Acyloxymethyl as a Drug Protecting Group: Part 4. The Hydrolysis of Tertiary Amidomethyl Ester Prodrugs of Carboxylic Acid Agents

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Purpose. Novel tertiary amidomethyl esters were synthesized and evaluated as potential prodrugs of carboxylic acid agents.

Methods. The hydrolyses of the title compounds in buffer solutions and in plasma were studied by UV spectroscopy and HPLC.

Results. Amidomethyl esters were hydrolyzed by acid-catalyzed, base-catalyzed and pH-independent pathways. Both the acid-catalyzed, k_{H^+}, and pH-independent processes, k_o, were strongly affected by the electronic and steric nature of the *N*-substituent in the pro-moiety. For both processes, the electronic effect exerted greater influence, and electron-withdrawing substituents retarded reaction. The pH-independent hydrolysis of amidomethyl esters were dependent on the pK_a of the carboxylate leaving group, giving a Brönsted β_{lg} value of -0.91. The base-catalyzed, k_{OH^-}, pathway was mainly affected by the steric bulk of the nitrogen substituents in the amide moiety, the reactivity being reduced with larger *N*-substituents. Hydrolysis in human plasma appeared to be mediated by enzymic processes and is dependent upon the steric bulk in the carboxylic acid moiety. Plasma hydrolysis rates were inversely dependent on the lipophilicity of the ester.

Conclusions. Derivatives containing the ethyl hippurate carrier are useful prodrugs for carboxylic acid-containing drugs with $pK_a > 3.5$, such as non-steroidal anti-inflammatory agents and valproic acid.

KEY WORDS: amidomethylation; NSAIDs; valproic acid; clofibric acid; probenecid; prodrugs; mechanism of hydrolysis.

INTRODUCTION

Drugs containing free carboxylic acid groups often have their therapeutic effectiveness reduced as a result of unfavourable physicochemical and/or biopharmaceutical properties. A useful approach to overcome this problem is to transform the drug molecule into a prodrug by linking the carboxylic acid to an inactive carrier (1). Indeed, the double prodrug concept has been widely used for improving the physicochemical and biological properties of a large number of drugs (2). In this context, *N*-(alkyl)amidomethyl esters, **1**, are of particular interest because they can be used as prodrugs of both secondary amides (3), including peptides (4), and of carboxylic acids (5,6). Such compounds offer potential advantages over the double esters by allowing both the rate of drug delivery and drug lipophilicity to be modulated through changes to the *N*-alkyl and *N*-acyl groups of the secondary amide pro-moiety.

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Recently, we found that *N*-(alkyl)amidomethyl esters of benzylpenicillin (*eg.* **1**, where R^2 is the benzylpenicillin fragment) undergo spontaneous hydrolysis by an S_N1 type mechanism, without suffering β-lactam ring opening to any detectable extent (7). However, these compounds hydrolyse very rapidly, the sole exception being the penicillate containing the ethyl hippurate carrier (**1**: $R^1 = CH_2CO_2Et$), which is *ca.* 100-fold more stable than its *N*-methyl counterpart (7). Such a difference in reactivity was ascribed to the electron-withdrawing effect of the *N*-CH_2CO_2Et group as compared to *N*-CH_3. Considering the potential utility of amidomethyl esters **1**, it was therefore necessary to determine the applicability of this prodrug approach with a wider range of carboxylic acid drugs and amide carriers in order to assess the factors affecting their chemical stability. Herein we report the synthesis of a series of amidomethyl esters **1** (Figure 1) and a kinetic study that evaluates the influence of the amide and carboxylic acid structures on the chemical reactivity and stability in plasma.

MATERIALS AND METHODS

Chemistry

Elemental analyses were obtained from ITQB (Oeiras, Portugal) and from MEDAC (Uxbridge, UK) laboratories. ^{1}H- and ^{13}C-NMR spectra were recorded as $CDCl_3$ solutions on Bruker MSX 300 or Jeol 400 spectrometers using TMS as internal standard. IR spectra were recorded on a Nicolet Impact 400. Melting points were recorded on a Buchi 510 and are uncorrected.

The synthesis of prodrugs **1** was achieved by amidomethylation of the carboxylic acid with the appropriate *N*-alkyl-*N*-chloromethylamides as described previously (6). The general procedure is decribed for compound **1i**; spectroscopic and physical parameters were as expected for all new compounds (detailed data are available upon request from the corresponding authors).

*N-(Ethoxycarbonylmethyl)benzamidomethyl benzoate (**1i**)*

A solution of *N*-chloromethyl-*N*-(ethoxycarbonylmethyl)-benzamide (6) (1.4 g, 5.5 mmol) in dry THF (5 ml) was added dropwise to a stirred suspension of sodium benzoate (0.72 g, 5 mmol) in dry THF (5 ml) at room temperature. After 2h, the solvent was removed under reduced pressure and the residue treated with water (50 ml) and extracted with dichloromethane (2 × 50 ml). The combined extracts were washed with sodium bicarbonate, water and then evaporated to afford the crude product which was purified by column chromatography on silica gel using diethyl ether as eluent. Evaporation of the solvent afforded (**1i**) as an oil (0.76 g, 46 %).

Kinetic Measurements

The hydrolyses were carried out using a Shimadzu UV-160 spectrophotometer fitted with a cell compartment coupled to a Grant thermostat water-bath. The ionic strength was maintained at 0.5 M with $NaClO_4$. A 40–50 μl aliquot of a 10^{-2} M stock solution of substrate in acetonitrile was added to the cuvettes containing 3 ml of the required buffer solution. The

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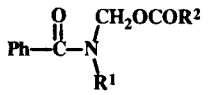

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	R^1	R^2		R^1	R^2
a	CH_3	CH_3	**k**	CH_2CO_2Et	Naproxen
b	CH_3	C_6H_5	**l**	CH_2CO_2Et	Diclofenac
c	C_2H_5	C_6H_5	**m**	CH_2CO_2Et	Valproic acid
d	n-C_3H_7	C_6H_5	**n**	CH_2CO_2Et	Probenecid
e	i-C_3H_7	C_6H_5	**o**	CH_2CO_2Et	Clofibric acid
f	n-C_4H_9	C_6H_5	**p**	CH_2CO_2Et	Penicillin G
g	$CH_2C_6H_5$	C_6H_5	**q**	CH_2CO_2Et	Dicloxacillin
h	C_6H_5	C_6H_5	**r**	CH_3	Ibuprofen
i	CH_2CO_2Et	C_6H_5	**s**	CH_3	Naproxen
j	CH_2CO_2Et	Ibuprofen	**t**	CH_3	Valproic acid

Fig. 1. Structures of the amidomethyl esters used in the present study.

reaction was monitored at fixed wavelength by following the decrease in absorbance. The experimental rate constants, k_{obs}, were determined from plots of $\ln(A_t - A_\infty)$ *versus* time.

Alternatively, reactions were monitored using HPLC, following either the loss of substrate or the formation of products. Reactions were initiated by injecting *ca.* 100 μl of the appropriate substrate stock solution to 10 ml of the buffer solution. At regular intervals, samples of the reaction mixture were analyzed using the following system: a Shimdazu LC-9 isochratic HPLC system coupled to a Shimadzu SPD-M6A diode-array detector; a Merck LiChrospher® 100 RP-8 5 μm 125 × 4 mm column; mobile phase, acetonitrile-water containing 0.2 M sodium acetate buffer (55:45 to 70:30%) with a 1.0 $ml.min^{-1}$ flow. Quantitation of the prodrug and corresponding parent drug was obtained from measurements of the peak areas in relation to those of corresponding standards chromatographed under the same conditions. There is good agreement (±5%) between the rate constants determined by both UV and HPLC methods.

Plasma Studies

The hydrolyses of prodrugs in human plasma were studied by the HPLC method described above. Plasma was obtained from heparinized blood of healthy donors, pooled, and frozen at −70°C before use. The prodrugs were incubated at an initial concentration of 10^{-4} M at 37°C in human plasma diluted to 80%(v/v) with pH 7.4 isotonic phosphate buffer. At appropriate intervals, 200 μl aliquots were added to 400 μl of acetonitrile in order to quench the reaction and deproteinize the plasma. These samples were centrifuged for 5 min at 13000 rpm and the supernatant was analyzed by HPLC for the presence of the substrate and parent drug.

Partition Coefficients

For the prodrugs **1h–o** these were determined in octanol-pH 7.4 phosphate buffer at 22°C. Each phase was mutually saturated before the experiment. The volumes of each phase were chosen so that solute concentration in the aqueous phase after distribution could readily be measured (typically, a ratio of 1:2 of octanol/buffer was used). The compounds were dissolved in octanol and the octanol-pH 7.4 phosphate mixtures were shaken for 30 min to reach an equilibrium distribution; each phase was analyzed separately by the HPLC method described above. Each experiment was repeated three times; reproducibility was ±5%. The partition coefficients, *P*, were calculated from the ratio of the peak area in octanol to the peak area in buffer.

The lipophilicity of compounds **1** was also evaluated by reverse-phase HPLC, which, for compounds **1p–t**, was the only method available as their reactivity precluded measurement of partition coefficients. In this HPLC method the capacity factors, log k′, were determined using eq 1:

$$\log k' = \log [(t_r - t_o)]/t_o \quad (1)$$

where t_r is the retention time of the compound under study and t_o is the retention time of methanol. Chromatography was carried

out using a RP-8 column and methanol-water 60/40% as eluent at a rate of 1 ml.min^{-1}.

RESULTS AND DISCUSSION

pH-Rate Profiles

The influence of pH on the rates of hydrolysis of compounds **1d**, **1i** and **1t** is shown in Figure 2. These pH-rate profiles are marked by a broad U-shape indicative of the presence of acid-catalyzed, k_{H^+}, base-catalyzed, k_{HO^-}, and pH-independent, k_0, processes, corresponding to Eq. 2. Similar pH-rate profiles with a broad plateau have been previously described for *N*-(methyl)benzamidomethyl benzoates and *N*-(alkyl)benzamidomethyl penicillates (5,7).

$$k_{obs} = k_o + k_{H^+}[H^+] + k_{HO^-}[HO^-] \quad (2)$$

The rate constant for the pH-independent process, k_0, was determined from the pH-rate profile, while the catalytic second-order rate constants, k_{H^+} and k_{HO^-}, were obtained from the plots of k_{obs} *versus* $[H^+]$ and $[HO^-]$, respectively. The intercepts of these plots were identical to the pH-independent rate constant, k_0, obtained from the pH-rate profile. Values of these derived rate constants for the model amidomethyl esters **1a–1i** are listed in Table I. The rates of hydrolysis were found not to be subject to general or nucleophilic catalysis by buffers over at least a 10 fold concentration range.

Structural Effects of the Amide Moiety on Chemical Stability

pH-Independent Pathway

Inspection of the kinetic data in Table I reveals that the pH-independent pathway rate constant, k_0, is increased by the electron-donating *N*-R^1 substituents. Moreover, the extension of the plateau in the pH-rate profile is clearly dependent on the polar effect of the nitrogen substituent. Thus, the plateau for the *N*-propyl derivative, **1d**, extends from pH 2 to pH 11 while for the ethyl hippurate derivative, **1i**, the plateau extends only from pH 3 to pH 8. This reflects the effect of the *N*-substituents upon the relative rates of the acid- and base-catalysed processes (see below).

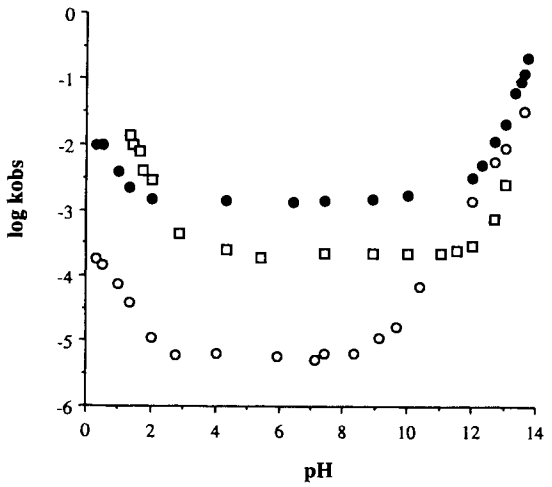
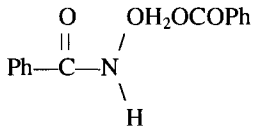


Fig. 2. pH-rate profiles for the hydrolysis of compounds **1d** (●) and **1i** (○) at 25°C, and of compound **1t** (□) at 37°C.

Using the data at 37°C, a correlation is obtained between log k_o and the values of the Taft σ^* and Charton's steric parameter, ν, for the *N*-R^1 substituents in the amide moiety (Eq. 3). This correlation includes compound **2**, which was shown also to present a pH-independent pathway from pH *ca.*2 to pH *ca.*5 (5,8).

$$\log k_o = (-1.8 \pm 0.2) - (2.8 \pm 0.2)\sigma^* - (1.0 \pm 0.3)\nu$$

$$(n = 9, R^2 = 0.97, s = 0.20) \quad (3)$$



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Of course, a dual-parameter equation encompassing only nine data points lacks rigorous statistical significance. However, we do believe that the signs and the relative magnitudes of the dependencies upon σ^* and ν provide mechanistic insights (9). Thus, the negative sign and magnitude of the inductive term indicates that chemical stability can be achieved only with electron withdrawing substituents on the amide nitrogen. Indeed, the negative sign and magnitude of the dependence upon the inductive parameter for the *N*-substituent is fully consistent with the S_N1-type mechanism previously proposed by us for this type of compound. This mechanism involves the rate-limiting formation of an iminium ion such as **3** (Figure 3).

The negative sign of the steric term in Eq. 3 indicates that the hydrolysis reaction is retarded by steric crowding of the amide nitrogen group. This is an unusual effect for an S_N1 type reaction, for which release of steric strain in the formation in the transition state usually leads to rate enhancement (10). We suspect that hydrogen atoms of the methylene group in the iminium ion are involved in unfavourable steric interactions with the *N*-substituent. However, it is clear from the magnitudes of the σ^* and ν terms that the electronic effect has by far the greater influence.

Acid-catalyzed Pathway

Inspection of the data in Table I also suggests that the polar effect exerted by the nitrogen substituent in the amide moiety is the major factor controlling the acid catalyzed pathway. Again, the dual parameter approach results in a correlation between log k_{H^+} values and the Taft σ^* and Charton ν values (Eq. 4).

$$\log k_{H^+} = (-1.3 \pm 0.1) - (2.1 \pm 0.1)\sigma^* - (0.6 \pm 0.2)\nu$$

$$(n = 9, R^2 = 0.98, s = 0.11) \quad (4)$$

The dependence upon σ^* and ν is similar to that for k_0, the negative sign for the σ^* term indicating that the acid-catalyzed reaction is also accelerated by electron-donating substituents. Again this is consistent with the mechanism of hydrolysis postulated previously for the *N*-(methyl)benzamidomethyl benzoates, involving a pre-equilibrium protonation followed by rate-limiting formation of the iminium ion (5). As with the pH-independent pathway, the steric effect can be ascribed to the increase in steric crowding in the iminium ion.

Table I. Second-Order Rate Constants, k_{H^+} and k_{HO^-}, for the Acid- and Base-Catalyzed, and Pseudo-First-Order Rate Constants, k_o, for the pH-independent, Hydrolysis for *N*-(alkyl)benzamidomethyl Benzoates **1a-i**

Compound	$10^2 k_{H^+}$/dm^3 mol^{-1}s^{-1}	k_{HO^-}/dm^3 mol^{-1}s^{-1}	$10^5 k_o$/s^{-1} 25°C	$10^5 k_o$/s^{-1} 37°C
1a	—	—	—	124
1b	1.94	1.00	99.0[a]	391
1c	3.41	0.206	165	626
1d	3.34	0.165	135	496
1e	5.46	0.0836	422	1700
1f	3.73	0.181	113	433
1g	0.460	0.119	14.1	55.2
1h	0.0891	0.120	1.98	4.67
1i	0.0536	0.0800	0.646	2.81

[a] From reference 5.

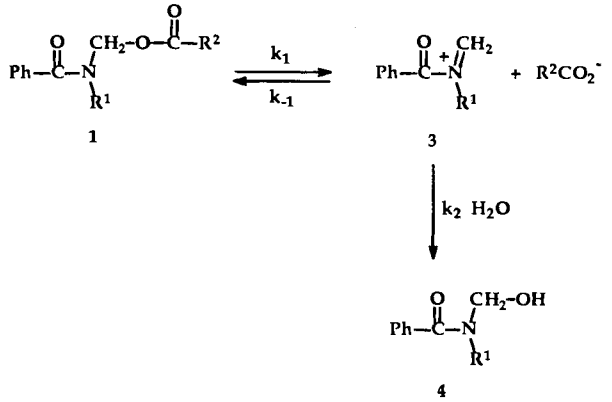
Base-catalyzed Pathway

The dependence of log k_{HO^-} upon the σ^*/ν parameters results in a poor correlation (Eq. 5). Nevertheless, a trend is clear; the large negative correlation with ν (as compared to the somewhat smaller values observed for the pH-independent and acid-catalysed pathways) indicates that the rate of alkaline hydrolysis decreases sharply with increased steric crowding in the nitrogen substituent. Indeed, steric effects appear to be much more important than electronic effects. This is consistent with nucleophilic attack of OH- at the ester carbonyl carbon atom. Similar steric dependence was obtained for the alkaline hydrolyses of methyl esters (at 20°C) and of ethyl esters at (25°C) with susceptibility parameters of −2.0 and −2.6, respectively (11).

$$\log k_{HO^-} = (-1.53 \pm 0.7) - (0.6 \pm 0.2)\sigma^* - (3.5 \pm 1.0)\nu$$
$$(n = 8, R^2 = 0.74, s = 0.21) \quad (5)$$

Structural Effects of the Carboxylic Acid Moiety on Chemical Stability

To evaluate the influence of the carboxylic acid structure on reactivity, derivatives **1j–q**, containing ethyl hippurate as the most promising pro-moiety, were synthesized. These were chosen specifically because (i) they represent derivatives of a range of commonly used drugs that contain the carboxylic acid group, and (ii) the pK_a of the carboxylic acid group spans a wide range, from 2.8 for the penicillins to 5.2 for ibuprofen.



1 3 4

Fig. 3. Mechanism for the pH-independent hydrolysis of amidomethyl esters **1**.

The pH-independent pathway rate constants of amidomethyl esters **1i–q** (Table II) display a high dependence on the pK_a of the carboxylate leaving group, with a Bronsted β_{lg} value of −0.91 (Figure 4). Together with the absence of any steric hindrance exerted by the carboxylic acid moiety on the reactivity, this value is consistent with the S_N1 mechanism for the pH-independent hydrolysis. A similar β_{lg} value of −1.06 was previously reported for a series of *N*-(methyl)-benzamidomethyl esters (7).

The rate data presented in Table II indicate that those prodrugs of carboxylic acid agents with $pK_a > 4$ based on the ethyl hippurate carrier are very stable in aqueous buffers. For example the half-life of the naproxen ester **1k** in pH 7.4 phosphate buffer is 42 h at 37°C and >200 h at 25°C. In contrast, the *N*-methylbenzamide naproxen prodrug **1s** has a half-life of hydrolysis of 14 min. These results show that it is readily feasible to prepare esters which are stable in aqueous solutions through the careful choice of the amide carrier.

Lipophilicity of Amidomethyl Esters

The octanol-water partition coefficients, log *P*, of the amidomethyl esters **1**, along with their reversed-phase HPLC capacity factors, log *k'*, are presented in Table II. The log *P* values for the derivatives **1r–t** were calculated by the fragment method. As reported for other classes of compounds, a reasonable linear relationship between log *P* and log *k'* was obtained for the esters **1** (Eq. 6) (12). This equation was used to calculate the log *P* value for the unstable dicloxacillin derivative **1q**.

$$\log P = (0.7 \pm 0.4) + (1.9 \pm 0.3)\log k'$$
$$(n = 12, R^2 = 0.81, s = 0.37) \quad (6)$$

Plasma Hydrolysis

As can be seen from Table II, the amidomethyl esters are hydrolysed in 80% human plasma with vastly different rates. In this medium, reactivity appears to be dependent on the pK_a of the carboxylic acid in the parent drug, the size and number

Table II. Lipophilicity Data, Rate Data for the pH-Independent Pathway at 37°C and Half-Lives in Human Plasma at 37°C and pH 7.4 for Compounds **1h-t**

Compound	log *P*	log k′	10^5 k_o/s^{-1}	$t_{1/2}$/min	
				pH 7.4 buffer	80% Human plasma
1h	3.62	1.27	4.67	246	40
1i	1.63	0.79	2.81	414	12
1j	3.86	1.92	0.548	2100[a]	720
1k	3.39	1.40	0.464	2520[a]	78
1l	4.17	1.78	1.34	900	162
1m	3.85	1.50	0.24	4920	1320
1n	3.00	1.06	12.7	90	46
1o	2.73	1.14	8.33	138	120
1p	1.60[b,c]	0.60	50.0	23[b]	5.1
1q	2.87[d]	1.15	46.1	25	10.1
1r	3.25[c]	1.38	62.4	18	162
1s	2.95[c]	0.94	81.8	14	72
1t	3.00[c]	1.34	20.1	58[b]	384

[a] From reference 6.
[b] From reference 7.
[c] Calculated by the fragment method.
[d] Calculated from Eq. 6.

of the α-substituents in the ester moiety, as well as on the lipophilicity of the compounds, as shown by the following:

1. The benzoates **1i** and **1n** are rapidly hydrolyzed (half-lives of 12 and 46 min, respectively) whereas the much more sterically hindered ibuprofen and valproate derivatives, **1j** and **1m**, are hydrolyzed at significantly lower rates (half-lives of 720 and 1320 min, respectively). Moreover, the diclofenac ester **1l**, which contains an *ortho* 2,6-dichloroanilino substituent, has a half-life of hydrolysis of 162 min. Since diclofenac has a similar pK_a to benzoic acid (4.5 *vs* 4.2) the greater than 10-fold difference in reactivity may be ascribed to the greater steric bulk of the ester carbonyl in diclofenac. The clofibric acid ester, **1o**, is highly hindered containing a tertiary centre α- to the ester carbonyl carbon. This compound reacts faster than most other hindered substrates, but this can be ascribed to the low pK_a of the acid leaving group (pK_a 3). Indeed, the rate of hydrolysis in plasma is almost identical to that in pH 7.4 phosphate buffer, from which we conclude that hindered derivative **1o** does not undergo hydrolysis catalysed by plasma enzymes but by the pH-independent mechanism described earlier. A similar reduction of plasma activation of sterically-hindered prodrugs has been reported elsewhere (12,13).

2. The hydrolysis of esters linked to *N*-methylbenzamide, **1r–t**, is at least 5-fold slower in plasma than in pH 7.4 phosphate buffer. This might imply that these derivatives bind strongly to non-catalytic proteins, thus preventing their hydrolysis (14).

3. Finally, a linear relationship between log $t_{1/2}$ values in human plasma and log *P* values was obtained, which indicates that the rates of hydrolysis are reduced with increasing lipophilicity (Figure 5). Thus, the high reactivity of the sterically-hindered benzylpenicillin and dicloxacillin esters **1p–q** can be ascribed to their low lipophilicity. Moreover, the 10-fold differ-

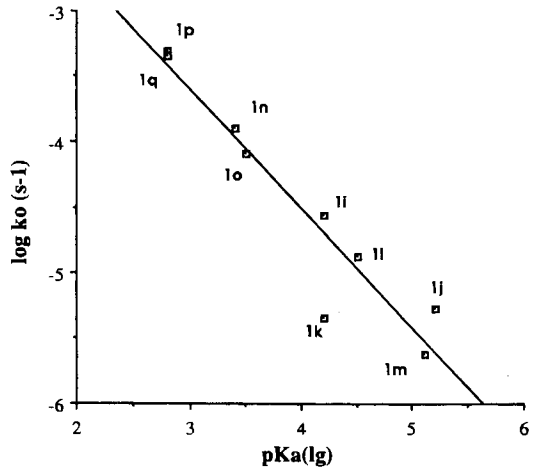


Fig. 4. Bronsted plot of log k_o *vs.* the pK_a of the carboxylate leaving group for the amidomethyl esters **1i–q**.

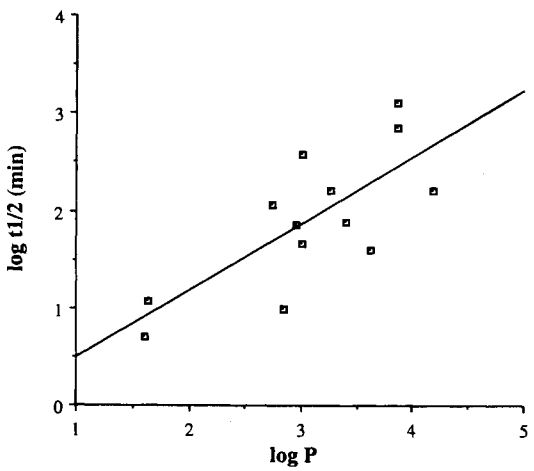


Fig. 5. Plot of log $t_{1/2}$ *vs* log **P** for the hydrolysis of amidomethyl esters **1h–t** in 80% human plasma at 37°C.

ence in reactivity between the the ibuprofen **1j** and naproxen **1k** derivatives, both containing a methyl group in the α-position to the ester, is partly consistent with the lower lipophilicity of the latter.

The large reduction of reactivity in human plasma observed for those derivatives α-substituted in the ester moiety (**1j**, **l**, **m**, **o**) appears to parallel the reduction in reactivity of *N*-(methyl)benzamidomethyl esters towards alkaline hydrolysis with the increasing of the steric crowding in the carboxylate moiety reported previously (7). These results suggest that the mechanism of hydrolysis in human plasma is different to that of the pH-independent pathway, and that catalysis is mediated by esterases. It has been put forward elsewhere that there is an analogy between HO^--catalyzed and esterase-catalyzed ester hydrolysis, the latter involving nucleophilic attack of an enzyme nucleophile in the active site (presumably, a serine residue) at ester carbonyl (15). As an aside, it is worth noting that the plasma-catalyzed hydrolysis of the ibuprofen ester **1j**, which is a racemic mixture, follows strict first-order kinetics. This suggests that the enzymic hydrolysis of this amidomethyl ester is not stereoselective, which contrasts with the recently reported enantioselective differences in the plasma-catalyzed hydrolysis of some esters of ibuprofen and flurbiprofen (16).

Relevance to Prodrug Design

Amidomethylation is an attractive approach to potentially useful prodrug systems for carboxylic acid drugs. The structure-reactivity relationships for the pH-independent pathway clearly indicate that stability can be achieved with amide carriers containing electron-withdrawing and bulky *N*-substituents. Preferably, the carboxylic acid agent should have a pKa > 4 in order to be stable in aqueous solutions. Hydrolysis of this new ester prodrug type in plasma is strongly reduced by the steric hindrance exerted by α-substituents in the carboxylate moiety. The prodrugs of carboxylic acids with pKa > 4 based on the ethyl hippurate carrier combine an high stability in aqueous buffers together with a high susceptibility to enzyme-catalyzed hydrolysis. Moreover, hippurate is an endogeneous system analogous to glycine, and is not reported to have any harmful effects.

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9. The ranges of the σ^* and ν values used are $-0.19 - 0.82$ and $0 - 0.76$. respectively. There is a slight negative intercorrelation for these two variables, such that $\nu = 0.6 - 0.3\sigma^*$. However, the R^2 value for this correlation is 0.25.
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