The Oxidative Addition Reaction between Compounds of Resorufin (7-Hydroxy-3*H*-phenoxazin-3-one) and 2-Mercaptoethanol

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2-Mercaptoethanol reacts with the methanesulphonate, the dimethylcarbamate, and the ethyl ether of resorufin (7-hydroxy-3*H*-phenoxazin-3-one) at 25°C and pH 7.4 to give colored products with maximal absorbances at 433, 442, and 481 nm, respectively. NMR spectra of the products show that the hydrogen atom on C-2 has been specifically replaced by the -SCH$_2$CH$_2$OH group. It is concluded that the thiol adds to the quinonimine ring and that the first-formed product then undergoes spontaneous oxidation to reform the resorufin structure. The implications of these studies toward biochemical work with resorufin derivatives is discussed.   © 1998 Academic Press

INTRODUCTION

In our recent work using resorufin acetate (see Scheme 1, X = CH$_3$CO) as a substrate for the esterase action of chymotrypsin (*1*) and aldehyde dehydrogenase (*2, 3*), it was assumed that no other reaction occurred but hydrolysis of the ester group, liberating the highly chromophoric resorufin anion. However, in subsequent work with a variety of resorufin derivatives in which the X group is not as susceptible to nucleophilic attack, it became evident that covalent interaction involving the resorufin ring system itself was occurring (see the accompanying paper, *4*). In particular, since the active site nucleophile of aldehyde dehydrogenase is Cys-302 (*5*), it was of interest to examine what chemical reactions may possibly occur between thiol groups and resorufin derivatives. To this end, the present work presents a study of the reaction between 2-mercaptoethanol (a conveniently water-soluble, small-molecular thiol) and the three resorufin derivatives listed in Scheme 1. As will be discussed below, the results of experiments using UV/visible spectro-photometry and NMR spectroscopy have enabled the identification of the products of these reactions. This information has proved invaluable in interpreting our studies with aldehyde dehydrogenase (*4*) and should be of interest and importance to any workers who use resorufin compounds as probes of enzymic activity (e.g., *6–10*).

The structures of the resorufin derivatives used in this work are given in Scheme 1 (resorufin methanesulphonate, X = CH$_3$SO$_2$; resorufin dimethylcarbamate, X = (CH$_3$)$_2$NCO; resorufin ethyl ether, X = CH$_3$CH$_2$). The letters label the various hydrogen atoms that are discussed in relation to the NMR spectra.
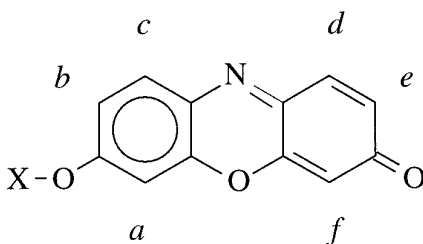
SCHEME 1

MATERIALS AND METHODS

Preparation of Resorufin Derivatives

The compounds shown in Scheme 1 were prepared by reacting the anion form of resorufin (Aldrich Chemical Co.) with the appropriate halogen compound, X-Hal. As only small quantities of material were required for the experiments carried out in this work, no attempt was made to optimize the yield of the preparations.

Resorufin dimethylcarbamate was prepared by mixing the sodium salt of resorufin (approx. 2.1 mmol) with dimethylcarbamyl chloride (2.4 mmol) in pyridine (25 ml). After 3 h at room temperature with occasional shaking, the pyridine was evaporated. The resulting orange powder was washed with water and dried. The product was recrystallized from ethanol/acetone as a microcrystalline orange-brown solid. m.p. 250–253 (dec.). $C_{15}H_{12}N_2O_4$ confirmed by mass spectrometry ($m/z = 284.080078$). The peaks in the NMR spectrum ($CDCl_3$) due to the protons on the resorufin ring system are shown in Fig. 3a. The spectrum also showed a doublet due to the $(CH_3)_2NCO$ group at 3.051/3.137 ppm.

Resorufin methanesulphonate was prepared by mixing the sodium salt of resorufin (approx. 1.1 mmol) with equimolar amounts of triethylamine and methanesulphonyl chloride in chloroform (25 ml). The mixture was refluxed briefly, filtered, and evaporated to dryness. The solid was dissolved in chloroform and purified by passage down a column of silica gel. A large amount of red coloration developed at the top of the column presumably due to hydrolysis of the product, reforming the resorufin anion. The orange solution that eluted was collected and evaporated to dryness, giving a small amount of an orange powder. m.p. 208–210 (dec.). $C_{13}H_9NO_5$ confirmed by mass spectrometry ($m/z = 291.019882$).

Resorufin ethyl ether was prepared according to the method of Nietzki *et al.* (*11*). m.p. 225°C (dec.) The NMR spectrum of the resorufin protons was essentially the same as that for resorufin dimethylcarbamate (Fig. 6a) except that the peaks due to the *a* and *b* protons (those closest to the ethoxy group, see Scheme 1) were significantly further upfield (at between 6.8 and 7.0 ppm). The spectrum also showed the expected resonances due to the ethyl group (a quartet and a triplet centred on 4.16 and 1.50 ppm, respectively).

Monitoring the Reactions of Resorufin Derivatives by UV/Visible Spectrophotometry

A Varian Cary 1 spectrophotometer was used to record the spectra of resorufin derivatives and to monitor changes in absorbance at particular wavelengths with time at 25°C. In all experiments, the bulk of the solution (total volume 3 ml) consisted of 50 mM sodium phosphate buffer, pH 7.4. The resorufin compounds were added as solutions in acetonitrile (0.2–0.4 ml) to give the final concentration after mixing that is noted in the figure legends. 2-Mercaptoethanol was added as a small volume of an aqueous solution. Ascorbic acid was added in 50 mM sodium phosphate buffer, pH 7.4. Hydrogen peroxide was added as a small volume of a concentrated aqueous solution (29–32% w/w).

Analyzing the Product of Reaction between Derivatives of Resorufin and 2-Mercaptoethanol by NMR Spectroscopy

Spectra were obtained using a Jeol GX 270 instrument operating at 270.198 MHz. Resorufin dimethylcarbamate (2.38 mg) or resorufin ethyl ether (2.0 mg) was dissolved in acetonitrile (20 ml) and 50 mM sodium phosphate buffer, pH 7.4 (180 ml), was added, followed by 2-mercaptoethanol (15 μl). The resulting concentration of the resorufin compound was 42 μM and that of the thiol was 1.1 mM. The reaction mixture was left at room temperature for 35 min in the case of the dimethylcarbamate and 9.5 h in the case of the ether. The mixture was then extracted with chloroform (20 ml); the chloroform layer was washed three times with water (50 ml) and then dried over MgSO$_4$. After filtration and evaporation of the solution, the resulting orange solid was dried overnight in a high-vacuum desiccator and then dissolved in CDCl$_3$ for NMR investigation.

RESULTS

Reaction of Resorufin Derivatives with 2-Mercaptoethanol

Figure 1a shows the UV/visible absorbance spectrum of resorufin methanesulphonate (approx. 40 μM) in 50 mM sodium phosphate buffer, pH 7.4. After the addition of 2-mercaptoethanol (to give a concentration of 1 mM) the solution was repetitively scanned over a period of 10 min, giving the results shown in Fig. 1b. The left-hand peak (λ_{max} = 353 nm) in the original spectrum disappears rapidly while a new peak grows at 433 nm. In an analogous experiment with resorufin dimethylcarbamate, very similar changes occur. The left-hand peak in the original spectrum (λ_{max} = 368 nm) shrinks and a new absorbance grows (λ_{max} = 442 nm); these changes are somewhat slower than with the methanesulphonate as Fig. 2 illustrates. With resorufin ethyl ether (40 μM) and 2-mercaptoethanol (1 mM) the λ_{max} shifts slowly from 486 to 481 nm over a period of 6 to 7 h with an increase in absorbance from 0.73 to 1.0.

Figure 1 shows that the rate of decline of the left-hand peak appears faster than the rate of growth of the new peak. This observation was substantiated by monitoring
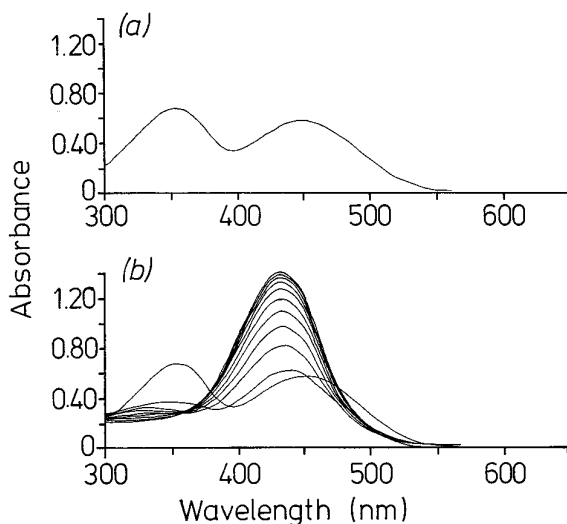
FIG. 1. Reaction of resorufin methanesulphonate with 2-mercaptoethanol. (a) The original spectrum of a solution of resorufin methanesulphonate (40 μM) in 50 mM sodium phosphate buffer, pH 7.4, at 25°C. (b) The results of scanning repetitively at regular intervals over 10 min after the addition of 2-mercaptoethanol (1 mM).
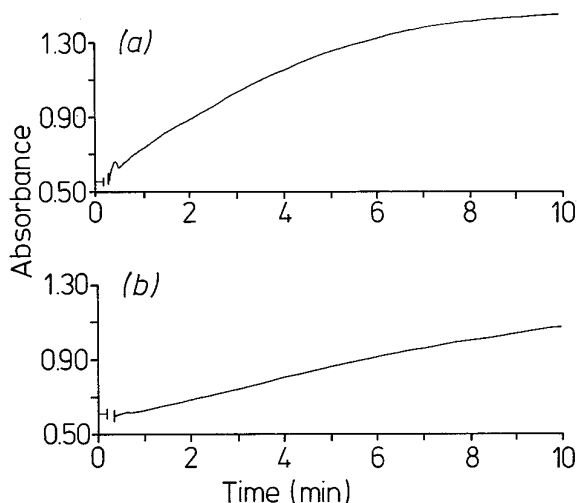
FIG. 2. Rate of appearance of product in the reactions of resorufin methanesulphonate and resorufin dimethylcarbamate with 2-mercaptoethanol. (a) The change in A_{433} when resorufin methanesulphonate (40 μM) reacts with 2-mercaptoethanol (1 mM) in 50 mM sodium phosphate buffer, pH 7.4, at 25°C. (b) The change in A_{442} when resorufin dimethylcarbamate reacts under the same conditions.

the relevant absorbances as a function of time, and suggests that resorufin methane-sulphonate is being destroyed relatively quickly to give some intermediate product that is then more slowly converted to the compound responsible for the final spectrum.

The product of the reaction between resorufin dimethylcarbamate and 2-mercaptoethanol was isolated as described under Materials and Methods and its ^{1}H-NMR spectrum was determined (Fig. 3b) and compared with that of the starting material (Fig. 3a). In these Figures, peaks are assigned to protons as labeled in Scheme 1; the large unlabeled peak at 7.26 ppm is due to chloroform. Peaks due to protons in a 1,2-relationship are mutually split by a relatively large amount (J = 8.5 to 9.8 Hz), whereas those in a 1,3-relationship are split to a lesser degree (J = 2.0 to 2.4 Hz); protons in a 1,4-arrangement have no measurable effect on each other's peaks with the instrumentation used here. Reaction with 2-mercaptoethanol has little effect on the three protons on the aromatic ring, *a*, *b*, and *c* (except that in Fig. 3b the doublet due to *a* and the doublet of doublets due to *b* are more clearly resolved than in Fig. 3a). The major difference in Figs. 3a and 3b is the replacement of the three resonances (with their characteristic splitting pattern) assigned to the protons on the quinonimine ring, *d*, *e*, and *f*, simply by two singlets; these two signals are assigned to protons *d* and *f*, as discussed below.

Although off-scale in Fig. 3, the resonance due to the dimethylcarbamate group (which is a doublet, owing to restricted rotation about the N-CO bond, at 3.0–3.1 ppm) is still present in the product, showing that this group is not displaced by reaction with 2-mercaptoethanol. (The spectrum of the product also shows the expected resonances due to the mercaptoethanol moiety).

The product of the reaction between resorufin ethyl ether and 2-mercaptoethanol was isolated and investigated by NMR in a similar way (spectra not shown). In this case (unlike with the dimethylcarbamate) it was clear that the product still contained an appreciable amount (about 30%) of unreacted starting material. However, by eliminating the peaks due to resorufin ethyl ether itself, it was not difficult to assign the remaining spectrum to the product; the conclusion here, as with the dimethylcarbamate, is that the -SCH$_2$CH$_2$OH effectively substitutes for the *e* proton. With the product from the ethyl ether, the two singlet peaks assigned to protons *d* and *f* have chemical shifts of 7.159 and 6.429 ppm, respectively; for the dimethylcarbamate the corresponding values are virtually identical, 7.150 and 6.432 ppm.

Redox Properties of Resorufin Derivatives

Spectrum 1 in Fig. 4a is that of resorufin dimethylcarbamate (30 μM), whereas spectrum 2 shows the results after a 10-min incubation at 25°C with 10 mM ascorbic acid. The dimethylcarbamate is almost completely reduced to a colourless product under these conditions. Spectrum 3 shows the result of a 10-min incubation after the addition of 10 μl of hydrogen peroxide solution (to give approx. 32 mM) to the solution that previously had spectrum 2. Obviously the reduced colorless product is capable of being reoxidized to resorufin dimethylcarbamate. However, it was found that complete reoxidation (reproducing spectrum 1) could not be achieved this way. Incubation for a longer time or with a higher concentration of hydrogen