Triazene Drug Metabolites: Part 15. Synthesis and Plasma Hydrolysis of Anticancer Triazenes Containing Amino Acid Carriers

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Purpose. The synthesis of chemically stable triazene prodrugs capable of hydrolysing under physiological conditions to liberate cytotoxic monomethyltriazene alkylating agents.

Methods. A series of 3-aminoacyl-1-aryl-3-methyltriazenes was synthesised through reaction of 1-aryl-3-methyltriazenes with N-BOC protected amino acids using the DCC method of activation, followed by deprotection of the amino function using HCl in nitromethane. Half-lives for the hydrolysis of these compounds to the corresponding monomethyltriazenes at 37°C in isotonic phosphate buffer and in 80% human plasma containing 20% phosphate buffer were determined by HPLC.

Results. The aminoacyltriazene prodrugs hydrolyse in isotonic phosphate buffer with $t_{1/2}$ values ranging from 26 to 619 minutes. In human plasma, several decompose at the same rate as in phosphate buffer whereas those containing more lipophilic groups decompose more slowly. A β-alanyl derivative was found to be more stable in phosphate buffer ($t_{1/2}$ = 180 minutes) than in plasma ($t_{1/2}$ = 53 minutes). An N-acetylated α-alanyl derivative was found to be chemically stable in phosphate buffer ($t_{1/2}$ = 10 hours) but liberated the cytotoxic drug in $t_{1/2}$ = 41 minutes in plasma, demonstrating its ability to act as a substrate for plasma enzymes.

Conclusions. Aminoacyltriazenes are prodrugs of the antitumour monomethyltriazenes hydrolysing in human plasma with a range of reactivities. The acylation of the α-amino group seems to be an effective and simple means of reducing the chemical reactivity of the α-aminoacyl derivatives while retaining a rapid rate of enzymatic hydrolysis.

KEY WORDS: prodrugs; triazenes; antitumour drugs; amino acid derivatives; hydrolysis kinetics.

INTRODUCTION

1-Aryl-3,3-dimethyltriazenes, (e.g. **1** in Figure 1), have been the subject of study by chemists and biologists for many years. Interest in these compounds is due to their antitumour activity and one, dacarbazine (Figure 2), has proven to be of therapeutic value against several tumours and especially malignant melanoma (1).

The basis for the biological action of the 1-aryl-3,3-dimethyltriazenes **1** lies in their capacity to alkylate DNA. They suffer metabolic oxidation by cytochrome P450 enzymes to give hydroxymethyltriazenes **2**, which, by loss of formaldehyde, generate the cytotoxic monomethyltriazenes **3**. These are known alkylating agents, capable of methylating DNA and RNA (Figure 1) (2,3). Dacarbazine is known to be poorly metabolised by humans; no more than 20% of the drug is metabolised to its active monomethyltriazene metabolite (4). Therefore, an active area of triazene research has been the search for prodrugs capable of liberating the active metabolites **2** and **3** without the need for metabolic oxidation. Many different types of prodrug of the hydroxymethyltriazene have been synthesised, such as esters (5), ethers (6), and thioethers (7). None of these possess ideal prodrug characteristics. More recently, an imidazotetrazine—temozolomide—has been found to be active against several tumours in phase I clinical studies. Its activity is thought to be due to the generation of an alkylating monomethyltriazene by reaction with water (8).

The aminoacyltriazenes (Figure 3) provide an alternative strategy as potential prodrugs of the cytotoxic methylating agents **3.** We have previously synthesised and studied the deacylation of the R = alkyl and R = aryl analogues of **4** in basic ethanolic media (9) and in sulfuric acid solutions (10) and showed that they decompose to give **3**, an observation consistent with the possibility that they indeed may be prodrugs of **3**. However, these compounds are poorly soluble in water and have high stability at pH = 7.4. We report herein both the synthesis of a more suitable series of triazene prodrugs **4a-j**, derived from α-amino acids, that circumvent the problems of solubility and stability, and also their stability in isotonic pH 7.4 phosphate buffer and plasma.

MATERIALS AND METHODS

Apparatus

Melting points were determined in Kofler camera Bock-Monoscop "M" and are uncorrected. IR spectra were recorded using a Perkin Elmer 1310 spectrophotometer. The ^{1}H-NMR spectra were recorded using either a Jeol PMX-60 or a Jeol FX-90 Q spectrometer. Mass spectra were recorded in a VG Mass Lab 25-250 spectrometer. High-performance liquid chromatography (HPLC) was performed using a Spectra-Physics system consisting of an isochrom L. C: pump, a Lichrospher 100 RP-8 (5 μm) column, a UV spectrachrom 100 detector, and an SP4270 integrator and a Merck-Hitachi AS-2000 autosampler. Elemental analysis was obtained from Medac Ltd., Brunel University, Uxbridge, U.K.

Chemicals

The N-BOC-L-aminoacids and the racemic N-acetylalanine were purchased from Sigma. All chemicals were reagent grade except those for kinetic studies and HPLC which were analytical or LiChrosolv (Merck) grade.

Warning. 3-Methylltriazenes are powerful carcinogens and 3-aminoacyl-1-aryl-3-methyltriazenes should be treated as such. Direct contact should be avoided.

1-Aryl-3-methyltriazenes were synthesised by previously described methods (11).

General Procedure for the Synthesis of N-BOCaminoacyltriazenes. DCC (1.7 mmol) was added to a solution of the N-BOC amino acid (1.3 mmol) in CH_2Cl_2 (5 ml). The mixture

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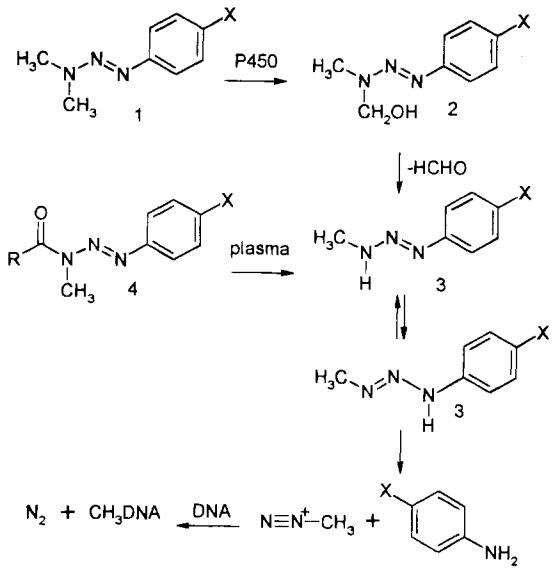
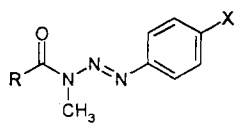


Fig. 1. Pathways for the metabolism of both 1-aryl-3,3-dimethyltriazenes and aminoacyltriazenes.



	R	X
4a	$CH(CH_3)NH_2$	-CN
4b	$CH(CH_3)NH_2$	$-COCH_3$
4c	$CH(CH_3)NH_2$	-COOEt
4d	$CH(CH_3)NH_2$	$-CONH_2$
4e	$CH(CH_3)NH_2$	-Br
4f	$CH(CH_3)NH_2$	$-CH_3$
4g	$CH(CH_2Ph)NH_2$	-CN
4h	CH_2NH_2	-CN
4i	$CH_2CH_2NH_2$	-CN
4j	$CH(CH_3)NHAc$	-CN

Fig. 3. Structures of the aminoacyltriazenes synthesised in the present study.

was stirred at room temperature for 30 minutes and then 4-dimethylaminopyridine (0.13 mmol) and the appropriate 1-aryl-3-methyltriazene (1.3 mmol) dissolved in CH_2Cl_2 (5 ml) were added. After 48 h at room temperature, N,N-dicyclohexylurea was removed by filtration and the solvent removed by evaporation under reduced pressure. The resultant mixture was then subjected to chromatography (silica gel 60 230-400 mesh ASTM, Merck) using petroleum ether/diethyl ether (for compounds **N-BOC 4a**, **b**, **c**, **e**, **f**, and **g**), CH_2Cl_2 (for compounds N-BOC **4c**, **h**, and **i**) and ethyl acetate (for compounds **N-BOC 4d** and **j**). The isolated N-BOCaminoacyltriazenes were recrystallised from petroleum ether/diethyl ether or petroleum ether/CH_2Cl_2 mixtures.

General Procedure for the Deprotection of N-BOC Aminoacyltriazenes. Anhydrous gaseous HCl was passed through a solution of N-BOCaminoacyltriazene (0.08 M) in nitromethane. The 3-aminoacyltriazene hydrochlorides precipitated from solution were recrystallised from acetonitrile/methanol affording the pure product.

Hydrolysis Studies

Plasma was derived from different individuals, pooled, and kept at −18°C until required. The 3-aminoacyltriazenes were incubated at 37°C in 80% human plasma diluted with pH 7.4 isotonic phosphate buffer (0.066 M) (the final pH of this mixture was 7.7). At appropriate intervals samples of the plasma reactions were withdrawn, diluted with acetonitrile (0.4 ml) and centrifuged at 13,000 rpm for 5 minutes. The clear supernatant was analysed by HPLC. The 3-aminoacyltriazenes were also incubated at 37°C in isotonic phosphate buffer (0.066 M) of pH 7.7. Samples of these reaction mixtures were analysed directly by HPLC. In both cases, the HPLC conditions were as follows: detector wavelength 290 nm; mobile phase acetonitrile; 0.01 M pH 6.5 phosphate buffer (40:60 to 60:40). All reactions, both in phosphate buffer and in plasma, followed pseudo-first-order kinetics up to at least 5 half-lives.

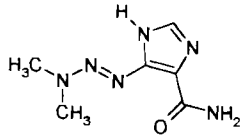


Fig. 2. Structure of dacarbazine.

RESULTS AND DISCUSSION

Synthesis

The N-BOC aminoacyltriazenes, and the N-acetylalanyl derivative **4j**, were synthesised by conventional coupling procedures involving reaction of the corresponding 1-aryl-3-methyltriazenes with DCC-activated N-BOC amino acids in the presence of 4-dimethylaminopyridine. The reaction yields, melting points, and IR and ^{1}H-NMR data for the N-BOC derivatives are contained in Table I. Triazenes are notoriously acid sensitive, and many of the common deprotection methods, e.g., CF_3COOH in CH_2Cl_2 and anhydrous HCl in either CH_2Cl_2 or CH_3COOH, failed due to decomposition of the triazene moiety. However, the use of anhydrous HCl in nitromethane enabled decomposition to be significantly avoided, presumably because in this solvent, deprotection resulted in the formation of insoluble hydrochlorides that precipitate from solution. Table II contains the yields, melting points, and analytical data for the desired aminoacyltriazenes **4** while Table III contains details of their IR and ^{1}H-NMR spectra. In the ^{1}H-NMR spectra, all the compounds, except compound **4c**, display a typical triazene N-methyl singlet ranging from δ 2.75 to 3.51 ppm. For **4c**, this peak appears as two singlets probably due to rotamers arising from restricted rotation about the triazene N-N bond caused by resonance delocalization of the triazene function with the ester carbonyl of the aryl ring substituent.

Table I. Yields, Physical and Spectral Data for the N-BOC Derivatives of Compounds **4a-i**

Compound	% Yield	m.p./°C	ν_{max}/cm^{-1}	δ/ppm
N-BOC - 4a	68	129-30	3400, 2210, 1700	1.44 (12H, s+d, Bu^t + α-Me), 3.47 (3H, s, N-Me), 5.37 (2H, q+br s, α-CH+NH), 7.72 (4H, dd, Ar)
N-BOC- 4b	41	161-2	3368, 1719, 1684	1.44 (12H, s +d, Bu^t + α-Me), 2.64 (3H, s, Ac), 3.47 (3H, s, N-Me), 5.40 (2H, q+br s, α-CH+NH), 7.68, 8.03 (4H, dd, Ar)
N-BOC - 4c	35	140	3360, 1715, 1709	1.42 (15H, m, Bu^t + α-Me), 3.47 (3H, s, N-Me), 4.40 (2H, q, OCH_2), 5.39 (2H, q+br s, α-CH+NH), 7.66, 8.12 (4H, dd, Ar)
N-BOC - 4d	51	93-95	3393, 3331, 3190, 1695, 1686, 1655	1.44 (12H, s+d, Bu^t + α-Me), 3.45 (3H, s, N-Me), 5.40 (2H, m, α-CH+NH), 6.06 (1H, s br, NH), 6.37 (1H, s br, NH), 7.78 (4H, dd, Ar)
N-BOC - 4e	47	189-10	3346, 1690	1,43 (12H, s+d, Bu^t + α-Me), 3.44 (3H, s, N-Me), 5.38 (2H, m, α-CH+NH), 7.53 (4H, dd, Ar)
N-BOC - 4f	45	163-4	3453, 3389, 1716, 1702, 1679	1.45 (12H, s+d, Bu^t + α-Me), 2.39 (3H, s, ArMe), 3.43 (3H, s, N-Me), 5.40 (1H, quin, α-CH), 5.45 (1H, d, NH), 7.39 (4H, m, Ar)
N-BOC - 4g	38	139-41	3340, 2220, 1697, 1684	1.42 (9H, s, Bu^t), 3.06 (2H, d, CH_2), 3.39 (3H, s, N-Me), 5.35 (1H, d, NH) 5.68 (1H, q, α-CH), 7.12 (5H, m, Ph), 7.66 (4H, dd, Ar)
N-BOC - 4h	25	124-5	3416, 2232, 1736, 1708	1.48 (9H, s, Bu^t), 3.48 (3H, s, N-Me), 4.56 (2H, d, α-CH_2), 5.36 (1H, s br, NH), 7.71 (4H, dd, Ar)
N-BOC - 4i	48	127-8	3339, 2229, 1679, 1702	1.43 (9H, s, Bu^t), 3.14 (2H, t, CH_2CO), 3.46 (3H, s, N-Me), 3.55 (2H, q, NCH_2), 5.14 (1H, s br, NH), 7.73 (4H, dd, Ar)

Kinetic Studies

The aminoacyltriazenes hydrolyse to give the corresponding 1-aryl-3-methyltriazenes both in isotonic phosphate buffer and in 80% human plasma containing 20% isotonic phosphate buffer. Under the reaction conditions, the monomethyltriazenes are also unstable and hydrolyse further to the corresponding anilines. These reactions were easily followed by HPLC, by monitoring both the loss of starting material and the formation of products. The calculated pseudo-first-order rate constants, k_{obs}, and half lives are given in Table IV.

Hydrolysis in Phosphate Buffer. Inspection of Table IV reveals that the aminoacyltriazenes decompose at physiological pH with half-lives ranging from 26 to 180 minutes. The rate of the reaction is influenced by the structure of the substituent, −X, present in the triazene aryl group. Electron-attracting substituents increase the rate of hydrolysis, as expected for the hydrolysis of an amide. Compounds containing an amino group, e.g., **4a**, are more reactive than that, **4j**, containing an acetylated amino group. The position of the amino group is also important; the β-amino acid derivative **4i** is less reactive than any of the α-amino acid derivatives **4a–h**. Finally, the structure of the α-amino acid is also important, the alanine derivative being the most reactive. To understand these structure–reactivity relationships, we are currently undertaking a detailed kinetic study of the hydrolysis of these compounds and shall report our findings in a future paper.

Table II. Yields and Physical Data for Compounds **4a-i** (as the Hydrochlorides) and **4j**

	% yield	m.p. (°C)	C% (calc.)	H% (calc.)	N% (calc.)
4a	97	214-6	48.4 (47.74)	5.2 (5.46)	25.3 (25.31)[a]
4b	68	187-8	48.9 (49.02)	5.9 (6.13)	19.0 (19.06)[a]
4c	84	190-2	49.1 (49.61)	6.2 (6.08)	17.6 (17.80)
4d	66	205-7	43.7 (43.50)	5.8 (5.90)	22.6 (23.00)[b]
4e	84	175-6	35.3 (35.37)	4.7 (4.75)	17.1 (16.50)[b]
4f	50	162-4	47.6 (48.01)	7.1 (6.97)	20.8 (20.39)[b]
4g	77	147-8	55.2 (55.20)	5.6 (5.95)	18.7 (18.90)[c]
4h	77	226-8	44.4 (44.21)	5.2 (5.19)	25.5 (25.78)[b]
4i	68	210-2	48.8 (49.35)	5.3 (5.27)	26.1 (26.16)
4j	63	202-3	57.0 (57.13)	5.5 (5.53)	25.6 (25.63)

[a] Hemihydrate.
[b] Monohydrate.
[c] Dihydrate.

Hydrolysis in Human Plasma and in the Presence of Human Serum Albumin. Liberation of the active drug moiety is a *sine qua non* of a prodrug. We were interested, therefore, in examining the hydrolysis of the aminoacyltriazenes **4** in human plasma. Blood serum and plasma are known to contain a range of enzymes that catalyse the hydrolysis of esters and amides (12). Even human serum albumin is known to have esterase-like activity (13). Although aminoacyltriazenes contain the amide funcional group, we have shown elsewhere (9,10) that their reactivity is more akin to esters. Consequently, we would expect them to readily liberate the active monomethyltriazene, especially as, at pH 7.4, the aminoacyl group exists in its protonated form, conferring a resemblance to the substrates of cholinesterases.

Compounds **4a-b** hydrolyse in phosphate buffer and in 80% human plasma essentially at the same rate, from which we infer that they are not substrates for plasma enzymes. Com-

Table III. IR, ^{1}H-NMR and Mass Spectrometry Data for Compounds **4a-i** (as Hydrochlorides) and **4j**

Compound	ν_{max} cm^{-1}	δ^H/ppm[a]	FAB-MS
4a	2220, 1710	1.71 (3H, d, α-Me), 3.50 (3H, s, N-Me), 4.96 (1H, s br, α-CH), 7.75 (4H, dd, Ar), 9.06 (3H, s br, NH_3^+)	232 (MH+), 130 (ArN_2+), 102 (Ar^+)
4b	1702, 1680	1.70 (3H, d, α-Me), 2.65 (3H, s, Ac), 3.51 (3H, s, N-Me), 4.69 (1H, m, α-CH), 7.9 (4H, dd, Ar), 8.98 (3H, s br, NH_3^+)	249 (MH+), 147 (ArN_2+), 44
4c	3439, 3322, 1717, 1707, 1603	1.4 (3H, t, CH_3CH_2), 1.85(3H, d, α-Me), 3.38 and 3.40 (3H, 2 × s, N-Me), 4.39 (2H, q, OCH_2), 5.30 (1H, m, α-CH), 7.88 (4H, dd, Ar), 9.0 (3H, s br, NH_3^+)	279 (MH+), 177 (ArN_2+), 149 (Ar^+), 44, 29
4d	3385, 3203, 1707, 1656	1.60 (3H, d, α-Me), 3.47 (3H, s, N-Me), 4.95 (1H, q, α-CH), 7.86 (4H, dd, Ar), 8.6 (3H, s br, NH_3^+)	250 (MH+), 148 (ArN_2+), 120 (Ar^+), 44
4e	3513,3419, 3347 (weak), 1687	1.67 (3H, d, α-Me), 3.46 (3H, s, N-Me), 4.88 (1H, q, α-CH), 7.58 (4H, dd, Ar), 8.90 (3H, s, NH_3^+)	287/285 (MH+), 213, 185/183 (ArN_2+), 157/155 (Ar^+), 44
4f	3518, 1694	1.84 (3H, d, α-Me), 2.38 (3H, s, ArMe), 3.36 (3H, s, N-Me), 5.22 (1H, q, α-CH), 7.37 (4H, dd, Ar), 8.99 (3H, s, NH_3^+)	221 (MH+), 119 (ArN_2+), 91 (Ar^+)
4g	3375, 2224, 1712	2.65 (2H, dd, CH_2 Ph), 3.26 (3H, s, N-Me), 5.23 (1H, br m, α-CH), 7.06 (5H, m, Ph), 7.6 (4H, dd, Ar), 8.98 (3H, s, NH_3^+)	308 (MH+), 130 (ArN_2+), 102 (Ar^+)
4h	3322, 3229, 2226, 1724	2.87 (3H, s, N-Me), 3.72 (2H, s, α-CH_2), 7.14 (4H, dd, Ar), 8.12 (3H, s, NH_3^+)	218 (MH+), 130 (ArN_2+), 102 (Ar^+), 30
4i	3198, 3122, 2221, 1724	2.53 (2H, t, CH_2CO), 2.69 (2H, t, CH_2N), 2.75 (3H, s, N-Me), 7.17 (4H, dd, Ar), 7.48 (3H, s, NH_3^+)	232 (MH+), 130 (ArN_2+), 102 (Ar^+)
4j	3318, 3234, 2223, 1720, 1647	1.50 (3H, d, α-Me), 2.05 (3H, s, Ac), 3.45 (3H, s, N-Me), 5.60 (1H, quin, α-CH), 6.39 (1H, d, NH), 7.73 (4H, m, Ar)	274[b](M+), 160, 130 (ArN_2+), 114, 102 (Ar^+), 86, 44

[a] DMSO.
[b] EIMS for **4j**.

Table IV. Pseudo-first Order Rate Constants, k_{obs}, and Half-lives, $t_{1/2}$, at 37 °C

	Phosphate buffer[a]		Plasma [b]	
	$k_{obs}/10^{-4}$ s^{-1}	$t_{1/2}$min	$k_{obs}/10^{-4}$ s^{-1}	$t_{1/2}$min
4a	4.50	26	4.78	24
4b	3.69	31	3.61	32
4c	3.68	31	2.49	47
4d	3.12	37	2.72	43
4e	2.38	49	0.86	134
4f	1.10	105	0.28	412
4g	2.09	55	0.98	119
4h	1.44	80	2.17	53
4i	0.64	180	2.21	53
4j	0.19	619	2.82	41

Note: For the hydrolysis of **4** in isotonic phosphate buffer and 80% human plasma containing isotonic phosphate buffer.
[a] Mean value ± 4%. Number of determinations: 2.
[b] Mean value ± 15%. Number of determinations: at least 3.

pounds **4c-g** hydrolyse more slowly in the presence of human plasma. This effect is often observed for lipophilic compounds and may be due to their increased affinity for the plasma proteins. Indeed, serum albumin has been reported to increase the half-lives for hydrolysis of the parent monomethyltriazenes (14). Likewise we have found that the half-lives for the hydrolysis of compound **4f** in phosphate buffer increased with increasing concentrations of serum albumin present in the buffer (Table V). A similar protective role of human serum albumin towards the hydrolysis of the lactone ring in several anticancer camptothecins (15), and of the ester group in aspirin (16), has been

Table V. Effect of Human Serum Albumin on the Pseudo-first-order Rate Constants, k_{obs}, and Half-lives, $t_{1/2}$, for the Hydrolysis of **4f** in pH 7.4 Isotonic Phosphate Buffer at 37°C

[HSA]/10^{-4} g dm^{-3}	$k_{obs}/10^{-4}$ s^{-1}	$t_{1/2}$min
0	1.10	105
11.0	1.00	116
41.4	0.66	176
55.2	0.61	188
276	0.30	380

noted. Human serum albumin (HSA), which has an average M_r of 68 kD, is present in blood at a level of 3000–4000 mg/100 cm^3 (17). This corresponds to a concentration of 0.66 mM, which, from Table V, would give a half-life of approximately half that observed for **4f** in plasma. Clearly, the stability of **4f** in plasma arises from more than binding to HSA.

The hydrolysis of compound **4h**, the glycyl derivative, is slightly faster in plasma than in isotonic phosphate buffer, and the hydrolysis of compound **4i**, the β-alanyl derivative, is three times faster in the presence of plasma. These effects are small in comparison with the effect observed for compound **4j**, the N-acetyl derivative of alanine, which hydrolyses 15 times faster in plasma.

Aminoacyltriazenes as Prodrugs. In contrast to simple acyltriazenes, the α-aminoacyltriazenes are relatively unstable chemical entities that hydrolyse in isotonic phosphate buffer with $t_{1/2}$ values ranging from 26 to 105 minutes. A similar behaviour has been found for short-chain aliphatic esters of α-aminoacids (18). For these esters, this enhanced reactivity has been attributed to the strong electron-withdrawing effect of the protonated amino group which activates the ester linkage towards hydroxide ion attack (18,19). Our results for triazenes, which, as activated amides, are known to behave like esters (10), agree with this proposal. Thus, the rate of hydrolysis falls with increasing distance of the amino function from the carbonyl group, while acylation of the amino group, which precludes protonation at pH 7.7, significantly enhances stability. As a consequence, the most reactive of these compounds are probably too unstable to be developed as prodrugs. In contrast, the most stable, **4e** and **4f**, decompose too slowly in plasma ($t_{1/2}$ = 134 and 412 minutes). The β-alanyl derivative **4i** has better characteristics for a prodrug: chemical stability ($t_{1/2}$ = 180 minutes) with enzymatic lability ($t_{1/2}$ = 53 minutes). In this respect, the most promising derivative is the N-acetylated derivative **4j** which has a half-life of 10 hours in phosphate buffer but hydrolyses rapidly in plasma ($t_{1/2}$ = 41 minutes). Thus, acylation of the amino group seems to be an effective and simple means of reducing the chemical reactivity of the α-amino acid derivatives yet retaining a rapid rate of enzymatic hydrolysis. Studies are under way to explore potential applications of α-(acylamino)acyl derivatives as prodrugs for other amines and alcohols.

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