Photo-oxidation of 6-Mercaptopurine in Aqueous Solution

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Irradiation of oxygenated aqueous solutions of 6-mercaptopurine with near-u.v. light leads to purine-6-sulphinate as primary product. The sulphinate is itself unstable to u.v. light and is further oxidized to purine-6-sulphonate. Hypoxanthine is a minor product (<10%) of direct photolysis, but also arises from degradation of the sulphonate. Sensitized photo-oxidation using dyes (Methylene Blue, Rose Bengal, or Rhodamine B) gives the same products, and reaction in D_2O occurs at a greater rate, indicating that the primary photoreaction occurs *via* addition of singlet molecular oxygen.

6-Mercaptopurine [purine-6(1*H*)-thione] (1) is an antineoplastic agent used in the treatment of several types of leukemia; however, its use is restricted because of associated hepatotoxicity.[1] The molecule exists principally as the thione tautomer,[2] with strong u.v. absorption around 320 nm, and as a consequence is susceptible to near-u.v. light. In 0.1M-NaOH or as a suspension in distilled water it gives hypoxanthine when irradiated for 72 h.[3] Purine and hypoxanthine are the major products of irradiation in t-butyl alcohol.[4] Additionally, 6-mercaptopurine is susceptible to the photodynamic action of the dye Methylene Blue, but the products have not been identified.[5]

We report here the primary products of both near-u.v. and dye-sensitized photolysis of 6-mercaptopurine in oxygenated aqueous solution. The application of high-performance liquid chromatography (h.p.l.c.) has enabled the identification of a metastable product of the photodegradation.

Experimental

Irradiation Procedure.—A stock solution of 6-mercaptopurine monohydrate (Sigma Chemical Co.) in 0.05M-potassium hydroxide was diluted with water to a final concentration of 1.4×10^{-4}M with pH 10.2. When another pH was required a buffer solution of the appropriate pH was used for the dilution. The solution was irradiated in a cylindrical Pyrex vessel (30 ml), 40 mm from a Hanovia 125 W medium-pressure mercury arc in a thermostat at 30 °C. Oxygen was bubbled through the solution for 15 min prior to, and throughout, the irradiation.

For dye sensitization experiments, the solution also contained 5μM-Methylene Blue (Sigma), Rose Bengal (Koch-Light Laboratories), or Rhodamine B (Sigma). For irradiation of these solutions, a yellow glass filter (Corning 3-71, cut-off 470 nm) was positioned between lamp and vessel. Irradiation of the dyes or of 6-mercaptopurine alone behind the yellow filter produced no reaction.

Samples were withdrawn after various times and analysed variously [(i)—(iv)] by (i) h.p.l.c. with an Altex 330 isocratic system consisting of a model 110A pump and a u.v. detector (fixed wavelength, 254 nm) The column–mobile phase combinations (A)—(D) were used [(A) reverse phase 10 μm C-8 column, 250 × 4 mm (Brownlee RP-8), mobile phase 0.05M-phosphate buffer pH 7.3, 1 ml min^{-1}; (B) column as in (A), mobile phase 0.05M-phosphate buffer pH 7.3 containing 5mM-tetrabutylammonium phosphate (Ajax Chemicals) as ion-pair reagent; (C) reverse phase 10 μm C-18 (Partisil-ODS) column, 250 × 7 mm, mobile phase freshly distilled water, 1 ml min^{-1}; (D) 10 μm silica (Partisil) column, 250 × 4 mm, mobile phase chloroform–ethanol (76 : 24)]; (ii) fluorimetric measurements with a Perkin-Elmer MPF 44B spectrofluorimeter fitted with a 150 W xenon source (corrected fluorescence readings obtained with the Differential Corrected Spectra Unit DCSU-2); (iii) polarograms recorded after dilution of the test sample in a buffer of the desired pH, followed by deoxygenation (15 min bubbling with nitrogen), using a PAR model 364 polarographic analyser in the differential pulse mode, scan rate 2 mV s^{-1}; a three-electrode system was employed, consisting of a dropping mercury electrode with a controlled drop time of 1 s, platinum counter electrode, and saturated calomel reference electrode; (iv) u.v. spectra recorded after five-fold dilution in pH 7.3 phosphate buffer, with a Varian model 634 spectrophotometer.

Mass spectra were obtained with a Finnigan 6110-9500 system in the chemical ionization mode with a direct insertion probe. [8-^{14}C]-6-Mercaptopurine (specific activity, 30 mCi $mmol^{-1}$) was from CEA (France). Radioactivity was measured with a Packard Tri-Carb model 3255 liquid scintillation spectrometer with a toluene–Triton scintillation fluid.

For comparison purposes, purine-6-sulphinate (potassium salt), purine-6-sulphonate (potassium salt), and dipurin-6-yl disulphide were prepared from 6-mercaptopurine.[6] 6-Thiouric acid was prepared from 2,4-diamino-6-hydroxypyrimidine.[7] 8-Hydroxy-6-mercaptopurine was a gift from Dr. G. Elion (Wellcome Research Laboratories). All products had the expected u.v. spectra and microanalytical figures and were eluted as single peaks in h.p.l.c. systems (A) and (B). Hypoxanthine (Calbiochem), purine (Sigma), sodium azide (B.D.H.), and 1,4-diazabicyclo[2.2.2]octane (DABCO) (Aldrich) were used as received. All other chemicals were reagent grade.

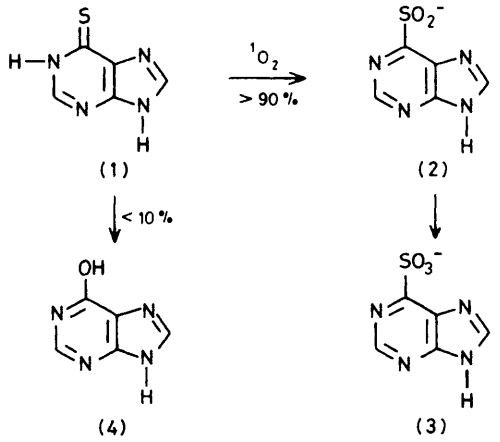
Heavy water (D_2O; 99.75%) was obtained from the Australian Atomic Energy Commission.

In experiments involving D_2O, the irradiations were performed with the light intensity reduced to 10% of that used in the earlier irradiations by means of a Corning 7-51 filter and increased separation of lamp and reaction vessel.

The quantum efficiency of the photochemical reaction was determined by ferrioxalate chemical actinometry.[8] The rate of oxygen consumption in irradiated solutions was determined using a Radiometer E5046 polarographic oxygen electrode as previously described.[9]

Results and Discussion

6-Mercaptopurine (1) is susceptible to oxidative degradation in aqueous solution when irradiated with near-u.v. light. The quantum yield for the disappearance of (1) was found to be $(1.15 \pm 0.03) \times 10^{-2}$ at pH 7. H.p.l.c. analysis of the irradiated solution as a function of time (Figure 1) indicated the presence of an intermediate (2) and two stable photoproducts (3) and (4). When oxygen was rigorously excluded from the solution these products were not found, nor were any other



Scheme.

changes evident in the solution after 4 h irradiation under the same conditions.

When [8-^{14}C]-6-mercaptopurine was added to the solution for irradiation, 91 ± 5% of the original radioactivity attributed to (1) was contained in the material represented by the four peaks in Figure 1 (inset).

The photodegradation is postulated to occur as shown in the Scheme. The photoproducts were identified as purine-6-sulphinate (2), purine-6-sulphonate (3), and hypoxanthine (4) by a combination of co-chromatography, fluorimetry, and polarography.

The presence of acidic photoproducts was indicated by a decrease in the pH of an aqueous unbuffered solution following irradiation. For example, 1.4 × 10^{-4}M-6-mercaptopurine had reacted completely after 120 min, during which time the pH fell from 10.2 to 8.7. This was accompanied by a loss of the strong absorption band at 323 nm (ε 1 920 m^2 mol^{-1} at pH 7.3) and the appearance of a new peak at 275 nm with approximately ⅓ of the original intensity.

The h.p.l.c. profile determined by u.v. detection was identical with that obtained by measurement of the radioactivity of fractions collected from irradiated [8-^{14}C]-6-mercaptopurine (Figure 1 inset). The comparison implies that the purine chromophore was still intact following irradiation. Photosensitized oxidation of xanthine, theophylline, and uric acid in alkaline solution has been shown to result in loss of C-8.[10]

The solution, which was non-fluorescent prior to irradiation, developed strong fluorescence provided the pH was above 8 ($\lambda_{ex.}$ 285 nm, $\lambda_{fluor.}$ 392 nm). These fluorescence. characteristics resembled those obtained following oxidation of 6-mercaptopurine by alkaline permaganate, thereby producing purine-6-sulphonate.[11] Purine-6-sulphonate, prepared according to the method of Doerr *et al.*[6] was found to run concurrently with peak (3) in both h.p.l.c. systems (A) and (B). The presence of the ion-pair reagent tetrabutylammonium phosphate in system (B) had the effect of lengthening the retention times of peaks (2) and (3), suggesting these to be anionic species.

Further evidence of the identity of (3) was obtained by virtue of its ready degradation to hypoxanthine when the irradiated solution was acidified (*cf.* ref. 6) and from the close agreement in polarographic behaviour. Half-wave potentials ($E_{\frac{1}{4}}$) for (3) in pH 5.0 buffer were −0.87, −1.05, and −1.27 V. The same values were recorded for a 120-min-irradiated solution adjusted to pH 5.0. Similar agreement was found at pH 7.3. The stable form of (3) prepared was the potassium salt, a factor which precluded molecular weight confirmation by conventional ionization mass spectroscopy. Similarly, the

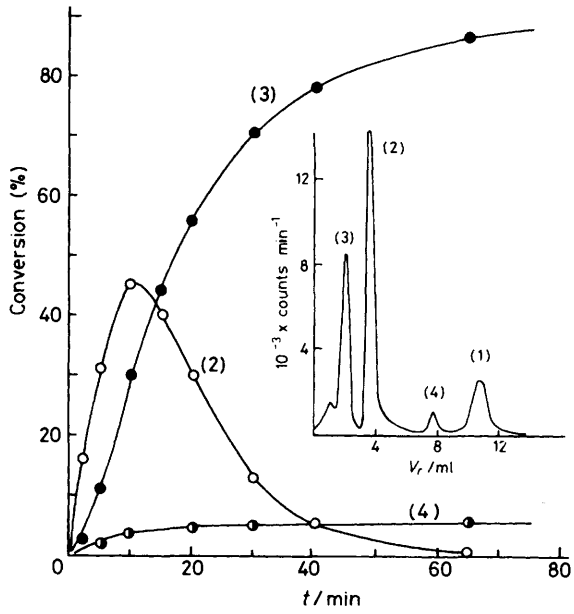


Figure 1. U.v. photodegradation products of 6-mercaptopurine (1.4 × 10^{-4}M) in oxygenated aqueous solution (initial pH 10.2) at 30 °C; products were separated by h.p.l.c. in system (A) (see Experimental section), and the detector response was calibrated with authentic samples. *Inset:* h.p.l.c. elution profile [system (A)] of 10-min-irradiated solution of [8-^{14}C]-6-mercaptopurine detected by scintillation counting (eluant collected in 0.2 ml fractions)

fraction collected from the h.p.l.c. peak (3) failed to yield a meaningful mass spectrum in our system. Attempts to separate the un-ionized sulphonic acid resulted in degradation to peak (4).

Hypoxanthine has been detected previously as a major product from irradiated 6-mercaptopurine.[3,4] Peak (4) was shown to be hypoxanthine by co-chromatography in h.p.l.c. systems (A), (B), (C), and (D), and by the molecular weight (136) for fractions collected from systems (C) and (D). Although a relatively minor product (Figure 1), it becomes increasingly important as the pH of the solution is decreased. When the irradiation was performed in 0.05M-H_2SO_4, (4) was the only product, indicating all others to be labile in acid solution. Compound (4) was also produced in higher yield (50%) from irradiation of (1) in methanol.

Peak (2) is the major product of short-time irradiations. Mass spectral analysis failed to determine a molecular weight for collected fractions, indicating that it was unstable and/or non-volatile. It was identified as purine-6-sulphinate following co-chromatography with an authentic sample in h.p.l.c. systems (A) and (B), and close similarity in polarographic behaviour at short irradiation times [$E_{\frac{1}{4}}$ for (2) at pH 5.0, −0.84 and −1.39 V]. When (2) was itself subjected to irradiation, (3) was the only product detected. The photolability is a consequence of (2) having a weak absorption at 340 nm. On the other hand, (3) does not absorb above 300 nm and is not degraded under the irradiation conditions used here. Both (2) and (3) absorb strongly at 275 nm, accounting for the observed shift in u.v. spectrum.

In the mass spectral analysis of fractions collected from h.p.l.c. system (C), a compound of molecular weight 120 was detected in the fraction corresponding to (3). The same compound was also detected using system (D), where its formation could be detected concurrently with the degradation of the preceding peak. This compound was identified as purine. Purine was reported[4] among the photo-oxidation

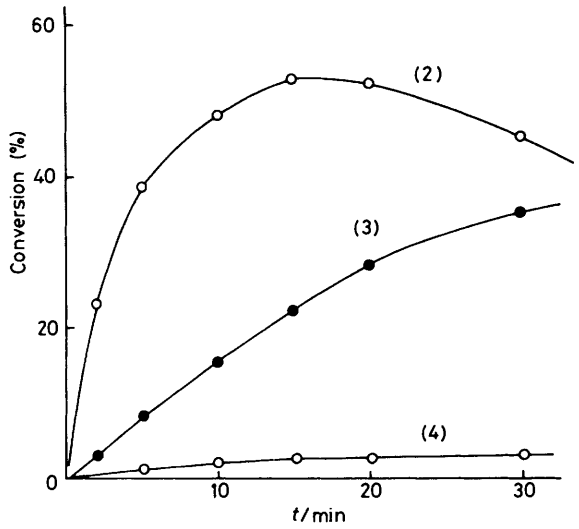


Figure 2. Photosensitized degradation of 6-mercaptopurine (1.4×10^{-4}M) with Rose Bengal (5 μM) at 30 °C; products separated by h.p.l.c. [system (A)]

products of (1) in t-butyl alcohol, although its production could not be explained *via* an oxidative pathway. A careful examination of our chromatograms failed to reveal any evidence of purine, which has a long retention time [18—22 min in systems (A) and (B)] as compared with the detected photoproducts [*e.g.* 4.2 min for (3) in system (A)]. Studies on the stability of (3) in the dark indicated breakdown to (4) and purine in aqueous solution over several days, with slightly faster reaction in methanol or ethanol. The dark-reaction products of (2) were found to be (3) and (4) but not purine.

Wenska and Paszyc[4] determined the photo-oxidation products of (1) in t-butyl alcohol to be (4) (36%) and purine (20%). Presumably (3) accounted for the remainder. We repeated the irradiation in t-butyl alcohol, propan-2-ol, and methanol. In each case, the major products were (3) (40%) and (4) (50%). Only very small amounts of (2) (<5%) were detected, and purine was a dark-reaction product occurring after work-up subsequent to the irradiation. Although (2) might be a relatively unstable intermediate, we were unable to demonstrate that degradation of (2) was more rapid in alcohol solutions than in water.

Oxygen uptake measured using a polarographic oxygen electrode was correlated with loss of absorbance at 323 nm (pH 7) and indicated that 1.0 ± 0.1 mole of O_2 was absorbed per mole of (1) for the initial stages of the reaction (<20% conversion). This result implies the direct addition of a molecule of oxygen to (1).

According to Doerr *et al.*[6] the product of mild chemical oxidation of (1) by iodine at pH 7.6 is dipurin-6-yl disulphide (R–S–S–R), which in the absence of O_2 undergoes reaction with hydroxide ion to return (1) as major and (2) as minor product by equation (i). In the presence of O_2, the sole product of disulphide decomposition is (2), by equation (ii).

$$2\ R\text{–}S\text{–}S\text{–}R + 4\ OH^- \longrightarrow RSO_2^- + 3\ RS^- + 2\ H_2O \quad (i)$$

$$R\text{–}S\text{–}S\text{–}R + O_2 + 4\ OH^- \longrightarrow 2\ RSO_2^- + 2\ H_2O \quad (ii)$$

On the other hand, biological oxidation of (1) has been shown to lead to 6-thiouric acid,[12] through 8-hydroxy-6-mercaptopurine.[13] Accordingly, a search was made using h.p.l.c. and authentic samples of dipurin-6-yl disulphide, 8-hydroxy-6-mercaptopurine, and 6-thiouric acid, but no evidence was found of their presence among the photo-products of (1).

The photo-oxidation of (1) is believed to proceed mainly by a singlet molecular oxygen mechanism. Supporting evidence was obtained as follows.

(a) Dye-sensitized photo-oxidation of (1) using the $^1O_2$ generating dye Methylene Blue, Rose Bengal, and Rhodamine B gave the products as for the direct photo-oxidation with a higher yield of (2) (see Figure 2).

(b) The yield of (2) in the direct photo-oxidation of (1) was diminished when an $^1O_2$ quencher [sodium azide (0.01M) or DABCO (0.001M)] was added. Similarly, in the dye-sensitized reaction the yield was reduced.

(c) The lifetime of $^1O_2$ in D_2O is 20 μs (*cf.* 2 μs in H_2O).[14] This difference may result in an increase in $^1O_2$-mediated reactions in D_2O *vs.* H_2O by a factor between 2 and 10.[15] We have observed an increase by nearly two-fold for formation of (2) from (1) but no effect on the photo-oxidation of (2) to (3) when each was irradiated at a concentration of 1.4×10^{-4}M.

The yield of (4) in aqueous reaction systems was small, suggesting that it may be a secondary product arising from decomposition of (2) and/or (3). However, when solutions of (2) or (3) were kept in the dark, at least 24 h were required before detectable amounts of (4) were seen, indicating relative thermal stability over the course of the irradiations. Nevertheless, some (4) was detected in the dye-sensitized photo-oxidation of (1), in conditions where the product (2) was not being irradiated. Also, when (2) was directly irradiated forming (3), no (4) was detected. We conclude that a small proportion (5—10%) of photoexcited (1) is degraded directly to (4) in alkaline solution.

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