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The reduction of azidoprofen: A model for a soft anti-inflammatory drug for topical application

A. Naik, W.J. Irwin and R.J. Griffin [1]

Drug Development Research Group, Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET (UK)

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Summary

Azidoprofen (*RS*-2-(4-azidophenyl)propionic acid) and a series of esters, together with the corresponding amino analogues, have been synthesised as a model anti-inflammatory agents susceptible to reductive bioinactivation. As monitored by ^{1}H-NMR and HPLC, azidoprofen undergoes a rapid, base-catalysed reduction by dithiothreitol (DTT) in vitro in aqueous solution at physiological temperature. This suggests that a thiol-mediated reduction, possibly induced by endogenous thiols acting as in vivo reducing agents, is a potential intracellular deactivation mechanism which may be utilised in the design of soft drugs. The conditions of the in vitro model with respect to DTT concentration and pH may be modified to facilitate kinetic modelling, and the base-dependence of the reaction supports the role of the nucleophilic thiolate anion (RS^-) as the predominant reducing species. In aqueous acetonitrile, reactions are significantly slower, oxidation of DTT becomes significant and anaerobic conditions are required for reliable rate estimates.

Introduction

Metabolites of arachidonic acid (AA) are implicated in the inflammatory manifestations of psoriasis. Many non-steroidal anti-inflammatory drugs (NSAIDs) limit cyclooxygenase transformation of AA to prostaglandins but such compounds have found little application in the treatment of psoriasis. Indeed, they may aggravate a number of dermatoses (Griffiths et al., 1985) although studies suggesting indomethacin-induced exacerbation of psoriasis (Katayama and Kawada, 1981; Ellis et al., 1983) remain controversial (Green and Shuster, 1987). In contrast, benoxaprofen therapy resulted in substantial improvement in psoriatic skin lesions (Allen and Littlewood, 1982; Allen and Littlewood, 1983; Kragballe and Herlin, 1983). It was proposed that benoxaprofen exerts this effect by inhibition of the competitive 5-lipoxygenase pathway which leads to leukotrienes, although later studies have questioned this mechanism since the drug does not appear to effectively inhibit the enzyme in vitro (Masters and McMillan, 1984) or in vivo (Salmon et al., 1984). The extended half-life and toxicity of benoxaprofen resulted in its withdrawal in 1982

Correspondence to: W.J. Irwin, Drug Development Research Group, Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, U.K.

[1] *Present address:* Dept of Chemistry, Bedson Building, The University, Newcastle upon Tyne NE1 7RU, U.K.

and indicates the need to minimise the systemic toxicity of such drugs. One strategy is to design soft drugs, which are therapeutically active compounds undergoing predictable and controllable in vivo metabolism to inactive, non-toxic moieties (Bodor, 1982). Soft drugs for topical use include the steroid fluocortin butyl (Kapp et al., 1977) and a series of NSAIDs (Coombs et al., 1973; Shen, 1977).

The basic structural features of anti-inflammatory agents based upon the arylalkanoic acids include an aromatic ring which has both a hydrophobic and a polar substituent. The latter is usually an acetic or an *S*(+)-α-propionic acid residue (Shen, 1981; Gund and Jensen, 1983). Studies have also revealed that the α-propionic acids may undergo a stereoselective inversion of the *R*(−) to the pharmacologically more active *S*(+) enantiomer in vivo and the presence of a hydrogen atom on the α-carbon atom also appears to be necessary for activity (Juby et al., 1972; Smeyers et al., 1985). In contrast, the nature of the hydrophobic substituent shows substantial variation and includes aliphatic or alicyclic residues which are able to adopt a non-planar conformation. Strategies involving modification of this hydrophobic residue could form the basis for the design of a soft drug, where biotransformation would yield an inactive, polar species ensuring rapid inactivation and elimination. Several arylalkanoic acids, including benoxaprofen, the most promising anti-psoriatic member of the group, contain a *p*-chlorophenyl residue in the hydrophobic region. The azido substituent (N_3) has certain properties such as lipophilicity and electronic characteristics in common with halogen atoms. For example, studies with dihydrofolate reductase inhibitors have demonstrated that the azido residue can be substituted for a chloro group while retaining activity (Bliss et al., 1987). The azide group may also provide a means of bioinactivation via a thiol-mediated chemical reduction to the corresponding amine in vivo and in vitro (Cartwright et al., 1976; Staros et al., 1978; Bliss et al., 1979; Slack et al., 1986; Stevens et al., 1987; Kamali et al., 1988; Baker et al., 1989, 1990). Alkyl azides are also readily reduced by thiols at room temperature (Bayley et al., 1978) with the antiretroviral drug, 3′-azido-3′-deoxythymidine (AZT) being assessed for its susceptibility to in vitro reduction by dithiothreitol, mercaptoethanol and the endogenous thiol glutathione (Handlon and Oppenheimer, 1988). Reduction of AZT was also observed in liver microsomal preparations (Cretton et al., 1991).

Extension of this concept to NSAIDs has led us to synthesise azidoprofen [**3**; *RS*-2-(4-azidophenyl)propionic acid; AZP] as a model azido-substituted NSAID in which the hydrophobic isobutyl residue is replaced with an azido moiety to provide the potential for cutaneous or systemic bioreduction to the inactive and polar amine metabolite. The sodium salt (**4**, AZP-Na), to overcome low aqueous solubility problems, and a series of ester derivatives, to provide a range of lipophilicities, have also been prepared. Here, we describe the reduction of the azido group of AZP and some alkyl esters with dithiothreitol (DTT). This reductant was chosen as significant rates of reduction are obtained and, although it is not a naturally occurring reducing agent, intracellular sources of thiols and dithiols, which include glutathione, lipoamide and the active sites of enzymes including ribonucleotide reductase, lipoamide dehydrogenase, and glutathione reductase (Handlon and Oppenheimer, 1988) are available.

Experimental

Apparatus

HPLC analyses were performed using a system constructed from an Altex 100A dual reciprocating, solvent-metering pump which delivered mobile phases at a flow rate of 1 ml min^{-1} to a stainless-steel column (10 cm × 4.6 mm) packed with 5 μm Hypersil-ODS (Shandon, U.K.) reversed-phase material. Samples were introduced through a Rheodyne 7120 injection valve fitted with a 10–100 μl loop as appropriate and UV detection was accomplished at 250 nm and 0.08–1.28 AUFS with a Pye Unicam LC3 variable wavelength UV detector equipped with an 8 μl flow cell. Chromatograms were recorded using

either a JJ instruments CR452 or an Omniscribe D5000 chart recorder operated at chart speeds of 12.0 and 12.5 cm h^{-1}, respectively. Mobile phases comprised aqueous acetonitrile (50–65%) containing 0.1% diethylamine with the pH adjusted to 2.5 with orthophosphoric acid.

^{1}H-NMR spectroscopy was conducted either at 60 MHz on a Varian EM 360A spectrometer or at 300 MHz on a Bruker AC 300 spectrometer in $CDCl_3$ or D_2O solvents with tetramethylsilane as reference. Mass spectra were recorded on a VG Micromass MM12 spectrometer, operating at an accelerating voltage of 4 kV, a trap current of 100 μA and a source temperature of 250°C, and infrared spectra were recorded either in KBr discs or in Nujol mulls on a Pye Unicam SP 200 infra-red spectrophotometer. All pH measurements were undertaken using a Radiometer PHM 62 Standard pH meter (2 decimal place display) or a WPA CD 660 Digital pH meter (3 decimal place display) in conjunction with a Gallenkamp combination glass electrode calibrated with Colourkey buffer solutions (BDH Ltd).

Methods

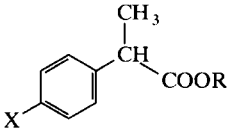
Synthesis of azidoprofen and esters

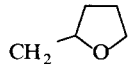
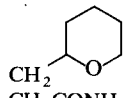
Compounds and analyses are identified in Table 1.

2-(4-Aminophenyl)propionic acid (1) To a solution of 2-(4-nitrophenyl)propionic acid (10.0 g, 51 mmol) in methanol (100 ml) was added 5% palladium on carbon (1 g), and the mixture was shaken overnight under hydrogen at atmospheric pressure. After removal of catalyst by filtration through kieselguhr, the solvent was evaporated in vacuo to furnish a cream solid, which was recrys-

TABLE 1

Azidoprofen and analogues (spectroscopic data were consistent with structures assigned



No.	X	R	Yield (%)	Formula	Elemental analysis % found (required)		
					C	H	N
1	NH_2	H	97	$C_9H_{11}NO_2$	–	–	–
2	NH_2	Na	45	$C_9H_{10}NO_2Na$	57.94 (57.75)	5.39 (5.35)	7.51 (7.49)
3	N_3	H	86	$C_9H_9N_3O_2$	–	–	–
4	N_3	Na	90	$C_9H_8N_3O_2Na$	50.70 (50.70)	3.77 (3.76)	19.60 (19.72)
5	N_3	Me	86	$C_{10}H_{11}N_3O_2$			
6	N_3	Et	78	$C_{11}H_{13}N_3O_2$			
7	N_3	*n*-Pr	75	$C_{12}H_{15}N_3O_2$			
8	NH_2	Me	60	$C_{10}H_{13}NO_2$			
9	NH_2	Et	77	$C_{11}H_{15}NO_2$			
10	NH_2	*n*-Pr	76	$C_{12}H_{17}NO_2$			
11	N_3	CH_2-CH_2-OH	95	$C_{11}H_{13}N_3O_3$			
12	N_3		92	$C_{14}H_{17}N_3O_3$	62.56 (62.28)	6.90 (6.57)	14.40 (14.53)
13	N_3		90	$C_{15}H_{19}N_3O_3$	61.05 (61.09)	6.47 (6.18)	15.10 (15.27)
14	N_3	CH_2CONH_2	77	$C_{11}H_{12}N_4O_3$	52.92 (53.22)	5.22 (4.84)	22.52 (22.58)
15	N_3	$CH_2CON(Et)_2$	62	$C_{15}H_{20}N_4O_3$	58.96 (59.21)	6.83 (6.58)	18.00 (18.42)

tallised from ethyl acetate-petroleum ether to give 8.2 g of the title compound. The sodium salt was prepared by dissolving the title compound (1.5 g) in sodium methoxide in methanol (0.1 g in 20 ml), removing the solvent in vacuo and triturating the resulting syrup with diethyl ether (30 ml), when a buff solid (2 g) deposited and was collected. Recrystallisation from aqueous ethanol afforded the required sodium 2-(4-aminophenyl) propionate (**2**) as cream microcrystals (1.01 g).

2-(4-Azidophenyl)propionic acid (3) A solution of 2-(4-aminophenyl)propionic acid (**1**) (4.0 g, 24.2 mmol) in 5 M hydrochloric acid (80 ml) was cooled to 0°C and aqueous sodium nitrite solution (2 g, 29 mmol, in 5 ml) was added dropwise over 15 min. After stirring for a further 30 min, sodium azide (6.4 g, 98 mmol) was added over 15 min with vigorous effervescence and the mixture was stirred for 1 h, poured into ice-water, and adjusted to pH 6.0 by the cautious addition of sodium bicarbonate. The solution was extracted with chloroform (2 × 50 ml) and, after washing with water (100 ml) and drying (Na_2SO_4), the solvent was removed in vacuo to give a pale yellow syrup (4.0 g) that slowly solidified on standing at 4°C. The sodium salt was prepared as described for (**2**) above, recrystallisation from aqueous propan-2-ol giving the required sodium 2-(4-azidophenyl)propionate (**4**) as photosensitive cream microcrystals.

Preparation of 2-(4-azidophenyl)propionic acid esters (General method A) To a suspension of 2-(4-azidophenyl)propionic acid (**3**) (0.5 g, 2.6 mmol) in methanol (50 ml) was added *p*-toluenesulphonic acid (1 g) and the mixture was heated under reflux for 12 h with protection from light. The yellow solution was cooled, triethylamine (2 g) was added, and the solvents were evaporated under reduced pressure to furnish a yellow syrup which was redissolved in ethyl acetate (50 ml). After washing with water (50 ml) and drying (Na_2SO_4) the ethyl acetate was removed to give methyl 2-(4-azidophenyl)propionate (**5**) as a yellow oil (0.46 g). Similarly prepared from 2-(4-azidophenyl)propionic acid and the appropriate alcohol were ethyl 2-(4-azidophenyl)propionate (**6**) and prop-1-yl 2-(2-azidophenyl)propionate (**7**).

Analogous reactions conducted with 2-(4-aminophenyl)propionic acid (**1**) and the appropriate alcohols afforded the methyl, ethyl and prop-1-yl esters (**8–10**) in comparable yield.

(General method B) A solution of 2-(4-azidophenyl)propionic acid (**3**) (1.0 g, 5.2 mmol) in dry tetrahydrofuran (30 ml) was stirred at room temperature, with protection from light, and thionyl chloride (1.0 g) was added. After the addition of dimethylformamide (2 drops) the mixture was stirred for a further 72 h at room temperature, dry methanol (5 ml) was added, and stirring was continued for an additional 2 h. Solvents were removed in vacuo to give a dark oil which was redissolved in ethyl acetate (50 ml), washed sequentially with saturated aqueous sodium bicarbonate solution (2 × 50 ml) and water (50 ml) and dried (Na_2SO_4). Removal of solvent afforded a pale yellow oil (1.1 g) which was purified by chromatography on silica gel, with 5% ethyl acetate in petroleum ether as eluent, to give methyl 2-(4-azidophenyl)propionate identical (TLC and NMR) to compound (**5**) prepared by method A above. Analogous reactions conducted with 1,2-ethanediol, tetrahydrofuranyl-2-methanol and tetrahydropyranyl-2-methanol furnished the requisite esters (**11–13**) in similar yields, and these were purified in the same manner.

2-(4-Azidophenyl)propionic acid glycolamide esters 2-[2-(4-Azidophenyl)propionyloxy]acetamide (**14**) was prepared essentially as described by Nielson and Bundgaard (1987) by refluxing a solution of sodium 2-(4-azidophenyl)propionate (**4**) (5 g, 23 mmol) in water (100 ml), containing 2-chloroacetamide (2.5 g, 27 mmol) and sodium iodide, for 6 h with protection from light. The dark oil that separated on cooling was extracted with ethyl acetate (2 × 100 ml), washed and dried as described above, and the solution was evaporated in vacuo to give 4.1 g of the glycolamide (**14**) as a pale yellow oil. Purification was achieved by chromatography on silica gel with 5% ethyl acetate in petroleum ether as eluent.

2-[2-(4-Azidophenyl)propionyloxy]-*N*,*N*-diethylacetamide (**15**) was synthesised as described for compound (**14**), and in comparable yield, by reacting 2-chloro-*N*,*N*-diethylacetamide with compound (**4**).

Reduction kinetics of azidoprofen

Effect of pH Phosphate buffer solutions (Perrin and Dempsey, 1974) were prepared in a pH range of 5.8–8.40 with a constant ionic strength of 0.5 M being maintained by the addition of potassium chloride. Values quoted in the text are final values in the reaction medium. A mixture comprising buffer (41 ml) and an aqueous stock solution (4 ml) containing dithiothreitol (22.6 mg ml^{-1}, 146.8 mM) was incubated in a reaction vessel, surrounded by a water jacket maintained at 37°C by a Churchill thermostatic pump. The mixture was stirred, protected from light, and, when equilibrated at 37°C, reaction was initiated by the addition of aqueous sodium azidoprofen (AZP-Na) stock solution (5 ml; 2.5 mg ml^{-1}, 11.7 mM). The final concentration of the reaction mixture was 1.17 mM and 11.7 mM with respect to AZP and DTT. The pH of the incubation mixture was monitored throughout the reaction and control experiments were also included with distilled water replacing the DTT solution.

Samples (0.5 ml) were withdrawn at appropriate time intervals and were diluted with an equal volume of buffer and butyl paraben solution (1 ml; 60 μg ml^{-1}, 309 mM) as internal standard. Aliquots (10 μl) of this solution were assayed by HPLC with a mobile phase containing 50% aqueous acetonitrile containing 0.1% diethylamine and acidified to pH 2.5 with phosphoric acid. Retention times were 3.1 min for AZP and 5 min for butyl paraben. Peak height ratios of AZP to internal standard were used to determine the percentage AZP remaining by reference to a calibration curve using standard solutions of AZP-Na over the same concentration range.

NMR analysis A McIlvaine buffer solution [$Na_2HPO_4 \cdot 2H_2O$ (24.42 mg ml^{-1}); citric acid dihydrate (6.597 mg ml^{-1}) (Perrin and Dempsey, 1974)] in deuterated water with a final pD of 6.95 (pH, 6.55) was prepared. To individual vials containing separately AZP-Na (10 mg) or DTT (72.3 mg, 10-fold molar excess; 144.6 mg, 20-fold; 361.5 mg, 50-fold) was added an aliquot of deuterated buffer (0.5 ml). The reaction was initiated by vigorously mixing AZP-Na and DTT solutions together. The resulting mixture was immediately transferred to an NMR tube, the final reaction concentration being 10 mg ml^{-1} (46.9 mM) in AZP-Na. For each kinetic experiment, the DTT concentration was varied (469.5 mM, 939.0 mM, 2.347 M). Spectra were collected at 23°C as shown in Table 2, using a Bruker AC 300 NMR spectrometer operating at 300 MHz. Results were analysed quantitatively by weighing the peak areas resulting from the aromatic protons for both AZP ($\delta_{Ar\text{-}H}$ 6.9, 7.2 ppm) and AMP ($\delta_{Ar\text{-}H}$ 6.7, 7.0 ppm). The percentage concentration of each species was derived by calculating each weight as a fraction of the total sum of areas for the two compounds.

TABLE 2

Conditions used to collect NMR spectra

DTT concentration (M)	Number of acquisitions per spectrum	Acquisition time (s)
0.4695	15	3.637
0.939	20	2.449
2.347	5	2.486

A comparative study between NMR and HPLC analysis was performed using stock solutions of AZP-Na (20 mg ml^{-1}, 93.9 mM) and DTT (144.6 mg ml^{-1}, 939.0 mM) in deuterated McIlvaine buffer (pD, 7.05) maintained at 23°C. For ^1H-NMR analysis, aliquots (0.5 ml) of each stock solution were thoroughly mixed together and transferred to an NMR tube. The final reactant concentrations were 10 mg ml^{-1} (46.9 mM) in AZP-Na and 72.3 mg ml^{-1} (469 mM) in DTT. Spectra were collected at intervals of 60 s, each consisting of 15 scans of 3.637 s and data were treated as above. For HPLC analysis, volumes (5 ml) of each stock solution were mixed together and maintained at 23°C, with constant stirring. Samples (100 μl) were withdrawn initially and at intervals of 2 min over a period of 30 min. Early samples (0–15 min) were immediately quenched by the addition of 0.1 M HCl (2 ml) followed by cooling to 0°C. Prior to analysis these samples were diluted (1:29) with butyl paraben solution (0.9 ml; 800 μg ml^{-1}, 4.12 mM) as internal standard. Samples removed after 15 min were quenched and diluted (1:14) by the addition of

0.1 M HCl (0.95 ml) and of internal standard (0.45 ml). The diluted samples (10 μl) were analysed by HPLC as above.

Reduction of azidoprofen propyl ester

Stock solutions for the propyl ester (2.50 mg ml^{-1}, 10.7 mM) and DTT (16.5 mg ml^{-1}, 107 mM) were prepared in a solvent composed of acetonitrile : 0.05 M phosphate buffer (50 : 50 v/v, final pH 7.4). An aliquot (5 ml) of the ester solution was introduced to a reaction mixture composed of solvent (40 ml) and the DTT stock solution (5 ml). The mixture, 1.07 mM in ester and 10.7 mM in DTT, was maintained at 37°C with stirring, while samples (0.5 ml) were removed at various intervals for a period of 84 h. The samples were quenched by the addition of 0.1 M HCl (1 ml), resulting in a final pH of approx. 1.0, and were then frozen. Prior to HPLC assay, the samples were diluted with a mixture of internal standards (0.5 ml) comprising ethyl salicylate (8 μg ml^{-1}, 48.2 μM) for the amino ester and propyl salicylate (80 μg ml^{-1}, 444 μM) for the azido ester. Two internal standards were necessary as the azido and amino derivatives were detected at different sensitivities, both at 245 nm, and were recorded on separate charts. Aliquots (100 μl) of the diluted solutions were analysed using a mobile phase comprising acetonitrile (50%) in aqueous sodium octane-1-sulphonate (0.1 M) with retention times of 14 min (propyl AZP), 4.3 min (propyl AMP), 5.8 min (ethyl salicylate) and 10 min (propyl salicylate). Concentrations were calculated by interpolation of the peak height ratios onto a calibration plot prepared from standards analysed under the same conditions. This experiment was repeated with a 50-fold molar excess of DTT with final concentrations of ester (291 μg ml^{-1}, 1.25 mM) and DTT (9.62 mg ml^{-1}, 62.5 mM). Samples (0.5 ml) were diluted with ethyl salicylate (240 μg ml^{-1}, 0.5 ml) as internal standard and 10 μl were assayed by HPLC with a mobile phase comprising 65% acetonitrile to monitor the degradation of the ester.

Anaerobic reduction of propyl AZP was followed by degassing a mixture of acetonitrile and 0.05 M phosphate buffer (50 : 50 v/v, final pH 7.4) with repeated boiling and cooling under a stream of nitrogen. Stock solutions of the ester (2.91 mg ml^{-1}, 12.5 mM) and DTT (19.25 mg ml^{-1}, 125 mM) were prepared in this solvent. An aliquot (5 ml) of the DTT stock solution was incubated with the degassed solvent (40 ml) purged with nitrogen and protected from light. The reaction was initiated by the addition of the ester stock solution (5 ml) to the reaction mixture, maintained at 37°C. Samples (0.5 ml) were taken at appropriate intervals and were suitably diluted with ethyl salicylate (240 μg ml^{-1} in 50% acetonitrile-buffer, 1.0 ml) as internal standard and solvent (50% acetonitrile-buffer, 0.5 ml) prior to HPLC analysis of 10 μl aliquots as above.

TABLE 3

Analytical conditions for the assay of AZP and esters

Compound	MeCN (%)	Internal standard (mg ml^{-1})	Retention time (min)		Sample diluent Internal standard : solvent (ml)	Sensitivity (AUFS)
			AZP derivative	IS		
AZP	50	Butyl paraben (0.08)	2.6	4.1	1.0 : 1.5	0.16
Methyl AZP	60	Propyl salicylate (1.2)	3.6	5.3	0.5 : 1.0	0.32
Ethyl AZP	60	Ethyl salicylate (0.3)	4.8	3.8	1.0 : 1.5	0.16
Propyl AZP	65	Ethyl salicylate (0.25)	5.3	3.4	1.0 : 1.0	0.16

Anaerobic reduction of azidoprofen and its alkyl esters

Stock solutions for the alkyl esters (methyl, ethyl, propyl) of AZP (12.5 mM in degassed acetonitrile) and DTT (125 mM in degassed phosphate buffer; pH, 6.65) were prepared. A solvent composed of acetonitrile (10 ml) and phosphate buffer (30 ml, pH 6.65) was degassed and equilibrated at 37°C. Aliquots (5 ml) of stock solutions of an ester and DTT were added with constant stirring. The reaction mixtures were 1.25 mM in ester and 12.5 mM in DTT, in a solvent composed of 30% v/v acetonitrile in buffer, final pH 7.4. The experiment was also repeated for AZP-Na but with the sodium salt dissolved in buffer and DTT in acetonitrile. Samples (0.5 ml) were removed at suitable time points. These were diluted with solvent and the required internal standard, as indicated in Table 3, prior to HPLC analysis of 10 μl aliquots using the appropriate concentration of acetonitrile in the mobile phase.

Results and Discussion

The incubation of azidoprofen with dithiothreitol (DTT) in the presence of base resulted in the conversion of the azide into 2-(4-aminophenyl) propionic acid (**1**; aminoprofen; AMP). Structural identification was confirmed by the synthesis of authentic material and comparison of chromatographic and spectroscopic data. This reaction was not evident in control experiments undertaken in the absence of DTT. The reduction of AZP in phosphate buffer at 37°C followed first-order kinetics over several half-lives throughout the pH range employed (pH 5.80–8.40) and rate constants (k) for the reduction and the corresponding half-lives ($t_{1/2}$) are reported in Table 4.

The rate of reduction increases exponentially with pH, indicating a base-catalysed reaction. Cartwright et al. (1976) observed a similar profile for the reduction of 8-azidoadenosine derivatives over this pH range, but reported a rate maximum at pH 10 with rates decreasing rapidly beyond this range. The observed rate constant, k_{obs}, for the reaction of an ionisable, anionic substrate is

TABLE 4

Effect of pH on the rate of reduction of azidoprofen at 37°C

pH	Rate constant (k) (min^{-1})($\times 10^3$)	Half-life ($t_{1/2}$) (min)
5.80	0.752	921.8
6.23	1.879	368.8
6.55	3.508	197.5
6.88	6.851	101.2
7.21	15.57	44.5
7.59	21.54	32.2
7.85	40.36	17.2
8.00	60.36	11.5
8.29	80.45	8.6
8.40	103.4	6.7

modelled by Eqn 1:

$$k_{obs} = \{k_1[H_3O^+] + k_2[H_2O] + k_3[HO^-]\} \cdot \{K_a/(K_a + [H_3O^+])\} + \{k_4[H_3O^+] + k_5[H_2O] + k_6[HO^-]\} \cdot \{[H_3O^+]/(K_a + [H_3O^+])\} \quad (1)$$

where k_1, k_2 and k_3 refer to the rate constants for proton, solvent and hydroxide catalysis, respectively, for the ionised species while k_4, k_5 and k_6 are the corresponding rate constants for the undissociated species. K_a is the dissociation constant for the acid and the terms $K_a/(K_a + [H_3O^+])$ and $[H_3O^+]/(K_a + [H_3O^+])$ refer to the fraction of the ionised ($[A^-]$) and unionised ($[HA]$) species, respectively. With a pK_a of 4.29 (Naik, 1990) AZP is predominantly in the ionised form over the pH range of this study and Eqn 1 reduces to:

$$k_{obs} = k_1[H_3O^+] + k_2[H_2O] + k_3[HO^-] \quad (2)$$

For reactions which are solely base-catalysed, or, indeed, in solutions of high pH where $[HO^-] \gg [H_3O^+]$, the $k_3[HO^-]$ term dictates the degradation rate and under such conditions Eqn 2 yields:

$$\log(k_{obs}) = \log(k_3 \cdot K_w) + pH \quad (3)$$

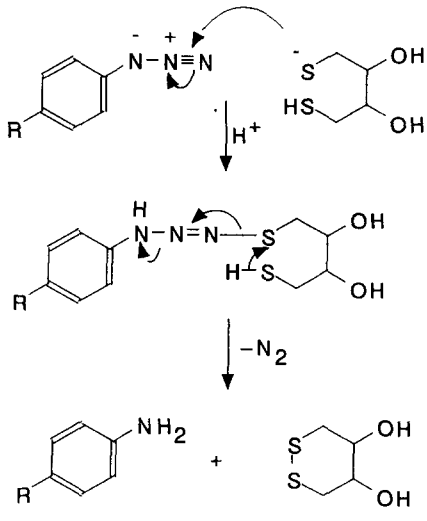


Fig. 1. Mechanism of reduction for aryl azides.

where K_w is the ionic product of water. Under these conditions a V-shaped dependence of log (rate constant) on pH is not observed and a plot of log k_{obs} vs pH provides a linear relationship with a slope equal to unity and an intercept equal to $\log(k_3 \cdot K_w)$. Analysis of the data in Table 4 gives a straight line ($r = 0.998$) with a positive slope of 0.8250 and a value for k_3 of 1.37×10^6 l mol^{-1} min^{-1}. This indicates that the reaction of AZP with DTT is exclusively base-catalysed although deviation of the slope from unity might imply that hydroxyl ion catalysis may not be the sole catalytic reaction influencing the reduction and other species, such as buffer components, may also be important. Indeed, phosphate ions are well known to complex with other organic and inorganic ions (Perrin and Dempsey, 1974). Alternatively, the ionisation of the DTT may be considered.

Fig. 1 illustrates a potential mechanism through which the reduction of azides is thought to proceed (Cartwright et al., 1976). Attack by the thiolate anion on the terminal nitrogen of the azide is followed by intramolecular cyclisation and loss of nitrogen to produce the cyclic disulphide and the arylamine. In this scheme, hydroxyl ions catalyse the reaction by ionising the thiol, rather than direct reaction with the azide group. Consequently, ionisation of the thiol moiety would be the base-dependent step. The pK_a values of DTT are 8.3 and 9.5 (Zahler and Cleland, 1968). Hence, over the pH range of study (5.80–8.40), the degree of ionisation of DTT and, therefore, the amount of thiolate anion available for nucleophilic attack, are sensitive to small pH changes. This indirect base catalysis may also explain the deviation from unity of the slope of the linear relationship between log k_{obs} and pH, since the degree of ionisation of the thiol as a function of pH will not be linear.

The rate of reaction (d[AZP]/dt) of azidoprofen is given by:

$$\frac{d[AZP]}{dt} = -k_2[AZP] \cdot [DTT^-] \quad (4)$$

where $[DTT^-]$ indicates the concentration of DTT monoanion and k_2 is the second-order reaction rate constant. On integration this leads to:

$$\ln\left(\frac{[DTT^-]_t}{[AZP]_t}\right) = \ln\left(\frac{[DTT^-]_0}{[AZP]_0}\right) + ([DTT^-]_0 - [AZP]_0) \cdot k_2 t \quad (5)$$

With the assumption that $[DTT^-] \gg [AZP]$ this will contract to the pseudo-first order case shown in Eqn 6.

$$\ln([AZP]_t) = \ln([AZP]_0) - [DTT^-]_0 \cdot k_2 t \quad (6)$$

This indicates that the first-order rate constant is related to the concentration of DTT monoanion by Eqn 7.

$$\log(k_{obs}) = \log(k_2) + \log([DTT^-]) \quad (7)$$

and a plot of log k_{obs} against log DTT monoanion concentration should be linear. The fraction of unionised (DTT^0), monoanionic (DTT^-) and dianionic (DTT^{2-}) forms of dithiothreitol are given by:

$$DTT^0 = [H_3O^+]^2/D \quad DTT^- = K_1[H_3O^+]/D$$

$$DTT^{2-} = K_1K_2/D$$

TABLE 5

Dependence of ionic composition of dithiothreitol on pH

pH	Ionic composition of DTT (%)		
	DTT^0	DTT^-	DTT^{2-}
5.80	99.68	0.32	
6.23	99.16	0.84	
6.55	98.25	1.75	
6.88	96.33	3.66	0.01
7.21	92.45	7.51	0.04
7.59	83.45	16.28	0.20
7.85	73.38	26.04	0.58
8.00	65.92	33.04	1.04
8.29	49.08	47.96	2.96
8.40	42.39	53.37	4.24

TABLE 6

Molar absorption coefficients for AZP and AMP under neutral and acidic conditions

Wavelength (nm)	Molar absorption coefficient ($l\ mol^{-1}\ cm^{-1}$)			
	AZP		AMP	
	EtOH (95%)	HCl (0.1 M)	EtOH (95%)	HCl (0.1 M)
210	15300	13960	7780	7430
220	4630	3420	4060	3750
230	3560	3560	8080	1700
240	6940	8970	11400	760
250	13480	14250	7730	510

where $D = [H_3O^+]^2 + K_1[H_3O^+] + K_1K_2$ and K_1 and K_2 are the successive dissociation constants of the dithiol. Solutions at the experimental pH values are presented in Table 5. Plotting these values according to Eqn 7 (standard errors in parentheses) gives:

$$\log k_{obs} = 0.934(\pm 0.0251)\ \log[DTT^-] - 0.999(\pm 0.0791)$$

The excellent linear relationship (Fig. 2; n, 10; r, 0.997; sum of squared deviations, 0.0276) and the closeness of the slope to unity confirms the validity of this analysis and indicates the importance of the thiolate monoanion in this reaction.

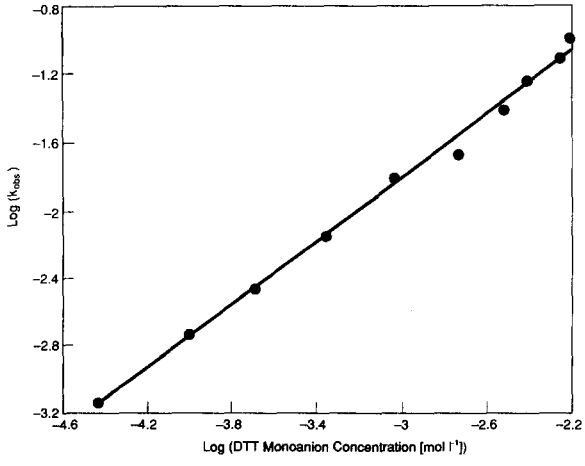


Fig. 2. Dependence of observed reduction rate constant on DTT monoanion concentration.

The HPLC method detected only AZP. The decomposition product AMP, as a result of increased polarity, eluted close to the solvent front and its formation could not be quantified under the same analytical conditions. Furthermore, at a mobile phase pH of 2.5 which was employed to ensure that AZP would be present in an unionised form, protonation of the amino (NH_2) residue to the NH_3^+ species occurred. This resulted in a lower molar absorption coefficient (Table 6) which further complicated attempts to measure simultaneously both AZP and AMP.

In contrast, ^{1}H-NMR spectroscopy enabled both AZP degradation and AMP formation (Fig. 3) to be observed. As AZP was reduced to AMP, an upfield chemical shift of both the *ortho* and *meta* protons (relative to the azido/amino substituent) on the aromatic ring was observed, due to the shielding effect produced by the electron-donating amino group. During a kinetic run, both species could be detected by the presence of the aromatic protons of AZP at 6.9 and 7.2 ppm and those of AMP at 6.7 and 7.0 ppm, respectively. To validate the spectroscopic assay, two analogous experiments, one using HPLC and the other NMR spectroscopy, were conducted. To minimise interference, reactions were performed in D_2O with the pD of the solution calculated as pD = pH meter reading + $429/T - 1.04$, where T is the absolute temperature (K). At 25°C this approxi-

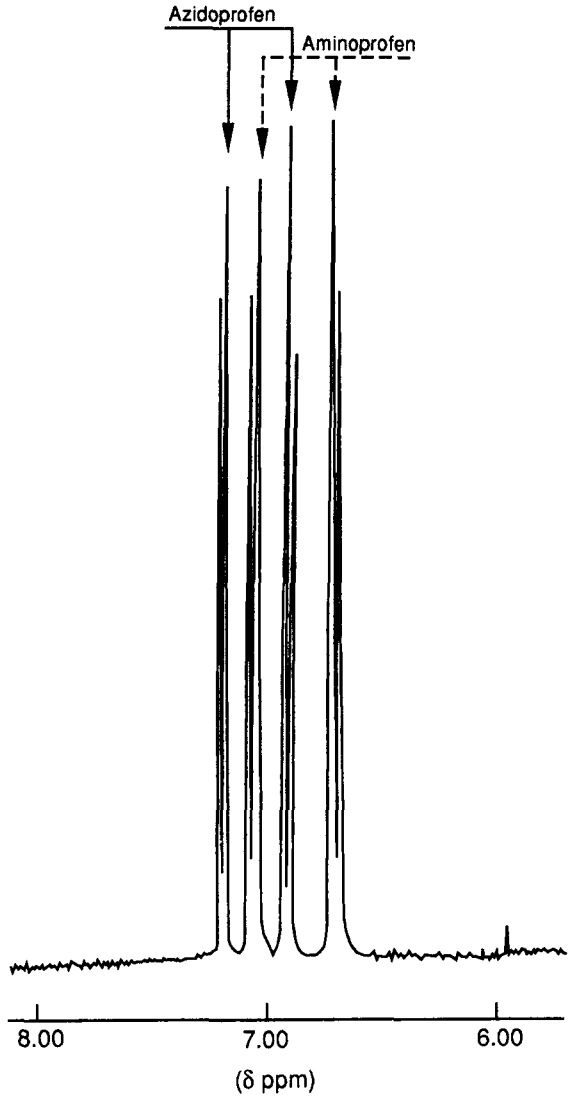


Fig. 3. NMR spectrum illustrating the upfield shift of the aromatic protons as AZP is reduced to AMP.

mates to an increase of 0.4 units (Fife and Bruice, 1961). Under these conditions the reduction profiles were almost superimposable and estimates for the rate constant ($k \pm$ SE, $n = 15$) were 1.64×10^{-3} ($\pm 1.95 \times 10^{-5}$) s^{-1} for the NMR method and 1.54×10^{-3} ($\pm 1.41 \times 10^{-5}$) s^{-1} by HPLC. The close agreement of the two values confirms the validity of the NMR method of analysis.

The effect of thiol concentration on the rate of AZP reduction was studied using NMR. All reduction profiles followed pseudo-first order kinetics with values of 1.003×10^{-3} s^{-1} (0.469 M DTT), 1.921×10^{-3} s^{-1} (0.938 M DTT) and 4.617×10^{-3} s^{-1} (2.33 M DTT) for the rate constants. These varied linearly with DTT concentration ($k = 1.924$ [DTT] $+ 1.074 \times 10^{-4}$, $r^2 = 1.000$). Following incubation of AZP (1.25 mM) with glutathione (GSH, 12.5 mM) in phosphate buffer (pH 7.4) at 37°C for 24 h, no azide degradation was observed. However, azide reduction was noticeable under more forcing reaction conditions (62.5 mM GSH, pH 8.4) over a period of 36 h. A rate constant of 3.09×10^{-2} was estimated but the aerobic conditions used may underestimate the true rate. Although reduction occurred at a much slower rate than with DTT, this preliminary study suggests that the potential does exist for intracellular conversion of azides by endogenous thiols such as glutathione.

Reduction of the alkyl esters of AZP with DTT was also studied. With these compounds the possibility exists for competitive reduction and hydrolysis leading to the kinetic scheme depicted in Fig. 4 (Irwin, 1990). HPLC methods were developed to detect the azido ester (A), amino ester (B) and azido acid (C) for all three esters (R = Me, Et, *n*-Pr), while the fourth component, AMP (D), was determined by mass balance. Preliminary experiments revealed no hydrolytic decomposition so that, in subsequent reduction studies, only the decomposition of the azido ester was kinetically modelled. Due to the low aqueous solubility of the esters of AZP, acetonitrile was employed as a co-solvent. This reduced the rate of degradation significantly with the rate constant

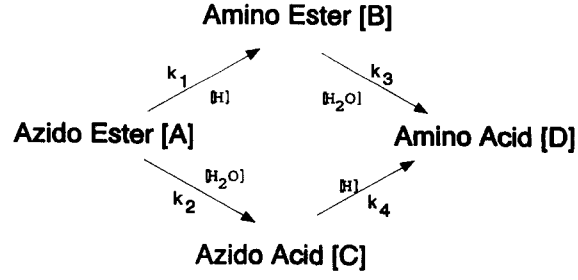


Fig. 4. Possible decomposition pathways for the alkyl esters of AZP.

for AZP in phosphate buffer at pH 7.4 being 1.74×10^{-2} min^{-1} while that in 30% acetonitrile-buffer at pH 7.4 was reduced to 3.64×10^{-3} min^{-1}. The consequent increased reaction times necessitated the introduction of an additional experimental precaution; that of performing the studies in the absence of oxygen.

Thiols are known to undergo oxidation on exposure to air (Capozzi and Modena, 1974). Disulphides are the dominant product, although prolonged reaction times cause oxidation beyond the disulphide level in aqueous solutions. Dithiothreitol degrades to the cyclic disulphide which may interfere with UV analysis of reaction mixtures unless extended chromophores are present (Baker et al., 1989). Both the rate (Wallace and Schriesheim, 1963a) and extent (Wallace and Schriesheim, 1963b) of oxidation are increased in dipolar-aprotic solvents under mild conditions. As reduction of the azido group is dependent on the presence of the un-oxidised monothiolate anion, the depletion of this species via aerobic oxidation during the course of the reaction could result in a reduced rate and extent of reduction. Furthermore, the presence of acetonitrile, a dipolar-aprotic solvent, may accelerate this process.

This possibility was supported by the reduction of the propyl ester in the presence of oxygen for a period of 84 h. The reaction failed to proceed beyond 80% completion and, as shown in Fig. 5, the rate of reduction (slope of plot) appears to decrease gradually with a plateau, indicating a cessation of the reaction process, occurring after 35 h. The possibility that a non-reducing impurity was present in the ester was eliminated by spectroscopy and chromatography. A 10-fold molar ratio was used for the reduction of the parent acid (AZP) to completion and this initial DTT concentration was sufficient to allow complete reduction of the ester. We have previously shown that the concentration of DTT governs the rate of reduction. Thus, when the DTT concentration was increased to provide a 50-fold molar excess the reaction then followed first-order kinetics over several half-lives (k, 0.0102 min^{-1}), as shown in Fig. 5. This suggests that the initial observations were associated with DTT depletion, probably by aerial oxidation. This was further confirmed by

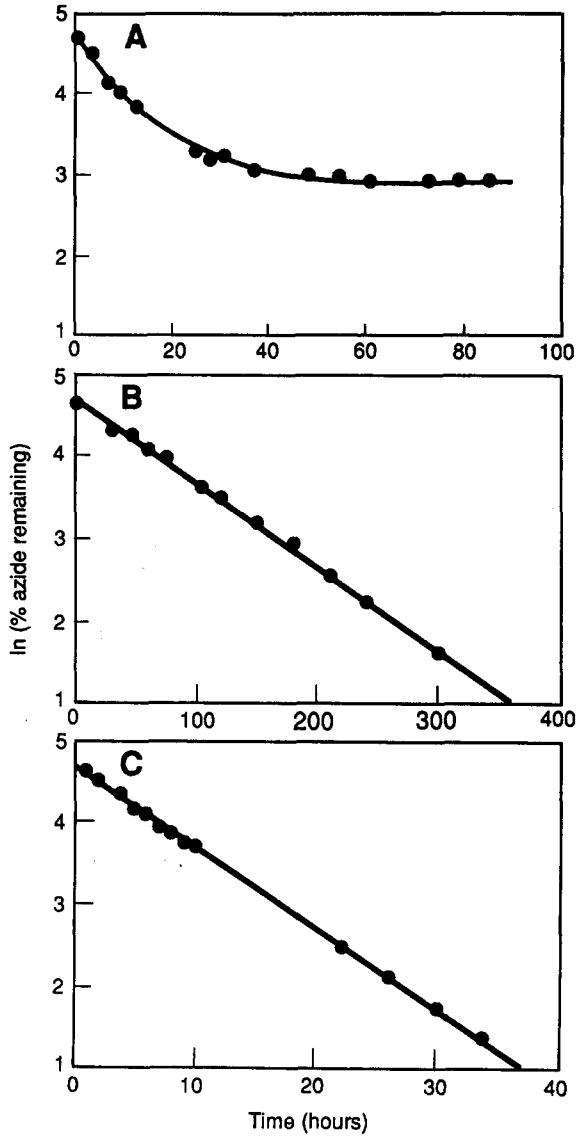


Fig. 5. Reduction of the propyl ester of AZP in 50% acetonitrile-buffer, pH 7.4 at 37°C. (A) Aerobic reduction with 10-fold molar excess of DTT; (B) aerobic reduction with 50-fold molar excess of DTT, $k = 0.0102$ min^{-1}; (C) anaerobic reduction with 10-fold molar excess of DTT, $k = 1.63 \times 10^{-3}$ min^{-1}.

repeating the reduction with only a 10-fold molar excess of DTT but in the absence of oxygen. Fig. 5 shows that the reaction now follows first-order kinetics with a reduction rate constant of 1.63×10^{-3} min^{-1} and $t_{1/2}$ of 7.14 h.

TABLE 7

Rate constants for the reduction of AZP and its alkyl esters in 30% acetonitrile-buffer, pH 7.4 at 37°C

Compound	Rate constant (k) (min^{-1})($\times 10^{-3}$)	Half-life ($t_{1/2}$) (min)
AZP	3.642	190.3
Methyl ester	8.050	86.1
Ethyl ester	7.242	95.7
Propyl ester	7.200	96.3

Thiol depletion by aerobic oxidation should therefore be regarded as an important phenomenon, particularly over prolonged reaction times. Subsequent reduction studies for the esters of AZP were performed under anaerobic conditions. The reduction kinetics for AZP and its methyl, ethyl and propyl esters show little difference in the pseudo-first order rate constants for the various esters, although the rank order of reduction rate constants (Table 7) is comparable to that of ester hydrolysis (Isaacs, 1987). AZP is reduced more slowly and this may be a consequence of its ionisation state. At a pH of 7.4 AZP is completely ionised and the electron-donating nature of the carboxylate anion may thus exert some stabilising influence on the azide moiety by rendering it less susceptible to nucleophilic attack by the thiolate ion. Charge repulsion between the two ionised species and ionic strength effects may also hinder reaction. In contrast, such effects are absent in the unionised esters. This picture is consistent with earlier findings (Staros et al., 1978; Baker et al., 1989) which reported faster rates of reduction for electron-deficient aryl azides, supporting a pathway involving rate-limiting nucleophilic addition to the azide group.

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