Photodissociable dimer reduction products of 2-thiopyrimidine derivatives.

ABSTRACT
Both 4,6-dimethyl-2-thiopyrimidine and its 1-methyl derivative undergo polarographic reduction in aqueous medium, via a 1e/1H reduction to a free radical which rapidly dimerizes to products isolated and identified as 4,4'-bi-(4,6-dimethyl-3,4-dihydropyrimidin-2-thione) and the corresponding 1-methyl dimer. The dimers may be oxidized electrolytically to regenerate the parent monomers. Both dimers also undergo photodissociation to quantitatively regenerate the parent monomers, in high quantum yield, 0.23 and 0.35 M/Einstein. The correlation between electrochemical and photochemical reductions of 2-thiopyrimidines are discussed, as well as the significance of the dimer photodissociation reactions in relation to nucleic acid photochemistry.

INTRODUCTION
In an earlier study on the mechanism of electrochemical reduction of biologically important pyrimidines and pyrimidine nucleosides,1,2 it was demonstrated that reduction of cytosine and cytidine led to formation of a dimer of reduced pyrimidone-2, characterized as 6,6'(4,4')-bi-(3,4(6)-dihydropyrimidone-2) and the corresponding nucleoside. Such dimers readily underwent photochemical dissociation to the parent pyrimidone-2 (or its nucleoside) in high quantum yield.

Apart from the intrinsic interest of the reduction reactions themselves, the ability of the dimer reduction products to photodissociate is of potential biological interest. This is further underlined by the fact that the dimer reduction product of pyrimidone-2 turned out to be an integral component of a tetramer photoproduct isolated from UV-irradiated DNA.3,4

The foregoing findings were subsequently extended to other pyrimidine and purine analogues. In particular, during the course of some studies on the electrochemical properties of the biologically important thio- and methylthio-uracils,5,6 examination of the reduction products of methylated 2-thiopyrimidines (Scheme 1) pointed to formation of a dimer analogous to that obtained from pyrimidone-2. This is the subject of the present communication.
The foregoing findings take on added significance in the light of recent work demonstrating a correlation between electrochemical and photochemical reductions and/or oxidations of such biologically important compounds as nucleic acid derivatives\textsuperscript{7} and nucleotide coenzymes.\textsuperscript{8,9}

**MATERIALS AND METHODS**

**4,6-dimethyl-2-thiopyrimidine (I):** This was obtained as the HCl salt by condensation of thiourea with acetylacetone as described by Hunt et al.\textsuperscript{10} in 87\% yield, m.p. 145\degree C, sublimation at 190-200\degree C. It was converted to the free base by deposition on a Dowex 50W (H\textsuperscript{+}) column, which was washed with water until the effluent was neutral, and the free base then eluted with 5 N NH\textsubscript{4}OH, and crystallized from ethanol-ether in the form of pale yellow needles, m.p. 209-212\degree C (lit. 210\degree C).\textsuperscript{11}

**1,4,6-trimethyl-2-thiopyrimidine (II):** The HCl salt was prepared in a manner analogous to I by modification of the procedure of Hele & Williams.\textsuperscript{11} To a solution of 1.8 gm (20 mmoles) N-methylthiourea in 50 ml ethanol was added 2.6 ml acetylacetone and 5 ml conc. HCl, and this was heated under reflux for 2 hrs. Crystallization commenced after 1.5 hrs heating. The reaction mixture was transferred to the refrigerator overnight, following which the deposited crystals were collected by filtration, and washed with ethanol and ether to yield 2.58 gm (70\%), m.p. 200-203\degree C (decomp.). This was converted to the free base as for I, above, but with the use of an excess of ether in the ethanol-ether mixture used for recrystallization, yielding long pale yellow needles, m.p. 159\degree C (lit. 156.5\degree C).\textsuperscript{11}

**Polarograms** were recorded on a Radiometer Polarizer PO4, using a dropping mercury electrode (DME) with a flow rate \( \nu = 2.54 \text{ mg sec}^{-1} \), and drop time \( t = 3.3 \text{ sec}^{-1} \) at open circuit in distilled water at a mercury...
pressure \( h = 60 \, \text{cm} \). All curves were recorded at \( 25^\circ \text{C} \), and potentials are referred to the saturated calomel electrode (SCE). Solutions were maintained at the desired pH with Britton-Robinson buffers at an ionic strength corresponding to 0.2 by addition of KCl. Oxygen was removed by bubbling nitrogen through the solutions as described by Meites.\(^{12}\)

**Controlled potential (preparative) electrolysis** was carried out with a mercury pool electrode (area \( 12 \, \text{cm}^2 \)), with a manually controlled cathode potential, and a silver coulometric system to determine the \( n \)-value (no. of electrons involved in reduction process). The cell was flushed continuously with nitrogen during electrolysis.

**Thin-layer chromatography** made use of silicagel GF\(_{254}\), using chloroform-methanol (9:1, v/v) for development.

**UV absorption spectra** were run on a Zeiss (Jena, GDR) VSU-2P spectrophotometer. Mass spectra were obtained with an LKB 9000 spectrometer at 70 eV and 270°C. A Radiometer PHM-4d compensating instrument, with semi-micro glass electrode, was employed for pH measurements.

UV irradiation of solutions in spectral 10-mm pathlength cuvettes was with a Philips TUV 6-W germicidal lamp (254 nm), the radiation from which was first filtered through a 5-mm layer of 30% aqueous acetic acid to remove traces of radiation below 230 nm.

**RESULTS**

**Polarography at DME**

4,6-dimethyl-2-thiopyrimidine: Over the pH range 1.8 to 11.5, 4,6-dimethyl-2-thiopyrimidine was found to give three polarographic waves, as illustrated in Fig. 1:

Wave I was a typical adsorption wave ("pre-wave") of the reduction product(s), based on the following criteria: (a) the linear dependence of the limiting current on concentration over the range 0.05 mM - 0.1 mM; at higher concentrations the wave height was constant, and concentration-independent; (b) the wave height was proportional to \( h_{\text{corr.}} \) (\( h_{\text{corr.}} \) is the mercury column pressure corrected for the back-pressure); (c) the temperature coefficient was of the order of 0.5%/°C; (d) addition of surfactants, such as Triton X-100 or gelatine (see below) resulted in complete suppression of this wave. The half-wave potential, \( E_{1/2} \), of wave I was pH-dependent, and the change in slope of the \( E_{1/2} \) vs pH curve in the neighbourhood of pH 3 (Fig. 1b) is in agreement with the reported pK, for protonation of the depolarizer, \( \sim 2.8 \).\(^{13}\)
Wave II is a typical reduction wave, and its height corresponds to the transfer of one electron. Its diffusion-absorption character at pH 9 is testified to by the linear dependence of limiting current on $h_\text{corr}$, the temperature coefficient of $2\% / ^\circ\text{C}$, and the proportionality of the wave height to concentration over the range 0.05 – 1.0 mM. At more elevated concentrations the wave is split into two, of which one is a "pre-wave". At pH 9.5 the wave is diffusion-like in nature. The height of wave II is pH-dependent; at about pH 10 it begins to decrease abruptly, to disappear completely at pH $\sim 11.4$ (Fig. 1a). The foregoing pH-dependence of wave height corresponds very well with the conversion of the neutral form of 4,6-dimethyl-2-thiopyrimidine to the polarographically inactive anion, in agreement with the reported pK for anion formation, pK$_2 \sim 8.5$. The change in slope of the $E_{1/2}$ vs pH curve of wave II in the neighbourhood of pH 7 (Fig. 1b) is also in accord with the pK$_2$ for monoanion formation of the depolarizer.

Wave III shows up only in acid medium (Fig. 1a). The remarkably high value of the current for this wave, which is very strongly pH-dependent, when considered in conjunction with its disappearance in the presence of surfactants such as Triton X-100, indicate that it is a surface catalytic wave.

1,4,6-trimethyl-2-thiopyrimidine: This compound, the 1-methyl derivative of 4,6-dimethyl-2-thiopyrimidine, which cannot undergo ionization to the anion, was selected for comparison purposes, and because of the
anticipated higher solubility of its reduction product (see below).

Its polarographic behaviour (Fig. 2) is similar to that of the parent 4,6-dimethyl-2-thiopyrimidine. Wave I is a typical adsorption wave of the reduction product. Its $E_{1/2}$ is pH-dependent (Fig. 2b) and the change in slope of the $E_{1/2}$ vs pH curve in the vicinity of pH 6 corresponds to protonation of the depolarizer and exhibits the characteristic alkaline shift with respect to $pK_a$ of the depolarizer, which is 3.15.

Wave II is the reduction wave of 1,4,6-trimethyl-2-thiopyrimidine. Its height corresponds to transfer of one electron and is independent of pH, in agreement with its inability to form the anion. Wave II is diffusion-like in nature, the limiting current being proportional to $h^{1/2}_{corr.}$ as well as to concentration over the range 0.05 to 0.75 mM, while the temperature coefficient is of the order of 1.8%/°C. At concentrations in excess of 0.75 mM, wave II undergoes splitting into two, of which one is a "post-wave" which is due to adsorption of the substrate in the electrode process.

The properties of wave III are virtually identical with those for the parent 4,6-dimethyl-2-thiopyrimidine.

Effect of surfactants: In the presence of 0.002% Triton X-100, both the compounds exhibit only reduction waves (Fig. 3). For 4,6-dimethyl-2-thiopyrimidine, the wave-height was pH-dependent, and disappears completely at pH 11.4 where only the polarographically non-reducible monoanion exists. The wave-height for 1,4,6-trimethyl-2-thiopyrimidine was,
as expected, invariant with pH.

For both derivatives $E_{1/2}$ was pH-dependent. In the case of 4,6-dimethyl-2-thiopyrimidine this dependence may be expressed in the form $E_{1/2}/V = -0.81 - 0.08pH$; for the 1-methyl analogue this is $E_{1/2}/V = -0.795 - 0.07pH$. These relationships hold in the pH range 0 - 11.5.

On the basis of a logarithmic analysis of the wave ("log-plot"), the values $\alpha_n^a$ were calculated and it was established that the electrode processes for both depolarizers were non-reversible. With the aid of the calculated values of $\alpha_n^a$ and the magnitudes of the changes in $E_{1/2}$ with pH, the electrode reaction was found in both cases to involve participation

![Figure 3](image)

Fig. 3. pH-dependence of limiting current and $E_{1/2}$ in the presence of 0.002% Triton X-100 for 0.5 mM solutions of: (a) 4,6-dimethyl-2-thiopyrimidine; (b) 1,4,6-trimethyl-2-thiopyrimidine.
of 1 proton per molecule depolarizer.

Attention was then directed to isolation of the reduction product(s) and their identification.

**Preparative electrolysis and coulometry**

4,6-dimethyl-2-thiopyrimidine, and its 1-methyl derivative, were each electrolyzed at a concentration of $5 \times 10^{-3}$ M and pH 6 - 7, at an applied potential corresponding to the initial limiting current of wave II, as well as wave III. In each case the course of electrolysis was accompanied by the gradual disappearance of waves I and II. Wave III remained unaltered, in agreement with its origin as the catalytic wave of the reduction products.

With each of the two starting compounds, electrolysis of wave II led to formation of a white precipitate, while coulometric measurements were consistent with a one-electron process. Each of the reduction products was collected by centrifugation and washed with water.

The reduction product of 4,6-dimethyl-2-thiopyrimidine was highly insoluble in a wide variety of solvents. An exception was DMSO in which, however, it underwent degradation. Its high insolubility in water facilitated its isolation in 65% yield directly from the electrolyzed solution. Following repeated washing with water and ethanol, the dried white product turned yellow at 315°C, but did not melt even at 360°C. Elementary analysis gave C, 50.65%; H, 6.32%; N, 19.77%, corresponding to the parent monomer plus 1 hydrogen; calculated for C$_{12}$H$_{16}$N$_4$S$_2$ (dimer consisting of two reduced rings): C, 51.05%; H, 6.43%; N, 19.85%. In the presence of 0.2% aqueous DMSO (essential to dissolve even the small quantity necessary for UV spectroscopy), it exhibited a single broad asymmetric absorption band with $\lambda_{max}$ 254 nm, $\varepsilon_{max}$ 20.4 $\times 10^3$. It was too unstable in acid and alkaline media to record spectra. The mass spectrum showed a peak at e/m 282 (1%, M$^+$), corresponding, like the dimer reduction product of pyrimidine-2, to a dimer consisting of two reduced rings of 4,6-dimethyl-2-thiopyrimidine. Additional intense peaks at e/m 141 (100%) and 140 (34%) corresponded to the protonated and neutral forms of the parent 4,6-dimethyl-2-thiopyrimidine, C$_6$H$_8$N$_4$S. Additional peaks at e/m 127 (6%), 125 (8%), 109 (5%), 82 (19%), 67 (8%), 42 (16%), represent fragmentation products, similar to those reported by Pfoertner for a similar dimer obtained by a photochemical route, but with a m.p. 274°C.

The reduction product of 1,4,6-trimethyl-2-thiopyrimidine was more soluble, particularly in methanol and ethanol. It crystallized from aqueous methanol as tiny needles, m.p. 215°C. Elementary analysis gave
C, 53.82%; H, 7.09%; N, 18.32%; calculated for C_{14}H_{22}N_{2}S (dimer consisting of two reduced rings): C, 54.14%; H, 7.14%; N, 18.05%. In aqueous medium at pH 6.5 the product exhibited a single broad, asymmetric UV band with $\lambda_{\text{max}}$ 258 nm, $\varepsilon_{\text{max}}$ 22.8 x 10^3 (see Fig. 5). In the mass spectrum there was a peak at e/m 310 (1%, M^+), corresponding to the dimer C_{14}H_{22}N_{2}S. Peaks at e/m 155 (100%) and 154 (29%) were for the protonated and neutral forms of the monomer, C_{7}H_{10}N_{2}. Additional peaks at e/m 139 (10%), 129 (9%), 128 (9%), 96 (6%), 94 (3%), 81 (4%), 66 (3%), 56 (10%) were products of fragmentation.

Note that the major intense peak corresponding to the dimer has a value e/m = 310, i.e. higher by 28 than the value for the 4,6-dimethyl-2-thiopyrimidine reduction product (e/m = 282), in accord with the presence of two additional methyls minus two hydrogens.

**Identification of dimer reduction products**

By analogy with the nature of the dimer reduction products of pyrimidone-2, it appeared likely that the products of reduction of 4,6-dimethyl-2-thiopyrimidine and its 1-methyl derivative were similar dimers. This is supported by elementary analysis, the mass spectral data for both dimer reduction products, as well as by the modifications in absorption spectra in going from the monomers to the dimers.

Dimerization may occur via formation of 4,4', 5,5' or 6,6' bonds (in the case of the dimer of 4,6-dimethyl-2-thiopyrimidine the 4,4' and 6,6' bonds are identical because of symmetry about the 2,5 positions). Unfortunately, poor solubility in various solvents, and instability in DMSO, precluded the running of NMR spectra. Attempts to obtain suitable crystals for X-ray diffraction have thus far been unsuccessful. But available data point to dimerization via 4,4'. For example, it is known that reduction of 2-thiopyrimidine occurs at the N=C double bond, since reduction of this bond in 2-thiouracil confers resistance to electrochemical reduction (M. Wrona, B. Czochralska & D. Shugar in preparation). Dimerization via a 4,4' linkage is observed in the electroreduction of pyrimidone-2 derivatives, and the dimer reduction product of pyrimidone-2, 6,6'(4,4')-bis-(3,6(4)-dihydropyrimidine-2) possesses properties similar to those of the reduction products of the 2-thiopyrimidine derivatives. The dimer reduction product of 4,6-dimethyl-2-thiopyrimidine would consequently be 4,4'-bis-(4,6-dimethyl-3,4-dihydropyrimidin-2-thione) (III), while that for the trimethyl derivative would be IV (Scheme 2). The one-electron reduction product of pyrimidine has been ascribed a similar structure on the basis of ESR.
spectroscopy. During the course of this study, Pfoertner reported that photochemical reduction of 4,6-dimethyl-2-thiopyrimidine in 2-propanol led to formation and isolation of a dimer identical to III.

The localization of the double bonds in III and IV, as shown, is based on analysis of the UV spectra, from which it follows that the C=S double bond is not conjugated with other double bonds. Such conjugation, as in 4-thiouracil and 2-thiopyrimidine, leads to a shift in the location of the principal long wavelength absorption band to the region above 300 nm. The absorption spectra of the dimer reduction products III and IV are, in fact, similar to that for 5,6-dihydro-2-thiouracil, in which the C=S bond is not conjugated.

Additional evidence for the structure of the dimers III and IV is provided by their ability to photochemically regenerate the respective parent monomers I and II, described in the next section.

**Dimer photodissociation**

Irradiation at 254 nm of a neutral aqueous solution (containing 0.2% DMSO to improve solubility) of the dimer reduction product of 4,6-dimethyl-2-thiopyrimidine led to a very rapid disappearance of the characteristic 254 nm absorption band of the dimer, and the simultaneous appearance of two new bands at 276 nm and 332 nm, corresponding to the absorption spectrum of the parent 4,6-dimethyl-2-thiopyrimidine (Fig. 4). The latter is itself slightly photosensitive, but its rate of photochemical transformation is much lower than that of the foregoing dissociation reaction. The isosbestic points at about 230 nm and 263 nm are consistent with transformation of the dimer reduction product to a single new photoproduct. Apart from the absorption spectrum, chromatography and polarographic behaviour further confirmed the identity of the photoproduct as 4,6-dimethyl-2-thiopyrimidine. The near quantitative nature of the reaction is testified to by the ratio of the optical densities of the
277 nm monomer band to the 255 nm dimer band, 1.65. This should be equal to the ratios of the extinction coefficients of these two bands, which is 1.75, a reasonably good agreement. The quantum yield for this photodissociation reaction was 0.35 M/Einstein.

Irradiation under the same conditions of the reduction product of 1,4,6-trimethyl-2-thiopyrimidine led to similar conversion to the parent monomer (Fig. 5). The isosbestic points at 230 nm and 266 nm again pointed to quantitative conversion; and the identity of the photoproduct with the parent monomer was confirmed by spectrophotometry, chromatography and polarographic behaviour. Following completion of photodissociation, the ratio of the optical densities of the monomer 280 nm absorption band to that of the dimer band at about 260 nm was 1.45. The ratios of the extinction coefficients for the corresponding bands is 1.48, in good agreement with quantitative photoconversion of one to the other. The quantum yield for this reaction was 0.23 M/Einstein.

Irradiation of the reduction product of 1,4,6-trimethyl-2-thio-

Fig. 4. Photochemical conversion, by irradiation at 254 nm, of dimer reduction product of 4,6-dimethyl-2-thiopyrimidine (III) to parent monomer, 4,6-dimethyl-2-thiopyrimidine (I), in neutral aqueous medium. The medium contained 0.2% DMSO to help dissolve the initial reduction product; 0', absorption spectrum of reduction product at time 0. 1', 2', 3', 5' refer to absorption spectra following 1', 2', 3' and 5' minutes irradiation. The absorption spectrum remained essentially unaltered on further irradiation, pointing to termination of the reaction. Note isosbestic points at 263 nm, and at ~230 nm (by extrapolation, since DMSO in the solution cut off at 235 nm).
pyrimidine in the presence of pure oxygen, or with exclusion of the letter
by bubbling argon through the irradiated solution, did not affect the rate
or quantum yield of the reaction.

Oxidation of dimer reduction products

Electrochemical oxidation of the dimer reduction products in
aqueous medium at concentrations of $5 \times 10^{-4}$ M required, in the case of the
4,6-dimethyl-2-thiopyrimidine reduction product, addition of 0.5% DMSO
to completely dissolve it under these conditions. For the trimethyl reduction
product, which is more unstable in the presence of DMSO, suitable solubility
was achieved by addition of 0.5% ethanol.

In the presence of 0.2 M phosphate buffer at pH 6.7, both dimer
reduction products gave an anodic wave with half-wave potentials of -0.035 V
for $6,6'(4,4')$-bis-(3,6(4)-dihydro-4,6-dimethyl-2-thiopyrimidine) and
-0.020 V for the trimethyl derivative. The heights of these waves in both
cases corresponded to the transfer of 3 or 4 electrons. Oxidation of the
dimers at a potential corresponding to the initial limiting current resulted,
in each case, in regeneration of the corresponding parent monomer, verified
spectrophotometrically and polarographically. The reactions were, however,

![Photochemical conversion, by irradiation at 254 nm, of dimer reduction product of 1,4,6-trimethyl-2-thiopyrimidine (IV) to parent monomer, 1,4,6-trimethyl-2-thiopyrimidine (II), in neutral aqueous medium; 0', absorption spectrum of reduction product; 1' to 10', absorption spectra following increasing times of irradiation, in minutes. After 10' irradiation, the reaction was essentially complete. Note isosbestic points at 230 and 266 nm, indicative of quantitative photoconversion from dimer reduction product to monomer.](image-url)
not quantitative, due to the fact that the monomers themselves undergo oxidation at the same potential. The oxidation products of the monomers have not as yet been identified.

**Mechanism of electrochemical reduction**

Both I and II exhibit identical mechanisms of reduction, as illustrated in Scheme 3, the protonated and neutral forms, but not the anions, being reducible. The reduction process is of the form $1e^-/1H^+$, with formation of a free radical which in turn dimerizes. The slope of the logarithmic plot points to the irreversibility of the reaction, undoubtedly as a result of the rapid rate of dimerization. The wave characteristics indicate that reduction occurs in the adsorbed state. The dimer reduction product is itself strongly adsorbed on the mercury electrode and catalytically evolves hydrogen.

The radical mechanism of reduction of 2-thiopyrimidine derivatives, leading to formation of photochemically reversible dimers of the form II (or III) is typical of the general mechanism of electrochemical reduction of pyrimidine derivatives with a 3,4 double bond. Dimers with such structures have been reported from pyrimidine, $^{15,18}$ pyrimidone-2, $^{1,2}$

![Scheme 3](image)

\[ R = H \text{ or } CH_3 \]

Scheme 3
cytosine and cytidine, $^1$ 4-methylthiocaracil, $^5$ and 2-chloropyrimidine. $^{19}$
Modification of the $C_2-O$ to $C_2-S$ does not alter the reduction pathway, and leads to formation of similar dimer reduction products. By contrast, saturation of the 3,4 bond, e.g. by introduction of a $C_4-O$, either alters the mechanism of electroreduction, $^{20}$ or confers resistance to reduction, as
for 2-thiouracil, uracil or isocytosine (M. Wrona & D. Shugar in preparation).

Similar radical reduction mechanisms, leading to formation of purine dimers, have been noted for purines in which the pyrimidine ring accords with the above conditions, such as 2-thiopurine and 2-ketopurine (B. Czochralska & D. Shugar in preparation).

Of particular interest is the fact that photochemical reduction of I under the influence of 254 nm radiation in the presence of a proton donor such as isopropanol and in the absence of oxygen, leads to formation of a dimer the structure of which is identical with that of III obtained by electrochemical reduction, but in considerably lower yield, about 6%. Under the same irradiation conditions 4,6-dimethyl-pyrimidone-2 photodimerizes to form a dimer in 50% yield, the structure of which is again identical with the electrochemical dimer reduction product of pyrimidone-2.1

Particular significance attaches to the photodissociation properties of the dimer reduction products III and IV, with regeneration of the parent monomers I and II, respectively, in relatively high quantum yields, previously discussed.1 Such photodissociation reactions of pyrimidine dimers have been considered a unique property of 5,5'-6,6'-cyclobutane photodimers formed in nucleic acid chains, the biological consequences of which are of considerable significance. It should, however, be noted that, in contrast to 5,5'-6,6'-cyclobutane photodimers, photodissociation of the 4,4'-dimers is a photooxidative process, which is being subjected to further study. The demonstration that a dimer of reduced pyrimidone-2 is an integral constituent of a tetramer photoproduct isolated from irradiated DNA,3,4 suggests also that such dimers may prove to be of wider biological significance.

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