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The reduction of aryl azides by dithiothreitol: a model for bioreduction of aromatic azido-substituted drugs *

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Summary

The relative rates of reduction of a series of simple phenyl azides by dithiothreitol (DTT) were studied and the results modelled using the Hammett equation. The rate of reduction was found to be dependent on electronic effects and the steric environment of the azido group, with electron-deficient aryl azides being reduced at a faster rate. These findings were extended to study the reduction rates of model azido-2,4-diaminopyrimidine drugs by DTT, and showed similar dependence on electronic and steric considerations. The findings have implications for the design of aromatic azido pro-drugs and "soft drugs", from the point of view of bioreductive susceptibility.

Introduction

Azido-substituted compounds have been shown to have biological effects ranging from analgesic (e.g. azidomorphine) to antimicrobial activity (e.g. azidocillin) (Breslow, 1984), as well as the recent development of the anti-AIDS drug azidothymidine (AZT). These examples are of aliphatic azides, but the aromatic azido group also has potential for use in drug design. The azido substituent has similar lipophilicity and electronic characteristics to, for example, the chloro-substituent, and it has been shown (Bliss et al., 1979) that aryl azides may be reduced, enzymatically or via circulating thiols, to the corresponding arylamine. It is therefore possible to envisage the use of aryl azides as pro-drug modifications of arylamines, or for biologically active aryl azides to be used as "soft drugs", which may then be inactivated by conversion to the arylamine.

Recent work has led to the development of the experimental antitumour agent, *m*-azidopyrimethamine (MZP, Fig. 1, **1**), a dihydrofolate reductase inhibitor, and an example of the use of aromatic azides as soft drugs (Stevens et al., 1987). This molecule has been shown to undergo bioreductive conversion to the more polar and inactive arylamine, *m*-aminopyrimethamine (MAP, Fig. 1, **2**), in vitro in murine tissues (Kamali et al., 1988) and in vivo in mice (Slack et al., 1986). This conversion should therefore minimise systemic toxicity due to the lipophilic parent compound MZP.

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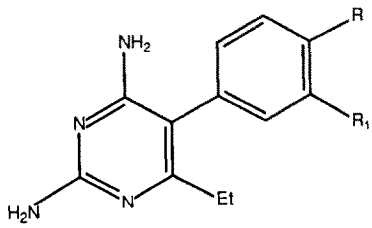

Compound	R	R_1
1	Cl	N_3
2	Cl	NH_2
3	F	N_3
4	MeO	N_3
5	EtO	N_3
6	NMe2	N3

Fig. 1. Structures of the substituted 2,4-diaminopyrimidines.

Staros and co-workers (1978) have shown that aryl azides are rapidly reduced to the corresponding amine by dithiothreitol (DTT) at physiological pH and temperature. It was shown that the common endogenous thiol glutathione also reduced aryl azides, but at a greatly reduced rate, and that relatively faster rates of reduction were observed for electron-deficient aryl azides. In order to investigate this phenomenon further the work presented here addresses the effect of differing substitution on the rate of reduction by DTT of a series of simple phenyl azides. The rate of reduction of MZP and analogous compounds (Fig. 1, 3–6) by DTT were also studied, with a view to optimising the susceptibility to bioreduction of lipophilic antifolates of this type.

Materials and Methods

Dithiothreitol was purchased from the Sigma Chemical Co. All chemicals and solvents were of HPLC or reagent grade as appropriate.

pH measurements were carried out using a Radiometer PHM62 Standard pH meter.

Synthesis of phenyl azide and substituted phenyl azides

The phenyl azides were prepared in yields of between 64% and 95% by the action of sodium azide on the corresponding diazonium salt of the arylamine (Smith and Boyer, 1963). All products were yellow/brown liquids with the exception of *p*-nitrophenyl azide, which was a yellow solid at room temperature. Briefly, the appropriate arylamine (2 g) was dissolved in 5 M HCl (100 ml) at $< 5\,^{\circ}$C. Sodium nitrite (1.1 M equivalents) in water (5 ml) was added over 5 min and the mixture stirred for between 15 and 30 min to form the diazonium salt. Sodium azide (4 M equivalents) was added cautiously over 15 min and the mixture stirred for 1 h, maintaining the temperature at $< 5\,^{\circ}$C. The mixture was poured onto ice and basified to ~ pH 12 with concentrated ammonia solution. The aqueous mixture was extracted with diethyl ether (3×50 ml). The ethereal phase was washed with 1 M hydrochloric acid (50 ml) and distilled water (50 ml), dried over sodium sulphate and the solvent removed in vacuo to give the product. Identity was confirmed by ^{1}H-NMR analysis using a Varian EM360A NMR spectrometer, and IR spectrophotometry using a Perkin Elmer 1310 IR spectrophotometer, where a strong band at ~2100 cm^{-1} confirmed azide formation. The products were stored in the dark in sealed containers.

Synthesis of MZP and analogous compounds

MZP and analogous compounds were synthesised according to a published method (Bliss et al., 1987).

Analysis

With the exception of *p*-nitrophenyl azide (see later), analysis of the phenyl azides was carried out by high-performance liquid chromatography (HPLC) using either of the following systems.

(i) An Altex Model 110A pump equipped with a Pye Unicam LC3 UV variable wavelength detector and a Rheodyne model 7125 injection valve fitted with a 100 μl injection loop. The column used, 10 cm $\times$ 4.6 mm i.d., was packed with Hypersil-5 ODS (Shandon, U.K.).

(ii) A Gilson Model 302 pump with Model 802 manometric module equipped with a Cecil Instruments CE 212 variable wavelength UV monitor and a Rheodyne model 7125 injection valve fitted with a 20 μl injection loop. Column as above.

The columns were eluted isocratically at ambient temperature at a flow-rate of 1 ml $\cdot$ min^{-1}

using a mobile phase consisting of acetonitrile (60%), 0.005 M K_2HPO_4 (40%) and diethylamine (0.05% v/v), adjusted to pH 4 with orthophosphoric acid. The column eluant was monitored at 250 nm. Adequate mixing and de-gassing of the mobile phase was achieved by sonication prior to use. Use of these conditions produced retention times of between 1.6 and 2.4 min for the series of phenyl azides studied. Sensitivity settings ranged from 0.08 to 0.15 AUFS. 1,2,4-Trichlorobenzene was included as an internal standard at a concentration of 43 $mg \cdot ml^{-1}$, and plots of peak height ratio against phenyl azide concentration were linear over the range 0.025–0.17 mM.

MZP and the ethoxy analogue (**5**) were analysed using system (i) as above, with a mobile phase comprising 40% acetonitrile in distilled water, with 0.1% v/v diethylamine, the final pH being adjusted to between 2.5 and 3.0 with orthophosphoric acid. A flow-rate of 1.5 $ml \cdot min^{-1}$ was employed and the column eluant was monitored at 250 nm. The 4′-fluoro substituted compound (**3**) was analysed using the same mobile phase at a flow-rate of 1.0 $ml \cdot min^{-1}$ and the methoxy analogue (**4**) was analysed using a mobile phase comprising 35% acetonitrile in distilled water at pH 2.5–3.0, with 0.1% v/v diethylamine as above. The dimethylamino analogue (**6**) was analysed using a Waters chromatographic system comprising a model 510 pump, a model 710B sample processor, a model 490 programmable multiwavelength detector, a system interface module and a model 840 data station. Separation from the corresponding amine was achieved using a Lichrosorb RP-Select B column (125 × 4 mm) and a mobile phase consisting of 65% methanol : 35% phosphate buffer (5 mM, pH 7.0), at a flow-rate of 1 $ml \cdot min^{-1}$. The column eluant was monitored at 272 nm with a sensitivity of 0.015 AUFS. Calibration plots of peak height against azide concentration were linear for all compounds over the range 2.5×10^{-5} to 2.5×10^{-6} M. Qualitative injections of the corresponding amines showed sufficient separation from the azide peak, the amine peak eluting close to the solvent front in all cases.

Analysis of *p*-nitrophenyl azide was conducted using a Hewlett Packard 8451A Diode Array Spectrophotometer. The conditions of reaction were the same as described below. The final reaction volume was 3.0 ml. The reaction cuvette was set up in the instrument's heated block and the reaction initiated by addition of the *p*-nitrophenyl azide solution. Absorbance readings at 310 nm were taken at 10 s time intervals, and the pseudo-first-order rate constants (k) were calculated using Eqn. 1:

$$\ln(A_t - A_\infty) = \ln(A_0 - A_\infty) - kt \qquad (1)$$

where t is the time in minutes, A_t is the absorbance reading at time t, A_0 is the initial absorbance reading, and A_∞ is the absorbance value when all the azide present has been converted to the corresponding amine. Since all solutions were made up in 95% ethanol for the kinetic studies on the series of phenyl azides, this solvent was used as the reference for the UV measurements.

Kinetics of reduction

Ethanolic solutions containing (a) 3.084 $mg \cdot ml^{-1}$ DTT (5.5 ml), and (b) 6.44 $mg \cdot ml^{-1}$ triethylamine and 10.94 $mg \cdot ml^{-1}$ 1,2,4-trichlorobenzene (7.5 ml) were pipetted into the reaction vessel, which consisted of an aluminium foil-encased sample tube surrounded by a water jacket maintained at 37 °C by a Churchill thermostatic pump. The DTT solution was stored at 4 °C and used within 48 h of preparation. Both the contents of the reaction vessel and the water jacket were stirred magnetically. After 15 min, to allow the reaction vessel contents to equilibrate at 37 °C, an ethanolic azide solution (1.5 mm, 1 ml) was added to initiate the reaction. These quantities represent a final molar ratio of approximately 230 : 100 : 1, triethylamine : DTT : phenyl azide. Samples (~ 0.1 ml) were taken at appropriate time intervals commencing 1 min after addition of the azide (to allow thermal equilibrium and adequate mixing to have been achieved) and injected directly onto the HPLC system previously described. The pseudo-first-order rate constant was calculated by following the decrease in peak height ratio using a first-order kinetic model.

The rates of reduction of MZP and its analogues were followed using solutions prepared in a phosphate buffer containing 0.005 M KH_2PO_4

(pH 7.4) in a similar manner to that described above, with the exclusion of triethylamine and the internal standard from the reaction mixture. An initial molar ratio of 100 : 1 DTT : azide was again employed.

Results and Discussion

The observed reduction of aromatic azides by DTT may be illustrated by the example chromatograms in Fig. 2. The peak corresponding to the azido compound can be seen to reduce in height with time while the amine peak, observable at the tailing edge of the solvent front, increases. The reaction was allowed to continue to completion in several cases, at which point no peaks other than those for the amine and internal standard were evident.

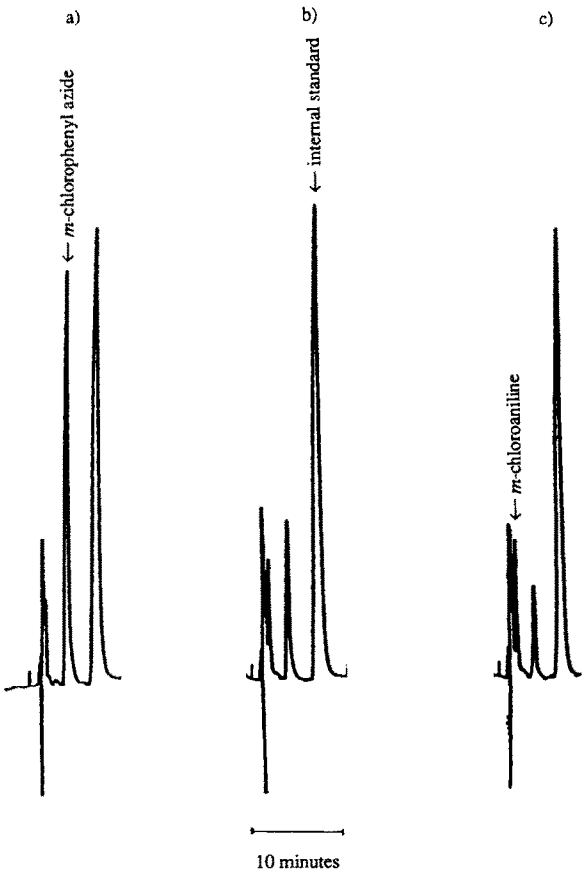

Fig. 2. Chromatograms of reaction mixture samples for *m*-chlorophenyl azide at time: (a) 1 min; (b) 60 min; and (c) 90 min. DTT was used as reductant under the conditions described in the text.

Due to insufficient aqueous solubility of the phenyl azides, an ethanolic system was employed using triethylamine as an organic modifier to provide a basic environment for nucleophilic attack by the DTT. In order to optimise conditions in terms of DTT concentration, triethylamine concentration and temperature, these factors were varied independently, and the effect of this on the rate of reduction of *m*-chlorophenyl azide measured. The rate was found to vary linearly with DTT concentration in the range 5–20 mM (50 : 1 to 200 : 1 molar ratio DTT : phenyl azide), and with triethylamine concentration rising to a maximum in the range 0.6–300 mM (5 : 1 to 2800 : 1 triethylamine : phenyl azide; results not shown). Subsequent to these studies, the conditions chosen for comparative purposes between the phenyl azides were 25.0 mM triethylamine, 10.7 mM DTT and 0.1071 mM azide, at 37 °C. This corresponds to a molar ratio of ~ 230 : 100 : 1 triethylamine : DTT : azide. Control experiments were performed, omitting the DTT in one, and the triethylamine in the other, from the reaction mixture. In both cases no reduction in azide peak height was observed after 1 h, showing that both are essential for reduction of the phenyl azides under these conditions.

p-Nitrophenyl azide is a special case in that it exhibited a very fast rate of reduction under the conditions stated, and therefore could not be monitored satisfactorily using the HPLC technique. It is amenable to UV analysis since the absorbance maximum at 310 nm is sufficiently separated from that of the corresponding amine (378 mm) or of the DTT and triethylamine in the reaction mixture (226 and 230 nm, respectively), as illustrated by Fig. 3. The presence of an isosbestic point in the spectra of the reaction mixture taken at time intervals indicates the conversion of one chromophoric species (*p*-nitrophenyl azide), into a species with a significantly altered spectrum (*p*-nitroaniline). The spectral differences observed in this case are pronounced and justify the use of spectrophotometry to quantify the reduction of

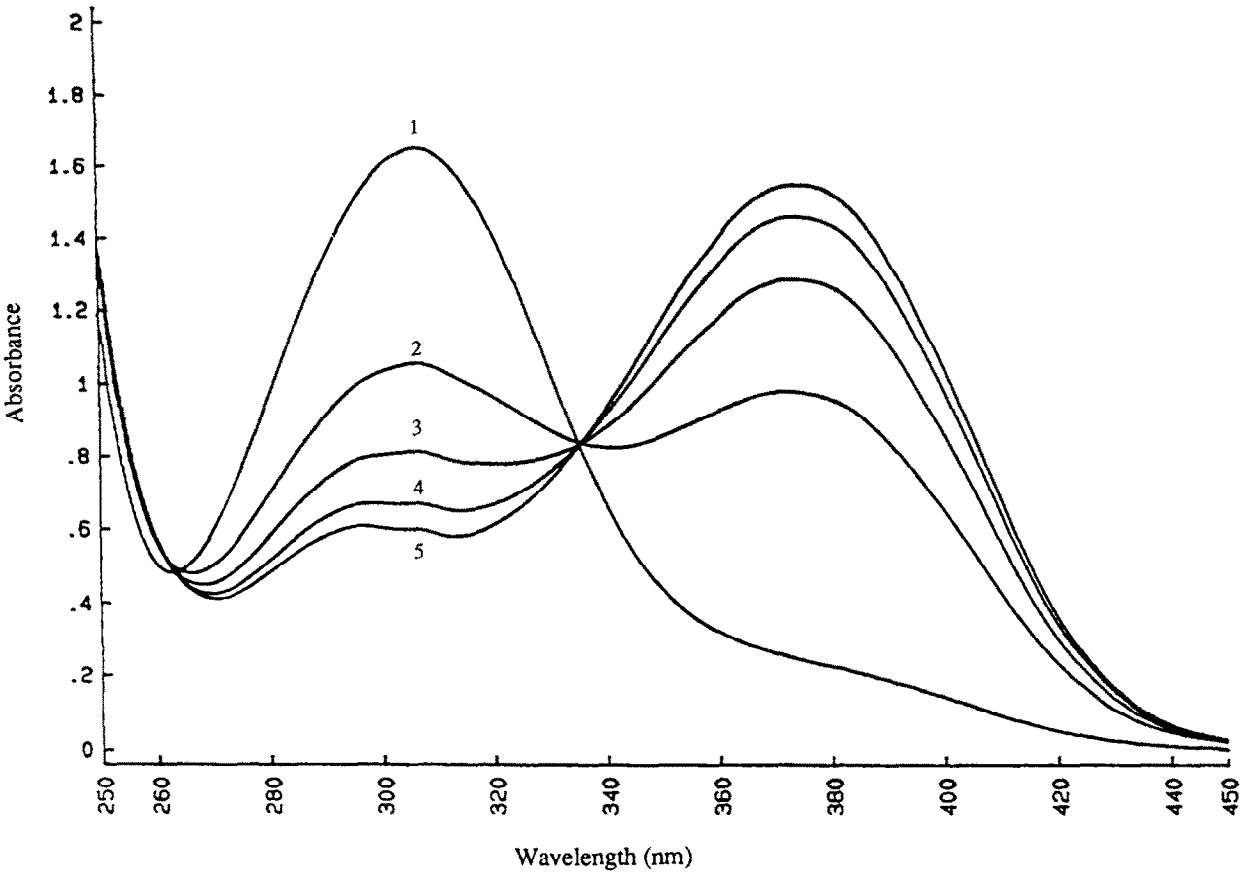

Fig. 3. Ultraviolet spectra of the reaction mixture for the reduction of *p*-nitrophenyl azide by DTT at: (1) 0, (2) 90, (3) 180, (4) 270 and (5) 360 s after initiation of the reaction.

p-nitrophenyl azide under the described conditions. The other phenyl azides have absorbance maxima which coincide with the dithiothreitol absorbance, and therefore cannot easily be quantified by this method. It is not possible to include the DTT in the reference cuvette, and thereby negate the interfering UV absorbance, since the oxidised disulphide of DTT has a significant absorbance maximum at 283 nm, i.e. at a higher wavelength than the reduced form (Cleland, 1964). The relative proportions of the two species will obviously change during a reduction reaction, and so the reference baseline would no longer be genuine. This emphasises the need for compounds to exhibit absorbance maxima at a minimum of 310 nm, and preferably a slightly higher value, to be appropriate for quantitation by this method.

Effect of the various substituents on the rate of reduction of the series of phenyl azides

The rates of reduction of the various substituted phenyl azides are summarised in Table 1, and example reduction profiles are shown in Fig. 4. It can be seen that those phenyl azides with electron-withdrawing substituents (e.g. the *meta* and *para* halo-, and the *p*-nitro substituents) have a much faster rate of reduction than that seen for the unsubstituted phenyl azide. This is consistent with the findings of Staros et al. (1978) who proposed that this observation lends support to a pathway involving rate-limiting nucleophilic addition to the azide group. Conversely, electron-donating *meta* and *para* substituents (e.g. methoxy) lead to a slower rate of reduction than the unsubstituted molecule. Cartwright and co-

TABLE 1

Effect of varying substituent on the rate of reduction of phenyl-azides ($R\text{-}C_6H_4\text{-}N_3$) by DTT.

R	k (min^{-1})	log k	$t_{1/2}$ [a]	σ [b]
H-	0.01463	−1.8348	47.4	0
o-chloro-	0.01274	−1.8948	54.4	−0.014 [c]
m-chloro-	0.05317	−1.2744	13.0	0.373
p-chloro-	0.04149	−1.3821	16.7	0.227
o-methoxy	0.001349	−2.87	513.8	−0.453 [c]
m-methoxy-	0.008832	−2.0539	78.5	0.115
p-methoxy-	0.007616	−2.1183	90.2	−0.268
p-bromo-	0.04911	−1.3088	14.1	0.232
p-nitro-	1.4391	0.1581	0.48	0.78
o-fluoro-	0.0142	−1.8477	48.8	0.008 [c]

[a] Half life in minutes.
[b] From Hammett, 1937 and Clark and Perrin, 1964.
[c] Calculated values using Eqn. 3 (see text).

workers (1976) proposed two possible mechanisms for the reduction of aryl azides by dithiols, involving attack by the dithiol on either the terminal (γ) or α-nitrogen atom of the azide group followed by intramolecular cyclisation and liberation of nitrogen to produce the cyclic disulphide and amino substituted compound.

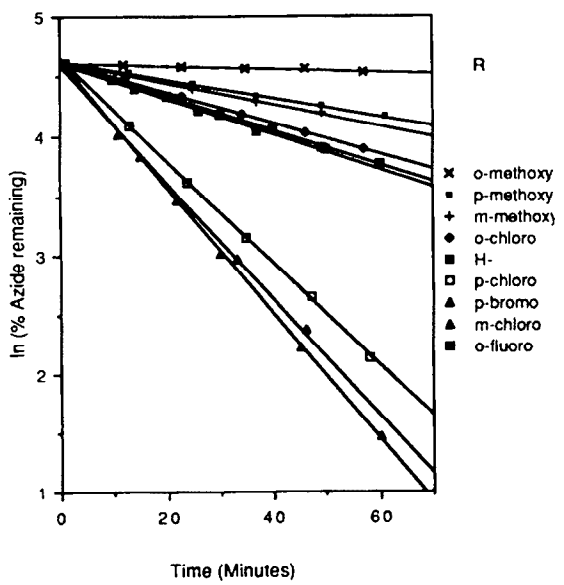

Fig. 4. Effect of varying substituent on the rate of reduction of the substituted phenyl azide series ($R\text{-}C_6H_4\text{-}N_3$) by DTT.

The Hammett linear free energy relationship for the effect of substituents on the rates of aromatic side-chain reactions (Hammett, 1937) is given by Eqn. 2:

$$\log k = \log k_0 + \rho\Sigma\sigma \qquad (2)$$

where k and k_0 are the rate constants for the reaction of the substituted and unsubstituted compounds, respectively, $\Sigma\sigma$ is the sum of the Hammett substituent constants which are determined by the nature of the substituents and are independent of the reaction, and ρ is the reaction constant which is dependent on the reaction, the conditions of the reaction, and the nature of the side-chains undergoing reaction. Thus a plot of log k against the summed Hammett constant values obtained from the literature (e.g. Clark and Perrin, 1964) is linear if this relationship is obeyed, with a slope of ρ. This can therefore be used to investigate the contribution of various substituents on rates of reaction. No true Hammett constants can be obtained for *o*-substituents, mainly because of variable steric and conjugative effects for the same groups in different reactions. Nevertheless apparent σ_{ortho} constants (valid only for the reaction conditions under which they are determined) can be used satisfactorily in conjunction with σ_{meta} and σ_{para} constants for prediction (Clark and Perrin, 1964). The plot of log k against the Hammett constant for the experimental data (Fig. 5) shows reasonable linearity with a slope ρ of 2.221 (r = 0.93); Eqn. 3:

$$\log k = -1.865 + 2.221\,\sigma \qquad (3)$$

The linearity demonstrates that for *meta*- and *para*-substituted phenyl azides, the reaction rate is strongly governed by electronic effects, whilst the positive value of ρ indicates that the reaction is aided by electron-withdrawing substituents. In the cases of the *ortho*-substituted compounds, slower rates than that of the unsubstituted phenyl azide were observed, suggesting that steric hindrance in the region of the azido group is also a contributory factor in governing the reduction rate. Calculated apparent σ_{ortho} constants for the *o*-chloro and *o*-methoxy groups are −0.014 and −0.453,

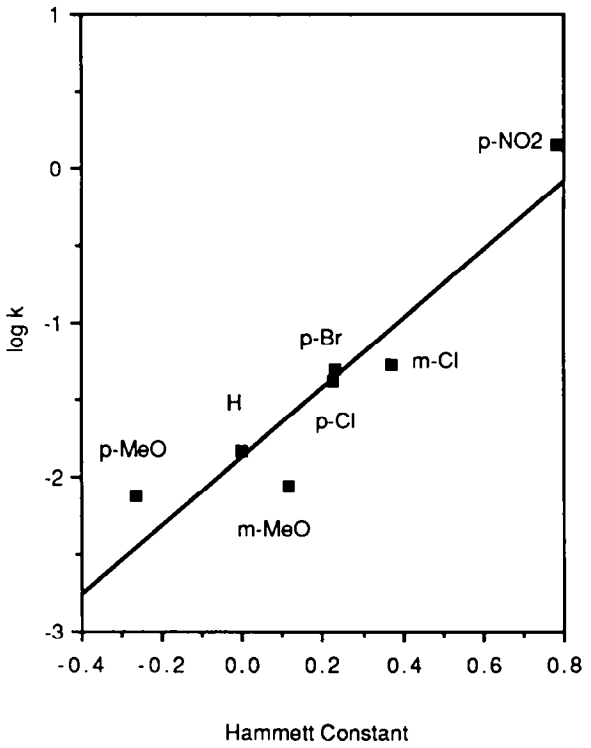

Fig. 5. Hammett plot for the phenyl azide series.

respectively. The experimental log k value of -1.8348 for the unsubstituted phenyl azide compares well with the intercept value of k_0 obtained by linear regression.

In order to extend the relationship between electronic factors and rate of reduction found in the series of model phenyl azides, the rate of reduction by DTT of the diaminopyrimidine MZP and its 4′-methoxy, 4′-ethoxy, 4′-fluoro and 4′-dimethylamino analogues were studied under aqueous conditions at 37°C and pH 7.4. The pseudo-first-order rate constants and half-lives are summarised in Table 2, and example kinetic profiles are shown in Fig. 6. As can be seen from these values the electron-withdrawing, and relatively small halo-substituents impose a fast rate of reduction in this system. The larger electron-donating alkoxy substituents impose significantly slower rates. In the case of the dimethylamino substituted analogue, the tertiary amino group will not be protonated at the studied pH, and therefore will have an electron-donating nature, stabilising the

TABLE 2

Kinetic data for the reduction of azido substituted 2,4-diaminopyrimidines by DTT

Compound	k (min^{-1})	$t_{1/2}$
1	0.0296	23.7
3	0.0169	41.0
4	0.0037	187.3
5	0.0034	203.8
6	0.0010	693.0

aromatic azide. This factor combined with the steric bulk of the substituent contributes to the observed slow rate of reduction for this compound.

The reduction of aryl azides by DTT can be seen to be an appropriate model for the bioreduction of aromatic azido drugs by the possible in vivo reductive system comprising the common cellular monothiol glutathione, which could reduce the azide grouping under biological conditions (Moldeus and Quanguan, 1987). The conditions of the reaction in terms of basicity, temperature and DTT concentration may be altered to give a reaction rate suitable for quantification and comparison between analogous compounds. The results

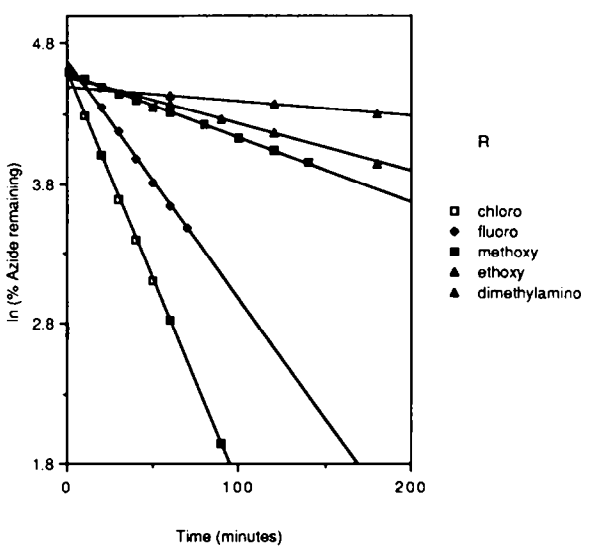

Fig. 6. Effect of varying substituent on the rate of reduction of the azido-2,4-diaminopyrimidine series. (R refers to Fig. 1., where $R_1 = N_3$.)

presented show that it may be possible to predict the rate of soft drug inactivation, or pro-drug activation, for compounds containing the azido moiety, by molecular modification.

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