of ca. 2 h [32]. Thus, the acylation of the  $\alpha$ -amino group seems to be an effective means of reducing the chemical reactivity of the  $\alpha$ -aminoacyl derivatives while retaining a rapid rate of enzymatic hydrolysis. Usually this kind of tunable dual reactivity is only achieved using the double prodrug strategy [33], but that involves formaldehyde liberation over which there toxicity concerns [34–36]. Additionally, these  $\alpha$ -(acylamino)acyl derivatives display log *P* values that suggest they should be well absorbed by biological membranes.

### 4. Experimental protocols

**Warning:** All triazenes used in this study should be considered as mutagenic and/or carcinogenic and appropriate care should be taken to handle them safely.

Melting points were determined using a Kofler camera Bock-Monoscop “M” and are uncorrected. IR spectra were recorded as KBr discs using a Perkin–Elmer 1310 spectrophotometer. <sup>1</sup>H NMR spectra were recorded in CDCl<sub>3</sub> solutions using a Jeol JNM LA-300 spectrometer; chemical shifts,  $\delta$ , are reported as parts per million from Me<sub>4</sub>Si, and coupling constants, *J*, in hertz. Mass spectra were recorded using a VG Mass Lab 20–250 spectrometer. High-performance liquid chromatography (HPLC) was performed using a Merck-Hitachi system consisting of an isochrom LCL-7100 pump, a Lichrospher 100 RP-8 (5  $\mu$ m) column, an UV L-7400 detector and a D-2500 integrator. Elemental analyses were obtained from Medac Ltd., Brunel Science Park, Englefield Green, Egham, Surrey, UK. All chemicals were reagent grade except those for kinetic studies and HPLC, which were of analytical or LiChrosolv (Merck) grade. 1-Aryl-3-methyltriazenes were synthesised by previously published methods [37]. The (*S*)-*N*-acetylamino acids were purchased from Sigma. *N*-Propanoyl- and *N*-butanoylalanine were synthesised by the reaction of *tert*-butyl (*S*)-2-aminopropanoate with the corresponding propanoyl and butanoyl chlorides followed by the conventional deprotection of the *tert*-butyl ester using trifluoroacetic acid [38].

#### 4.1. General procedure for the synthesis of $\alpha$ -(acylamino)acyltriazenes (**6a–l**)

*N,N'*-Dicyclohexylcarbodiimide (2.4 mmol) was added to a solution of the *N*-acylamino acid (1.9 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 ml). After 30 min at room temperature, triethylamine (0.19 mmol) and the appropriate 1-aryl-3-methyltriazene (1.9 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 ml) were added. After 48 h at room temperature, *N,N'*-dicyclohexylurea by-product was removed by filtration and the solvent removed under reduced pressure.

Table 2

Pseudo-first-order hydrolysis rate constants for **6a** in aqueous buffers at 25 °C

Buffer	Buffer/M	pH (pH <sub>calc</sub> )	$k_{\text{obs}}/10^{-5} \text{ s}^{-1}$	Buffer	Buffer/M	pH (pH <sub>calc</sub> )	$k_{\text{obs}}/10^{-5} \text{ s}^{-1}$
H <sub>2</sub> SO <sub>4</sub>	0.0946	(0.72)	0.102	Morpholine <sup>e</sup>	0.0010	7.53	0.711
	0.0085	(1.78)	0.013		0.0040	8.67	0.808
Formate <sup>a</sup>	0.0020	3.72	0.0055		0.0080	8.71	0.841
	0.0200	3.43	0.0057	Piperazine <sup>f</sup>	0.010	8.60	0.869
	0.0400	3.41	0.0058		0.01	9.21	12.6
	0.1000	3.44	0.0068		0.02	9.31	15.3
Pyridine <sup>b</sup>	0.01	5.73	0.0097		0.08	9.39	19.8
		5.78	0.0126		0.16	9.47	26.2
		5.78	0.0232		0.20	9.36	27.0
		5.72	0.0400	Piperidine <sup>g</sup>	0.01	10.84	477
	0.2	5.70	0.0625		0.05	11.11	830
Phosphate <sup>c</sup>	0.0063	6.62	0.0036		0.10	11.14	908
	0.0127	6.62	0.0041		0.30	11.15	2127
	0.0422	6.66	0.0045		0.40	11.17	2481
	0.8440	6.70	0.0048	2,2,4,4-Tetramethylpiperidine <sup>h</sup>	0.01	10.86	532
Imidazole <sup>d</sup>	0.01	6.90	0.0532		0.02	11.06	775
	0.08	7.01	0.1085		0.03	11.12	995
	0.16	7.06	0.171		0.04	11.14	1156
	0.20	7.02	0.191	NaOH	0.0006	(10.80)	454
	0.30	7.03	0.238		0.0011	(11.03)	710
					0.0013	(11.12)	1016
					0.0018	(11.26)	1265
					0.0024	(11.38)	1536
					0.0035	(11.55)	2107

<sup>a</sup> pK<sub>a</sub>' 3.61.<sup>b</sup> pK<sub>a</sub>' 5.09.<sup>c</sup> pK<sub>a</sub>' 7.35.<sup>d</sup> pK<sub>a</sub>' 7.09.<sup>e</sup> pK<sub>a</sub>' 8.47.<sup>f</sup> pK<sub>a</sub>' 9.95.<sup>g</sup> pK<sub>a</sub>' 11.26.<sup>h</sup> pK<sub>a</sub>' 11.21.

The crude residue was subjected to chromatography (silica gel 60, 70–230 mesh ASTM, Merck) using one of the following systems: hexane:diethyl ether (for **6f**), CH<sub>2</sub>Cl<sub>2</sub>:ethyl acetate (for **6e,g**), ethyl acetate (for **6a–d,h,i,k,l**) and ethyl acetate:–MeOH (for **6j**). The isolated *N*-(acylamino)acyltriazenes were subsequently recrystallised from hexane/ethyl acetate or hexane/CH<sub>2</sub>Cl<sub>2</sub>.

#### 4.1.1. 3-(2-(Acetylamino)propanoyl)-1-(4-cyanophenyl)-3-methyltriazene (**6a**)

Yield 34%; m.p. 203–204 °C;  $\nu_{\text{max}}/\text{cm}^{-1}$  3326, 3238, 2224, 1719, 1645;  $\delta_{\text{H}}$  1.47 (3H, d,  $J = 7.6$  Hz, MeCH), 2.07 (3H, s, Ac), 3.49 (3H, s, NMe), 5.64 (1H, m, CH), 6.37 (1H, br d,  $J = 7.5$  Hz, NH), 7.67–7.71 and 7.72–7.77 (4H, AA'XX',  $J_{\text{ortho}} = 8.7$  Hz, Ar);  $m/z$  (EIMS) 274 (MH<sup>+</sup>, 20%), 130 (7), 102 (20) and 114 (23). Found: C, 57.1; H, 5.6; N, 25.6; C<sub>13</sub>H<sub>15</sub>N<sub>5</sub>O<sub>2</sub> requires C, 57.13; H, 5.53; N, 25.63%.

#### 4.1.2. 3-(2-(Acetylamino)acetyl)-1-(4-cyanophenyl)-3-methyltriazene (**6b**)

Yield 56%; m.p. 172–173 °C;  $\nu_{\text{max}}/\text{cm}^{-1}$  3321, 3279, 2232, 1716, 1650;  $\delta_{\text{H}}$  2.13 (3H, s, Ac), 3.51 (3H, s, NMe),

4.68 (2H, d,  $J = 5.0$  Hz, COCH<sub>2</sub>N), 6.37 (1H, br s, NH), 7.66–7.71 and 7.72–7.76 (4H, AA'XX',  $J_{\text{ortho}} = 9.0$  Hz, Ar);  $m/z$  (EIMS) 259 (M<sup>+</sup>, 8%), 130 (70), 102 (100) and 100 (23). Found: C, 55.1; H, 5.3; N, 26.9; C<sub>12</sub>H<sub>13</sub>N<sub>5</sub>O requires C, 55.59; H, 5.05; N, 27.01%.

#### 4.1.3. 3-(2-(Acetylamino)-3-phenylpropanoyl)-1-(4-cyanophenyl)-3-methyltriazene (**6c**)

Yield 58%; m.p. 178–179 °C;  $\nu_{\text{max}}/\text{cm}^{-1}$  3281, 2221, 1715, 1641;  $\delta_{\text{H}}$  2.04 (3H, s, Ac), 3.12 (2H, m, CH<sub>2</sub>Ph), 3.40 (3H, s, NMe), 5.97 (1H, m, CH), 6.31 (1H, br d,  $J = 8.4$  Hz, NH), 7.00–7.15 (5H, m, Ph), 7.56–7.61 and 7.70–7.75 (4H, AA'XX',  $J_{\text{ortho}} = 8.7$  Hz, Ar);  $m/z$  (EIMS) 349 (M<sup>+</sup>, 6%), 148 (11), 102 (41) and 190 (50). Found: C, 65.2; H, 5.5; N, 20.4; C<sub>19</sub>H<sub>19</sub>N<sub>5</sub>O requires C, 65.32; H, 5.48; N, 20.04%.

#### 4.1.4. 3-(2-(Acetylamino)-3-methylbutanoyl)-1-(4-cyanophenyl)-3-methyltriazene (**6d**)

Yield 35%; m.p. 185 °C;  $\nu_{\text{max}}/\text{cm}^{-1}$  3371, 2222, 1700, 1670;  $\delta_{\text{H}}$  0.92 and 1.01 (each 3H, 2 × d,  $J = 6.8$  Hz, 2 × Me), 2.09 (3H, s, Ac), 2.16 (1H, m, Me<sub>2</sub>CH), 3.49 (3H, s, NMe), 5.71 (1H, m, COCHN), 6.19 (1H, br d,  $J = 9.2$  Hz,

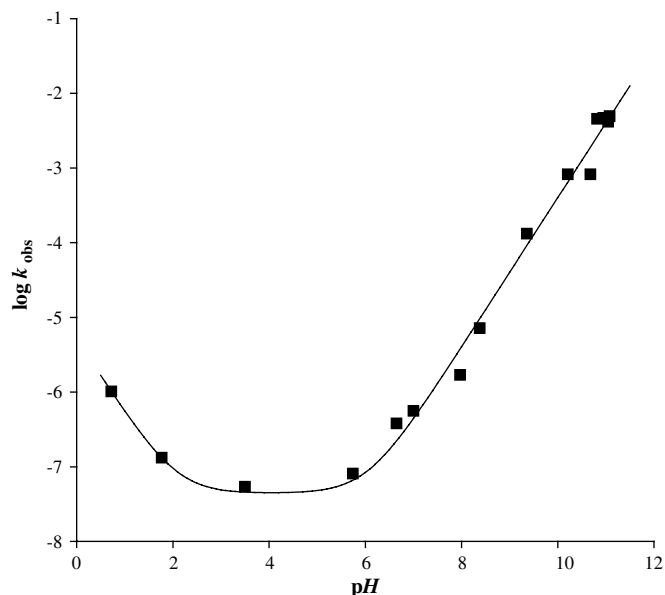


Fig. 3. pH-rate profile for compound **6a**. The points are experimental, the curve is the best fit using  $k_{\text{OH}} 5.7 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{\text{H}_2\text{O}} 4.4 \times 10^{-8} \text{ s}^{-1}$  and  $k_{\text{H}^+} 5.17 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ .

NH), 7.72–7.74 and 7.77–7.79 (4H, AA'XX',  $J_{\text{ortho}} = 6.0 \text{ Hz}$ , Ar);  $m/z$  (EIMS) 142 (39), 130 (17), 114 (59), 102 (21), 72 (100) and 43 (50). Found: C, 59.5; H, 6.4; N, 23.3;  $\text{C}_{15}\text{H}_{19}\text{N}_5\text{O}_2$  requires C, 59.79; H, 6.36; N, 23.24%.

#### 4.1.5. 1-(4-Cyanophenyl)-3-methyl-3-(2-(propanoylamino)propanoyl)triazene (**6e**)

Yield 51%; m.p. 189 °C;  $\nu_{\text{max}}/\text{cm}^{-1}$  3324, 3241, 2223, 1721, 1647;  $\delta_{\text{H}}$  1.16 (3H, t,  $J = 7.6 \text{ Hz}$ , Me), 1.43 (3H, d,  $J = 7.0 \text{ Hz}$ , Me), 2.27 (2H, q,  $J = 7.7 \text{ Hz}$ ,  $\text{CH}_2$ ), 3.46 (3H, s,

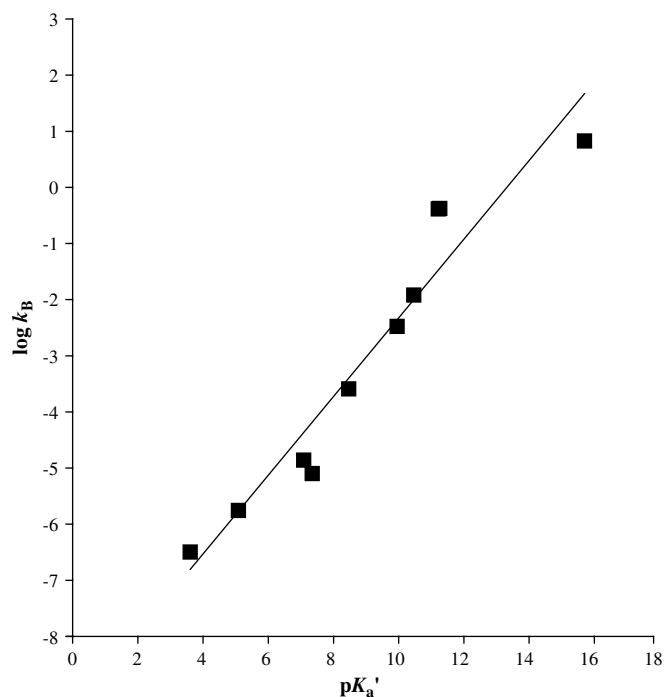


Fig. 4. Brønsted plot for compound **6a**.

Table 3

Solvent isotope effects for the hydrolysis of **6a** in pyridine and piperidine buffers

Buffer	pH/pD	$k_{\text{B}}/10^{-6} \text{ s}^{-1}$	$k_{\text{B}}^{\text{H}}/k_{\text{B}}^{\text{D}}$
Pyridine			
H <sub>2</sub> O	5.74	1.75	2.03
D <sub>2</sub> O	6.31	0.86	
Piperidine			
H <sub>2</sub> O	10.84	419 785	2.08
D <sub>2</sub> O	12.02	202 406	

NMe), 5.64 (1H, quin,  $J = 7.0 \text{ Hz}$ , CH), 6.30 (1H, br d,  $J = 7.0 \text{ Hz}$ , NH), 7.67–7.71 and 7.72–7.76 (4H, AA'XX',  $J_{\text{ortho}} = 9.0 \text{ Hz}$ , Ar);  $m/z$  (LCMS) 288 ( $\text{MH}^+$ , 5%), 130 (100), 102 (40) and 128 (7). Found: C, 58.5; H, 6.0; N, 23.9;  $\text{C}_{14}\text{H}_{17}\text{N}_5\text{O}_2$  requires C, 58.52; H, 5.96; N, 24.37%.

#### 4.1.6. 3-(2-(Butanoylamino)propanoyl)-1-(4-cyanophenyl)-3-methyltriazene (**6f**)

Yield 71%; m.p. 160–161 °C;  $\nu_{\text{max}}/\text{cm}^{-1}$  3326, 3242, 2223, 1722, 1644;  $\delta_{\text{H}}$  0.95 (3H, t,  $J = 7.0 \text{ Hz}$ ,  $\text{MeCH}_2$ ), 1.44 (3H, d,  $J = 7.0 \text{ Hz}$ , MeCH), 1.67 (2H, m,  $\text{CH}_2\text{Me}$ ), 2.22 (2H, t,  $J = 7.0 \text{ Hz}$ ,  $\text{COCH}_2$ ), 3.46 (3H, s, NMe), 5.64 (1H, quin,  $J = 7.0 \text{ Hz}$ , CH), 6.30 (1H, br d,  $J = 7.0 \text{ Hz}$ , NH), 7.66–7.71 and 7.72–7.76 (4H, AA'XX',  $J_{\text{ortho}} = 8.8 \text{ Hz}$ , Ar);  $m/z$  (LCMS) 302 ( $\text{MH}^+$ , 5%), 130 (38) and 142 (15). Found: C, 59.7; H, 6.4; N, 23.0;  $\text{C}_{15}\text{H}_{19}\text{N}_5\text{O}_2$  requires C, 59.79; H, 6.36; N, 23.24%.

#### 4.1.7. 3-(2-(Benzoylamino)propanoyl)-1-(4-cyanophenyl)-3-methyltriazene (**6g**)

Yield 21%; m.p. 196–197 °C;  $\nu_{\text{max}}/\text{cm}^{-1}$  3340, 2226, 1717, 1640;  $\delta_{\text{H}}$  1.59 (3H, d,  $J = 7.0 \text{ Hz}$ , MeCH), 3.53 (3H, s, NMe), 5.86 (1H, m, CH), 7.09 (1H, br d,  $J = 8.0 \text{ Hz}$ , NH), 7.46–7.57 and 7.84–7.86 (5H, m, Ph), 7.75–7.80 (4H, AA'XX',  $J_{\text{ortho}} = 6.6 \text{ Hz}$ , Ar);  $m/z$  (FAB<sup>−</sup> MS) 334 ( $\text{M}-\text{H}^+$ ), (EIMS) 130 (12%), 102 (38) and 176 (12). Found: C, 64.5; H, 5.1; N, 20.8;  $\text{C}_{18}\text{H}_{17}\text{N}_5\text{O}_2$  requires C, 64.47; H, 5.11; N, 20.88%.

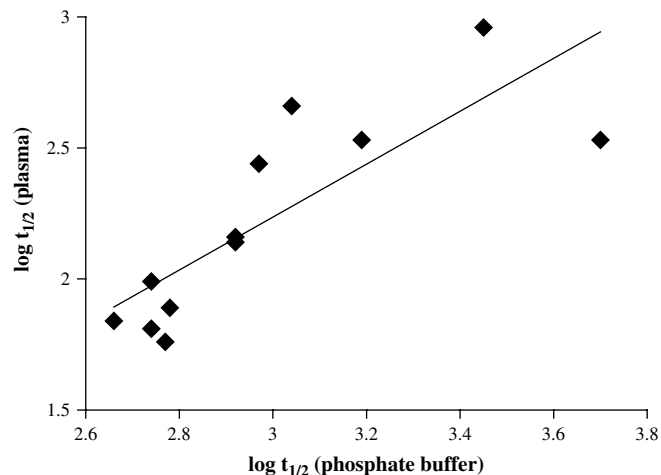


Fig. 5. Correlation of the plasma half-lives with the phosphate buffer half-lives for compounds **6a–l**.



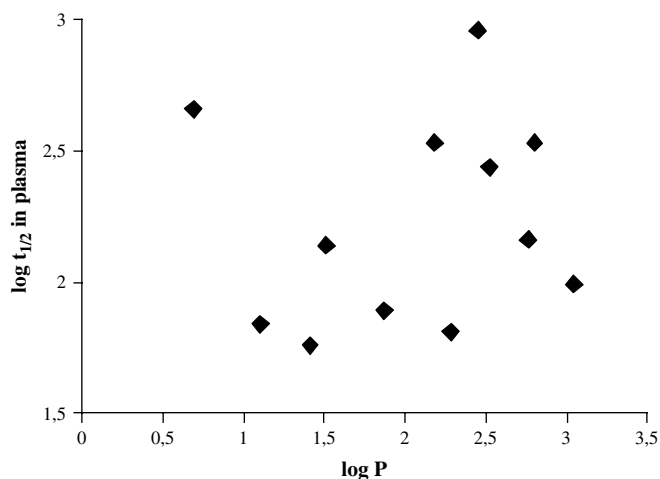


Fig. 6. Plot of the plasma half-lives versus lipophilicity for compounds **6a–l**.

#### 4.1.8. 3-(2-(Acetylamino)propanoyl)-1-(4-acetylphenyl)-3-methyltriazene (**6h**)

Yield 73%; m.p. 175–174 °C;  $\nu_{\max}/\text{cm}^{-1}$  3344, 1692, 1671, 1628;  $\delta_{\text{H}}$  1.45 (3H, d,  $J = 7.0$  Hz, MeCH), 2.04 (3H, s, Ac), 2.62 (3H, s, PhAc), 3.46 (3H, s, NMe), 5.62 (1H, quin.,  $J = 7.1$  Hz, CH), 6.39 (1H, br d,  $J = 7.5$  Hz, NH), 7.61–7.70 and 7.97–8.01 (4H, AA'XX',  $J_{\text{ortho}} = 8.8$  Hz, Ar);  $m/z$  (EIMS) 291 ( $\text{MH}^+$ , 49%), 147 (20) and 119 (50). Found: C, 57.9; H, 6.4; N, 18.8;  $\text{C}_{14}\text{H}_{18}\text{N}_4\text{O}_3$  requires C, 57.92; H, 6.25; N, 19.30%.

#### 4.1.9. 3-(2-(Acetylamino)propanoyl)-1-(4-ethoxycarbonylphenyl)-3-methyltriazene (**6i**)

Yield 48%; m.p. 142 °C;  $\nu_{\max}/\text{cm}^{-1}$  3530, 1717, 1648;  $\delta_{\text{H}}$  1.40 (3H, t,  $J = 7.0$  Hz, MeCH<sub>2</sub>), 1.45 (3H, d,  $J = 7.0$  Hz, MeCH), 2.04 (3H, s, Ac), 3.46 (3H, s, NMe), 4.38 (2H, q,  $J = 7.0$  Hz, CH<sub>2</sub>O), 5.62 (1H, quin.,  $J = 7.1$  Hz, CH), 6.39 (1H, br d,  $J = 7.5$  Hz, NH), 7.63–7.68 and 8.09–8.14 (4H, AA'XX',  $J_{\text{ortho}} = 8.8$  Hz, Ar);  $m/z$  (EIMS) 321 ( $\text{MH}^+$ , 10%), 177 (100) and 149 (50). Found: C, 56.2; H, 6.4; N, 17.2;  $\text{C}_{15}\text{H}_{20}\text{N}_4\text{O}_4$  requires C, 56.24; H, 6.29; N, 17.49%.

#### 4.1.10. 3-(2-(Acetylamino)propanoyl)-3-methyl-1-(4-amino carbonylphenyl) triazene (**6j**)

Yield 35%; m.p. 209–210 °C;  $\nu_{\max}/\text{cm}^{-1}$  3419, 3351, 3303, 1694, 1664, 1619;  $\delta_{\text{H}}$  1.34 (3H, d,  $J = 6.9$  Hz, MeCH),

1.93 (3H, s, Ac), 3.35 (3H, s, NMe), 5.50 (1H, quin.,  $J = 7.7$  Hz, CH), 6.85 (1H, br d,  $J = 8.0$  Hz, NH), 7.54–7.59 and 7.83–7.88 (4H, AA'XX',  $J_{\text{ortho}} = 9.0$  Hz, Ar);  $m/z$  (EIMS) 292 ( $\text{MH}^+$ , 23%), 148 (100), 120 (25) and 114 (3). Found: C, 53.0; H, 5.9; N, 23.7;  $\text{C}_{13}\text{H}_{17}\text{N}_5\text{O}_3$  requires C, 53.60; H, 5.88; N, 24.04%.

#### 4.1.11. 3-(2-(Acetylamino)propanoyl)-1-(4-bromophenyl)-3-methyltriazene (**6k**)

Yield 30%; m.p. 192–194 °C;  $\nu_{\max}/\text{cm}^{-1}$  3322, 1667, 1626;  $\delta_{\text{H}}$  1.43 (3H, d,  $J = 7.0$  Hz, MeCH), 2.03 (3H, s, Ac), 3.42 (3H, s, NMe), 5.59 (1H, quin.,  $J = 7.0$  Hz, CH), 6.41 (1H, br d,  $J = 7.7$  Hz, NH), 7.47–7.53 and 7.54–7.59 (4H, AA'BB',  $J_{\text{ortho}} = 9.0$  Hz, Ar);  $m/z$  (LCMS) 328/326 ( $\text{MH}^+$ , 17%), 185/183 (30), 157/155 (20) and 114 (8). Found: C, 44.2; H, 4.7; N, 17.0;  $\text{C}_{12}\text{H}_{15}\text{N}_4\text{O}_2\text{Br}$  requires C, 44.05; H, 4.62; N, 17.12%.

#### 4.1.12. 3-(2-(Acetylamino)propanoyl)-1-(4-tolyl)-3-methyltriazene (**6l**)

Yield 31%; m.p. 136–137 °C;  $\nu_{\max}/\text{cm}^{-1}$  3345, 1687, 1673;  $\delta_{\text{H}}$  1.44 (3H, d,  $J = 7.0$  Hz, MeCH), 2.03 (3H, s, Ac), 2.38 (3H, s, MePh), 3.42 (3H, s, NMe), 5.60 (1H, quin.,  $J = 7.0$  Hz, CH), 6.47 (1H, br d,  $J = 7.1$  Hz, NH), 7.18–7.29 and 7.52–7.54 (4H, AA'XX',  $J_{\text{ortho}} = 8.4$  Hz, Ar);  $m/z$  (EIMS) 262 ( $\text{M}^+$ , 6%), 119 (67) and 91 (100). Found: C, 59.9; H, 7.0; N, 20.9;  $\text{C}_{13}\text{H}_{18}\text{N}_4\text{O}_2$  requires C, 59.53; H, 6.92; N, 21.36%.

### 4.2. Experimental procedure for hydrolysis studies

Plasma was derived from seven different healthy individuals, pooled, and kept at  $-18$  °C until required. The 3-(acylamino)acyltriazenes were incubated at 37 °C in 80% human plasma diluted with 0.066 M pH 7.4 isotonic phosphate buffer. At appropriate intervals samples of the plasma reactions were withdrawn, diluted with acetonitrile (0.4 ml) and centrifuged at 15 000 rpm for 6 min. The clear supernatant was analysed by HPLC. The 3-(acylamino)acyltriazenes were also incubated at 37 °C in 0.066 M pH 7.7 isotonic phosphate buffer. Samples of these reaction mixtures were analysed directly by HPLC using a mobile phase of acetonitrile:water (20:80 to 50:50) and a detector wavelength of 290 nm. Values of  $t_{1/2}$  were calculated from the decrease in substrate concentration using

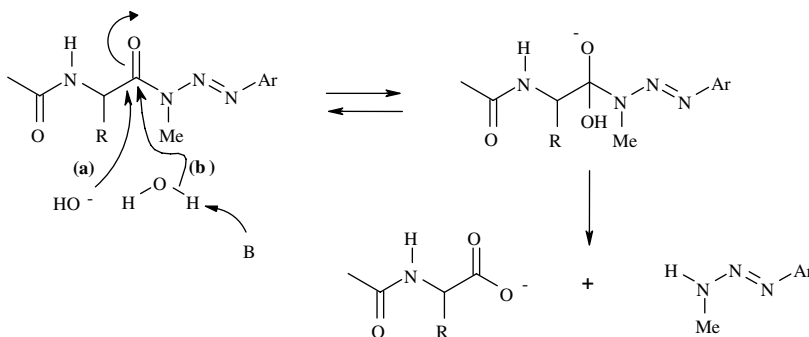


Fig. 7. Pathways of the hydroxide (a) and general base (b) catalysed hydrolyses of triazene prodrugs **6a–l**.

Enzfitter version C, 1987 (Elsevier-Biosoft). For **6g** samples of the reaction mixtures were also analysed by HPLC using pH 3.5 phosphate buffer containing 16% acetonitrile as eluant and a detector wavelength of 250 nm, this allowed the quantification of *N*-benzoylalanine.

#### 4.3. Experimental procedure for partition coefficients

For compounds **6a–l**, partition coefficients were determined in octanol-pH 7.4 phosphate buffer at 25 °C. Each phase was mutually saturated before the experiment. The compounds were dissolved in octanol and the octanol-pH 7.4 mixtures were shaken for 30 min to reach an equilibrium distribution; each phase was analysed separately by the HPLC method described above. The partition coefficients,  $P_{\text{exp}}$ , were obtained from the ratio of the peak area in octanol to the peak area in buffer. Calculated values of  $P$ , expressed as  $\log P_{\text{calc}}$ , were obtained using the software ChemsSketch version 3.60 from Advanced Chemistry Development Inc. (Advanced Chemistry Development Inc., 90 Adelaide St. West, Suite 702, Toronto, Ontario ONM5H 2L3, Canada). Calculated  $\log P$  values were typically ca. 0.5 ( $\pm 0.2$ ) units too large and therefore overestimated lipophilicity.

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