Investigation of *N*-[(Acyloxy)alkyl] Ester as a Prodrug Model for Drugs Containing the Phenyltetrazole Moiety

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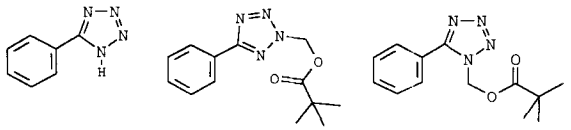
Abstract □ N-Acyloxyalkylation using 5-phenyltetrazole as a model compound was investigated as a general means of prodrug modification of the tetrazole ring with a view to change the physicochemical properties for improving biomembrane transport. Pivaloxymethylation gave a mixture of 1- and 2-[(pivaloxy)methyl]-5-phenyltetrazole isomers in 1:4 ratio. The structures of the compounds were established by NMR spectroscopy using nuclear Overhauser effect (NOE) difference and heteronuclear multiple bond correlation (HMBC) techniques. The second-order rate constants for hydroxide ion catalyzed hydrolysis of the ester functions of both the isomers were nearly the same, though the two isomers differ in the degree of conjugation between the tetrazole and the aromatic rings as a result of the differences in the extent to which the two rings can assume coplanarity. The values of the second-order rate constants were comparable to those reported for other *N*-[(pivaloxy)methyl]-substituted compounds, such as derivatives of 5-fluorouracil, indicating that the unique electronic properties of the tetrazole ring do not have a significant effect on the rate of base-catalyzed hydrolytic regeneration of 5-phenyltetrazole, or the potential stability of such prodrugs. However, the hydrolysis of the more sterically hindered 1-[(pivaloxy)methyl] ester in rat plasma was slower than that of the 2-substituted isomer.

The tetrazole ring is nearly as acidic as many carboxylic acids, probably because of resonance stabilization of its deprotonated form.[1] The apparent acidic dissociation constant of phenyltetrazole[2] ($pK_a = 4.5$), for example, is slightly greater that of benzoic acid ($pK_a = 5.1$). The tetrazole function is also believed to be more stable to metabolism.[3] These features have led to its increasing use as an isostere of carboxylic acid group[4] in the design of peptidomimetic drugs, such as nonpeptidic angiotensin II antagonists,[4-6] many of which carry a biphenyltetrazolyl or phenylheteroaryltetrazolyl[6] side chain. Analogs which carry an additional basic amino function elsewhere in the structure, thereby existing in a zwitterionic form, generally exhibit poor absorption. We were interested in a general prodrug approach to bioreversibly mask the phenyltetrazolyl side chain as a strategy to change the physicochemical properties of the molecule to improve the biomembrane transport.

Prodrug modification involving acylation[7] or acyloxyalkylcarbamylation[8-11] of the tetrazole ring was deemed nonviable in light of the facile rearrangement of acyltetrazoles to 1,3,4-oxadiazoles (the Huisgen reaction).[12-15] We investigated soft alkylation[16] as an approach to bioreversible derivatization of the tetrazole ring using commercially available 5-phenyltetrazole (1)as a model compound. Since the tetrazole group can exist in



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two tautomeric forms,[17] alkylation results in two isomeric monoalkylated phenyltetrazole derivatives. In this paper we describe the preparation, structure assignment, and a brief investigation on the base-catalyzed hydrolysis of the two isomeric *N*-(pivaloxy)methyl esters of 5-phenyltetrazole with the limited objective of finding out if the unique electronic properties of the tetrazole ring system might have any anomalous effects on the hydrolytic behavior of the *N*-[(acyloxy)alkyl] group.

Experimental Section

Ultraviolet spectral measurements were performed on a Shimadzu UV-2101PC spectrophotometer, equipped with a temperature-controlled sample compartment, at 23 °C using quartz cells of 1-cm path length. ^1H and ^{13}C NMR spectra were run on Bruker AC200 or AM500 instruments using tetramethylsilane as internal reference for protons and $CDCl_3$ as internal reference for carbons. IR spectra were taken on a Mattson Galaxy 5000 FTIR spectrometer. The pH measurements were made at room temperature on a Fisher Accumet Model 815MP pH meter using gel-filled Ag/AgCl combination electrodes. Melting points were determined on an Electrothermal 9200 capillary melting point apparatus and are reported uncorrected. High-performance liquid chromatographic measurements (HPLC) were performed on a system consisting of a SSI 300 pump equipped with a SSI 210 guardian and pulse damper, an ABI Analytical Spectraflow 757 variable-wavelength UV detector and a Rheodyne 7121 manual injector valve with a 20-μL sample loop. The column used for the buffer hydrolysis experiment was a 10 cm × 4.6 mm Brownlee Spheri-5 RP-8 cartridge with a 3 cm × 4.6 mm precolumn of the same packing material. For rat plasma hydrolysis experiments, a 10 cm × 4.6 mm Brownlee Spheri-5 RP-18 cartridge coupled with a 3 cm × 4.6 mm RP-18 precolumn was used. Microanalyses were performed by Oneida Research Services, Whitesboro, NY.

5-Phenyltetrazole was purchased from TCI America and was used as received. All other chemicals and solvents used were analytical or HPLC grade. Standard 1 N KOH solution was obtained from American Scientific Products. Carbonate (pH 10) and phosphate (pH 11) buffers were prepared by standard procedures.[18]

Preparation of 1- and 2-[(Pivaloxy)methyl]-5-phenyltetrazole Isomers—5-Phenyltetrazole (1) (1.46 g, 10 mmol) was dissolved in 25 mL of dimethylformamide and stirred with 1.83 g (12 mmol) of chloromethyl pivalate, 1.80 g (12 mmol) of sodium iodide, and 1.2 g (12 mmol) of potassium acetate at room temperature for 64 h. The reaction mixture was diluted with 50 mL of water and extracted with ethyl acetate. The ethyl acetate extract was washed with 1% sodium sulfite solution, water, and saturated sodium chloride solution. The organic extract was dried over sodium sulfate and evaporated to obtain 2.6 g of a mixture of two isomers. The two isomers were separated by preparative centrifugally accelerated radial thin-layer chromatography on a Chromatotron Model 7924T (Harrison Research, Palo Alto, CA) in three batches on 2-mm plates using hexane:ethyl acetate (7:3) as the mobile phase.

The isomer with the higher R_f value that eluted first weighed 1.68 g. It was crystallized from hexane to furnish 0.62 g of the analytically pure compound which was identified as 2-[(pivaloxy)methyl]-5-phenyltetrazole (2) (see Discussion), with the following physical properties: (plates) mp 64 °C; ^1H NMR ($CDCl_3$) δ 1.23 (s, 9H, t-Bu), 6.52 (s, 2H, CH_2), 7.48-7.51 (m, 3H, *m,p*-H), 8.16-8.21 (m, 2H, *o*-H); ^{13}C NMR ($CDCl_3$) δ 26.75, 38.81, 71.55, 126.74, 126.99, 128.88, 130.65, 165.74 (tetrazole C), 176.43; UV (MeOH) λ (ε, $M^{-1}cm^{-1}$) 202 (17 000), 237 (17 200), 275 (1000), 282 (650) nm; IR (KBr) ν 2975, 1740, 1531, 1451, 1390, 1144, 919, 907, 825 cm^{-1}. Anal. Calcd for $C_{13}H_{16}N_4O_2$: C, 59.98; H, 6.19; N, 21.52. Found: C, 59.90; H, 6.15, N, 21.50.

The second fraction (lower R_f) eluted from the Chromatotron weighed 0.46 g, which on crystallization from hexane gave 0.13 g of the pure

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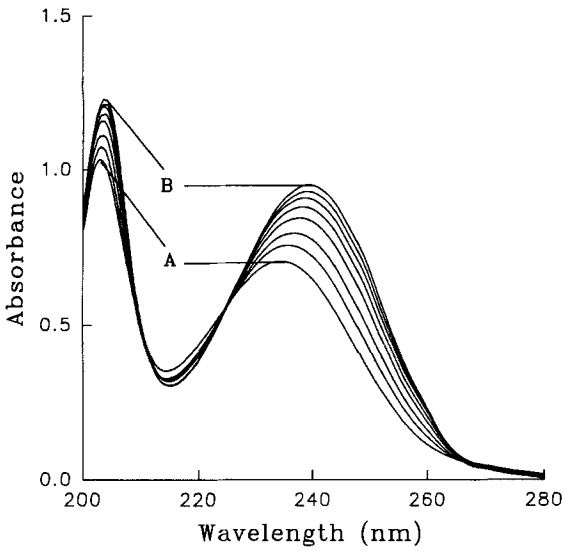


Figure 1—UV spectral changes which occurred on hydrolysis of a 7 × 10^{-5} M solution of **3** in pH 10 buffer containing 10% methanol: (A) beginning, (B) end of hydrolysis.

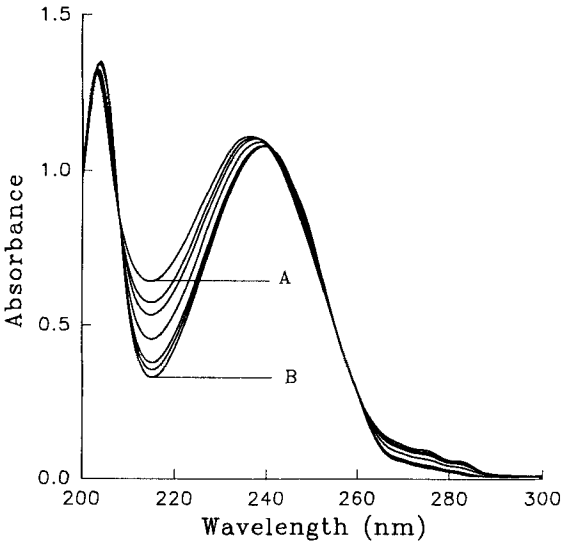


Figure 2—UV spectral changes which occurred on hydrolysis of a 7 × 10^{-5} M solution of **2** in pH 10 buffer containing 10% methanol: (A) beginning, (B) end of hydrolysis.

1-[(pivaloxy)methyl]-5-phenyltetrazole (**3**) (needles): mp 79 °C; ^{1}H NMR ($CDCl_3$) δ 1.21 (s, 9H, t-Bu), 6.32 (s, 2H, CH_2), 7.58–7.62 (m, 3H, *m,p*-H), 7.78–7.82 (m, 2H, *o*-H); ^{13}C NMR ($CDCl_3$) δ 26.74, 38.81, 68.25, 122.92, 128.71, 129.37, 131.86, 155.26 (tetrazole-C), 176.28; UV (MeOH) λ (ε, M^{-1} cm^{-1}) 202 (17 300), 233 (10 000) nm; IR (KBr) ν 1757, 1480, 1273, 1186, 1113, 1013, 729 cm^{-1}. Anal. Calcd for $C_{13}H_{16}N_4O_2$: C, 59.98; H, 6.19; N, 21.52. Found: C, 60.17; H, 6.08; N, 21.65.

Kinetic Measurements—The base-catalyzed hydrolysis of the *N*-[(pivaloxy)methyl] esters was studied in aqueous buffers containing 10% methanol as a cosolvent at 23 °C. Carbonate and phosphate solutions[18] were used as buffers at pH 10 and 11 at a buffer concentration of 0.05 M. At higher pH, 0.01, 0.02, and 0.05 M potassium hydroxide solutions were used and the ionic strength (μ) was adjusted to 0.1 M using potassium chloride. The hydrolysis reactions were followed in most cases by UV spectroscopy by recording absorbance changes at a wavelength that showed maximal change on hydrolysis. This corresponded to an increase in absorbance at 240 nm for the hydrolysis of 1-[(pivaloxy)methyl]-5-phenyltetrazole (**3**) with an isosbestic point observed at 225 nm (Figure 1). Spectral scans of 2-[(pivaloxy)methyl]-5-phenyltetrazole (**2**) exhibited an isosbestic point at 235 nm and showed a decrease in absorbance at 215 nm on hydrolysis, which was followed for the kinetic measurements (Figure 2). The reactions were initiated by adding 250 μL of a methanolic solution containing approximately 0.05 mg of the (pivaloxy)methyl ester to 2.5 mL of the buffer solution contained in a quartz cuvette to give a concentration of 7 × 10^{-5} M. The observed pseudo-first-order rate constants, k_{obs}, were obtained by fitting absorbance changes through four to five half-lives to standard first-order rate equations, using a personal computer. Sigmaplot version 5 (Jandel Scientific, San Rafael, CA) or Grafit version 2 (Erithacus Software Ltd., Staines, UK) softwares were used to fit the experimental data to the equations $y = Ae^{-kt} + C$ or $y = A(1 - e^{-kt}) + C$.

In order to determine if ester hydrolysis was the sole pathway for degradation of the (pivaloxy)methyl esters, the first-order rate measurements at pH 10 were also carried out by a reversed-phase HPLC method. In order to simultaneously measure the concentration of the starting material **2** and the hydrolysis product, 5-phenyltetrazole, a mobile phase consisting of 50% v/v of acetonitrile in water containing 1 mL/L of triethylamine, 1 mL/L of phosphoric acid (85%), and 1 g/L of tetrabutylammonium hydrogen sulfate, at a flow rate of 2 mL/min, at room temperature was used. The column effluent was monitored at 238 nm. Under these conditions, **1** and **2** had retention times of 0.95 and 3.46 min, respectively. The hydrolysis reaction was initiated by adding 1 mL of a freshly prepared methanolic solution of the ester containing 0.5 mg to 9 mL of pH 10 buffer at room temperature to get an initial concentration of 1.9 × 10^{-4} M. Aliquots of this solution were injected at appropriate time intervals directly into the HPLC for analysis. Pseudo-first-order rate constants were calculated from peak areas by the curve-fit method as described above. The quantitation of the amount of **1** formed by hydrolysis of **2** was achieved by generating a standard curve of the peak areas obtained on serial dilution of a methanolic solution of **1** under the chromatographic conditions described above.

Hydrolysis in Rat Plasma—The progress of hydrolysis of the *N*-[(pivaloxy)methyl] esters was followed by measuring the fraction remaining unreacted at 37 °C in neat rat plasma or in rat plasma that was diluted to 10% v/v concentration in pH 7.4 (0.05 M) phosphate buffer. The reactions were initiated by adding a dimethyl sulfoxide (DMSO) solution of **2** or **3** to the reaction medium pre-equilibrated at 37 °C in a thermostated water bath. The initial concentrations of the (pivaloxy)methyl esters were 3.8 × 10^{-3} M in the experiment using neat plasma and 4.2 × 10^{-4}, 2 × 10^{-4}, and 3.8 × 10^{-5} M in diluted rat plasma experiments. The concentration of DMSO in the neat rat plasma experiment was 10%, and that in the diluted plasma experiments was 1%. Samples (100 μL) were pipetted out at appropriate intervals and added to 200 μL of acetonitrile kept at −30 and −40 °C in a dry ice–acetone bath. After the cessation of sample withdrawals, the vials were thawed, vortex mixed for 30 s to precipitate the plasma proteins, and centrifuged at 10000*g* for 5 min. The supernatants were injected directly and analyzed by HPLC. The mobile phase used for the analysis of **2** was 50% v/v of acetonitrile in water containing 1 mL/L of 85% phosphoric acid and 1 mL/L of triethylamine at a flow rate of 2 mL per min. The retention time of **2** was 4.0 min. For the analysis of **3** the proportion of acetonitrile in the above mobile phase was decreased to 45% v/v and the flow rate was increased to 2.5 mL/min to get a retention time of 3.8 min.

Results and Discussion

Although the tetrazole moiety is used as a substitute for the carboxylic acid group because of the similarity in the acidity of the two functionalities, one property of the tetrazole group not present in the latter is the possibility of existing in two tautomeric forms. As a result, pivaloxymethylation of 5-phenyltetrazole yielded two isomeric (pivaloxy)methyl esters. The structure determination of the isomers was based on comparison of the NMR spectra of the two isomers to the data reported in the literature[19] for the 1- and 2-methyl-substituted 5-phenyltetrazole analogs and by nuclear Overhauser effect (NOE) difference[20] spectroscopy. Fraser and Haque[19] reported a downfield shift of 0.6–0.7 ppm in the *ortho*-protons of the phenyl ring relative to the *meta*- and *para*-protons in 2-methyl-5-phenyltetrazole, which was absent in the 1-methyl analog. This *ortho*-deshielding phenomenon, which arises from an upfield shift of the *meta*- and *para*-protons and a downfield shift of the *ortho*-protons, has been attributed to a combination of ring anisotropy, nitrogen anisotropy, and an electron-donating resonance interaction in

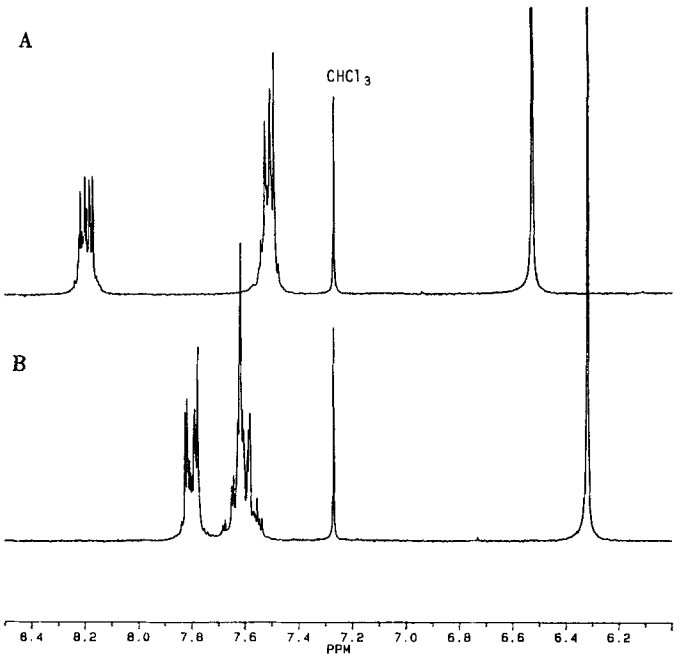


Figure 3—Aromatic region of the 200-MHz ^{1}H NMR spectra of (A) 2-[(pivaloxy)methyl]-5-phenyltetrazole and (B) 1-[(pivaloxy)methyl]-5-phenyltetrazole.

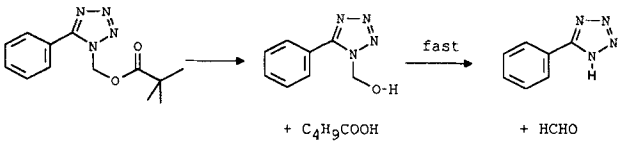
the 2-alkyl-substituted 5-phenyltetrazole, in which the tetrazole and phenyl rings can assume coplanarity. This effect has also been observed[21] in other *N*-(methylaryl)azole isomeric pairs such as 1- and 2-methyl-substituted phenylpyrazoles, -imidazoles, and -triazoles. A similar *ortho*-deshielding effect is seen in the NMR spectrum (Figure 3) of one of the two (pivaloxy)methyl isomers, viz **2**. This observation and the upfield shift of 0.2 ppm exhibited by the methylene protons of the other isomer (**3**), possibly as a result of noncoplanarity of the rings, which would place the methylene group of **3** in the shielding region of the aromatic ring, led to the assignments. The upfield shift of the methylene group in **3** may also be attributed to reduced resonance interaction between the phenyl and tetrazole rings, as result of noncoplanarity. This could also explain[2] the low absorption coefficient in the UV spectrum of **3** at 233 nm compared to the other isomer. Consistent with this assignment, irradiation at the resonance of the methylene protons of **3**, which are spatially closer to the aromatic ring, resulted in a positive NOE of 4.6% for the *ortho*-protons in NOE difference spectroscopy. The *ortho*-protons of the other isomer did not show detectable NOE under similar conditions.

The structure assignment was further confirmed by heteronuclear multiple bond correlation spectroscopy[22,23] (HMBC). This inverse-detected two-dimensional NMR technique allows the suppression of one-bond C–H couplings. Using appropriate ^{1}H and ^{13}C pulse sequences, two- and three-bond C–H coupling are used to generate multiple-bond heteronuclear multiple-quantum coherence, followed by removal of signals from protons that do not have long-range coupling to ^{13}C. On detection following an appropraite delay, this generates a heteronuclear shift correlation spectrum through long-range couplings. In the HMBC spectrum of **3**, a correlation peak showing three-bond ^{13}C–^{1}H coupling between C-5 of the tetrazole ring and the NCH_2 protons was observed, unequivocally establishing the substitution of the (pivaloxy)methyl group to the 1-position of the heterocyclic ring. The isomer **2** did not show such a correlation peak arising from long-range coupling, showing that the methylene protons are more than three bonds removed from the tetrazole carbon.

The kinetics of the hydrolytic breakdown of the (pivaloxy)-methyl esters **2** and **3** were measured in buffered aqueous

Table 1—First-Order Rate Data for the Hydrolysis of 1- and 2-[(Pivaloxy)methyl]-5-phenyltetrazole Isomers in Aqueous Buffer (0.05 M) at 23 °C

pH	Method of Measurement	Half-life (min) 2	3
10	UV spectroscopy	31	41
11	UV spectroscopy	6	7
10	disappearance by HPLC	28	
10	appearance of 1 by HPLC	29	



Scheme 1—Hydrolytic breakdown of (pivaloxy)methyl ester through the short-lived *N*-(hydroxymethyl) intermediate.

solutions (pH 10 and 11) at room temperature by UV spectroscopy. The change in absorbance observed was consistent with pseudo-first-order kinetics for four to five half-lives in every case. The half-lives are shown in Table 1. Since the changes in UV absorption observed during hydrolysis of the (pivaloxy)-methyl esters occur as a result of ionization of the 5-phenyltetrazole formed on hydrolysis in alkaline solution, and there is very little difference between the UV spectra of the esters and 5-phenyltetrazole in acidic solution, UV spectroscopy was most suitable for kinetic measurement in alkaline conditions. The mechanism of hydrolysis of *N*-[(acyloxy)methyl] esters of acidic amines and amides investigated previously in other systems, such as 5-fluorouracil,[24,25] phenytoin,[26] and chloroxazone,[27] are consistent with the two-step reaction as depicted in Scheme 1. Rate-determining hydrolysis of the (pivaloxy)methyl ester group would give rise to a relatively short-lived N-(hydroxymethyl) intermediate which would rapidly break down into formaldehyde and 5-phenyltetrazole, consistent with the behavior of other N-(hydroxymethyl) derivatives reported in the literature.[28] It has been shown,[29] for example, in the hydrolysis of 1-(butyroxymethyl)-5-fluorouracil at pH 7.4, that the rates of formation of formaldehyde and 5-fluorouracil were identical. A similar correspondence was also observed[30] for the rates of breakdown of the 1-butyroxymethyl ester and the formation of formaldehyde.

In order to establish if ester hydrolysis was the sole pathway for the breakdown of the (pivaloxy)methyl esters, the hydrolysis reaction in buffered aqueous solution at pH 10 was monitored by HPLC by following the degradation of **2** and the formation of 5-phenyltetrazole simultaneously. The results are shown in Table 1. The formation of 5-phenyltetrazole from **2** was quantitative. No other products having UV absorption at 238 nm, with retention times between those of the *N*-[(pivaloxy)-methyl] ester and 5-phenyltetrazole, could be detected. The half-lives measured for the disappearance of the ester and formation of 5-phenyltetrazole at pH 10 were consistent with that measured by UV spectroscopy within the limits of experimental error.

Having established the validity of the UV spectrometric method for the determination of the pseudo-first-order rate constants, this procedure was used for the determination of second-order rate constants for hydroxide ion catalyzed hydrolysis of **2** and **3**. The pseudo-first-order rate constants in 0.01, 0.02, and 0.05 M aqueous potassium hydroxide solution, at an ionic strength maintained at 0.1 M with potassium chloride, are shown in Table 2, with the corresponding second-order rate constants obtained from the slopes of plots of the observed first-order rate constants against hydroxide ion concentration. No

Table 2—Rate Constants for the Hydrolysis of 1- and 2-[(pivaloxy)methyl]-5-phenyltetrazole in Aqueous Potassium Hydroxide Solutions at 23 °C and μ = 0.1

Compd	KOH concn (M)	Rate Constant (min^{-1})	
		Pseudo-First-Order	Second-Order
2	0.01	0.9	71
	0.02	1.5	
	0.05	3.7	
3	0.01	0.66	81
	0.02	1.6	
	0.05	4.0	

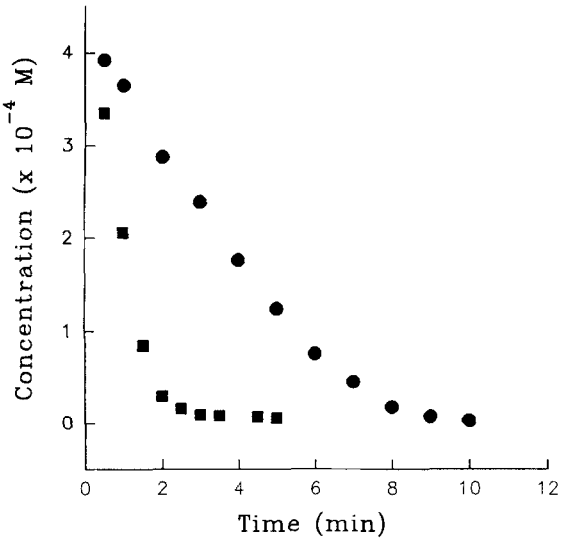


Figure 4—Concentration–time profile for the hydrolysis of (●) 1-[(pivaloxy)-methyl]-5-phenyltetrazole and (■) 2-[(pivaloxy)methyl]-5-phenyltetrazole in rat plasma diluted to 10% v/v in pH 7.4 phosphate buffer (0.05 M) at 37 °C. The initial concentration of the esters was 4.2 × 10^{-4} M.

significant difference was observed between the second-order rate constants for hydrolysis of the two isomers. This is probably because the ester carbonyls undergoing hydroxide ion attack during hydrolysis are insulated by the hydroxymethyl group from the resonance effects in the tetrazole moiety, which are believed to be different in the two isomers[2,19] as a result of differing degrees of conjugation between the tetrazole and aromatic rings. Steric differences arising as a result of phenyl substitution in the α- or β-position with respect to the pivaloxymethyl group also do not seem to have any significant effect on the hydrolysis rates. Bundgaard and co-workers[25] reported the second-order rate constants of 12 and 125 M^{-1} min^{-1} for 1-[(pivaloxy)methyl]-5-fluorouracil and 1,3-bis[(pivaloxy)methyl]-5-fluorouracil, respectively, at 37 °C and an ionic strength of 0.5 M, for hydroxide ion catalyzed hydrolysis of the (pivaloxy)-methyl ester. The second-order rate constants of 71 and 81 M^{-1} min^{-1} (at ionic strength of 0.1 M) obtained for **2** and **3**, respectively, are not substantially different from those reported for the 5-fluorouracil derivatives.

Evaluation of the susceptibility of **2** and **3** to esterases in rat plasma indicate that the isomers differ in their suitability as substrates for the plasma enzymes. The hydrolysis of both compounds deviated from first-order kinetics at 37 °C in neat rat plasma and in rat plasma that was diluted 10-fold in pH 7.4 phosphate buffer. A typical time versus concentration profile for both the isomers is shown in Figure 4. The initial rate of hydrolysis of the 2-[(pivaloxy)methyl] isomer **2** was faster than that of the corresponding 1-substituted isomer **3** in all the experiments. This could be attributed to the increased steric crowding in **3**. It is possible that such isomeric tetrazole prodrugs of more complex drug classes also might exhibit similar differences in their hydrolytic breakdown.

Conclusions

The observed product distribution in the acyloxyalkylation of 5-phenyltetrazole with (pivaloxy)methyl iodide, which showed preponderance of the 2-substituted isomer, is consistent with that reported[19] for alkylation with simple alkyl halides. Differences in the extent of conjugation arising from differing degrees of coplanarity between the phenyl and tetrazole rings in **2** and **3**, evident in the extinction coefficients in the UV spectra and the NMR spectra of the two compounds, do not lead to dissimilar hydrolytic rates for the two isomers under basic conditions. This is probably because the ester function undergoing hydrolysis is separated from the tetrazole ring by a methylene group. As a result, the characteristic electronic properties of the tetrazole ring do not have any apparent atypical effects on the hydrolytic behavior and stability of these *N*-[(pivaloxy)methyl] esters in the alkaline pH range. However, probably due to the increased steric hindrance in the 1-[(pivaloxy)methyl] substituted derivative, ester hydrolysis of this compound in rat plasma is slower than that of the corresponding 2-substituted isomer. The possibility of the formation of two *regioisomeric* prodrugs in unequal amounts and their differential susceptibility to hydrolytic enzymes may have implications in the applicability of this prodrug approach for more complex drugs containing the tetrazole moiety.

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