Bioorganic Chemistry **27,** 73–88 (2000)
doi:10.1006/bioo.2000.1164, available online at http://www.idealibrary.com on IDEAL®

Studies with Potential Reporter Group Reagents for Enzymes: 3,4-Dihydro-3-(2-Hydroxyethyl)-6-Nitro-2*H*-1,3-Benzothiazin-2-Thione and 6-Nitrochromone—Hydrolysis and Interaction with Chymotrypsin

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Received August 5, 1999

3,4-Dihydro-3-(2-hydroxyethyl)-6-nitro-2*H*-1,3-benzothiazin-2-thione (**II-HE**), a cyclic dithiocarbamate, has the potential to react with an esterase to furnish it with a *p*-nitrobenzenethiolate "reporter group." However, unlike a closely similar cyclic carbamate, **II-HE** is totally without reaction on chymotrypsin. Possible reasons for this major effect of substituting sulfur for oxygen are discussed. The results support the idea that chymotrypsin's "oxyanion hole" cannot properly accommodate a thioanion. **II-HE** undergoes an interesting intramolecular cleavage reaction under alkaline conditions. The mechanism of this process has been determined using evidence from NMR and mass spectrometry. 6-Nitrochromone (**6-NC**) likewise has the potential to modify an enzyme covalently and thereby act as a reporter group reagent. With chymotrypsin, **6-NC** reacts as predicted, except that the attached label is stable only at high pH; the labeling reaction slowly reverses at low pH. From the lack of effect on enzyme activity, it is clear that modification of chymotrypsin by **6-NC** does not occur at the active site.

INTRODUCTION

In previous work, the cyclic carbamate 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one (**I**)[1] was used to react with enzymes having an esterase function (including chymotrypsin and aldehyde dehydrogenase) in order to provide them with a coloured *p*-nitrophenoxy "reporter group" (*1–3*). Scheme 1 shows the chemistry of this process. The first part of the present work concerns analogous compounds with a dithiocarbamate instead of a carbamate group. It was thought that such a compound might either react with an esterase in just the same way as **I** (providing an alternative and more easily synthesised reporter group reagent) or it might show an interestingly different chemistry to that of **I.** Accordingly, the synthesis is reported here of 3,4-dihydro-3-

[1] Abbreviations used: **I,** 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one; **II-Me,** 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzothiazin-2-thione; **II-HE,** 3,4-dihydro-3-(2-hydroxyethyl)-6-nitro-2*H*-1,3-benzothiazin-2-thione; **6-NC,** 6-nitrochromone. The abbreviations **II-Me** and **II-HE** draw attention to the relevant sidechains, methyl and hydroxyethyl, respectively.

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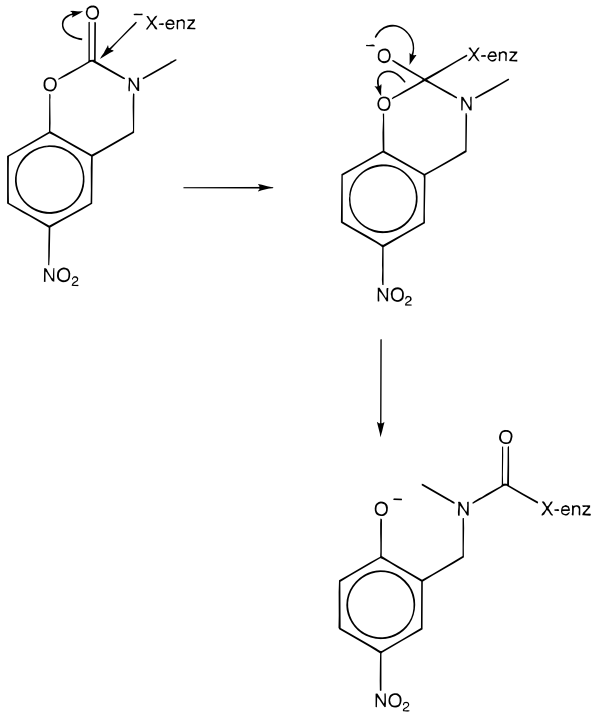


SCHEME 1. Reaction of **I** with an enzymic nucleophile to provide a *p*-nitrophenoxy reporter group.

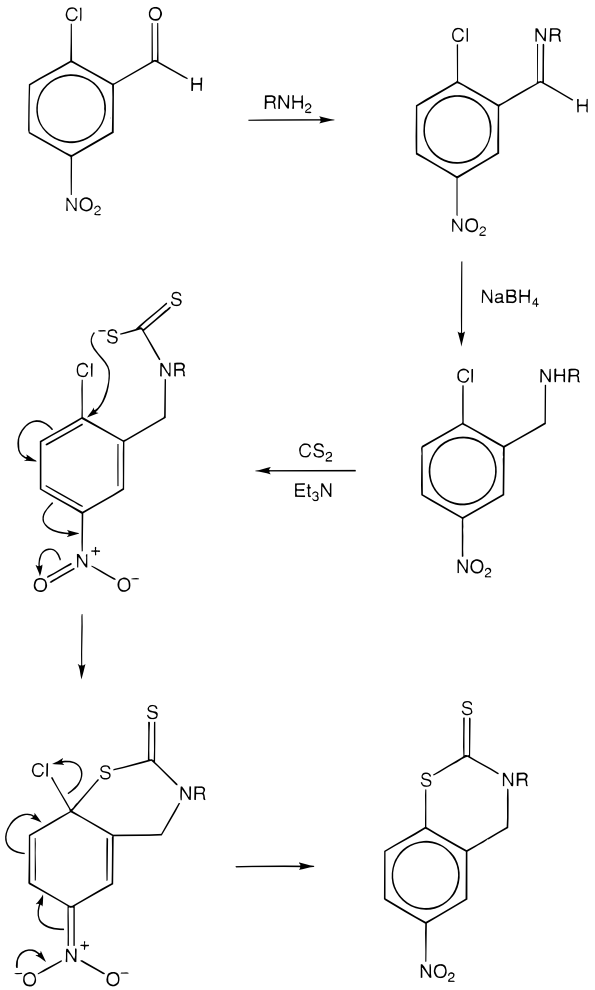
methyl-6-nitro-2*H*-1,3-benzothiazin-2-thione (**II-Me**) and 3,4-dihydro-3-(2-hydroxyethyl)-6-nitro-2*H*-1,3-benzothiazin-2-thione (**II-HE**); the route is shown in Scheme 2. The advantage of this synthesis over that of **I** (*1*) is that it is shorter and does not involve the dangerously toxic phosgene. The reactivity of these compounds toward chymotrypsin and to alkaline hydrolysis is described below.

The second part of the present work concerns an investigation of 6-nitrochromone (**6-NC,** 6-nitro-4*H*-1-benzopyran-4-one) as another potential reporter group reagent. This compound is of very similar size and shape to **I** and in principle could react with an enzymic nucleophile as shown in Scheme 3, again incorporating a covalently linked *p*-nitrophenoxy group. Chromone itself is known to react in similar fashion with diethylamine, giving 3-diethylamino-1-(2-hydroxyphenyl)propenone in good yield (*4*). **6-NC** is very simply prepared by the nitration of chromone; its reaction with chymotrypsin is described below.

MATERIALS AND METHODS

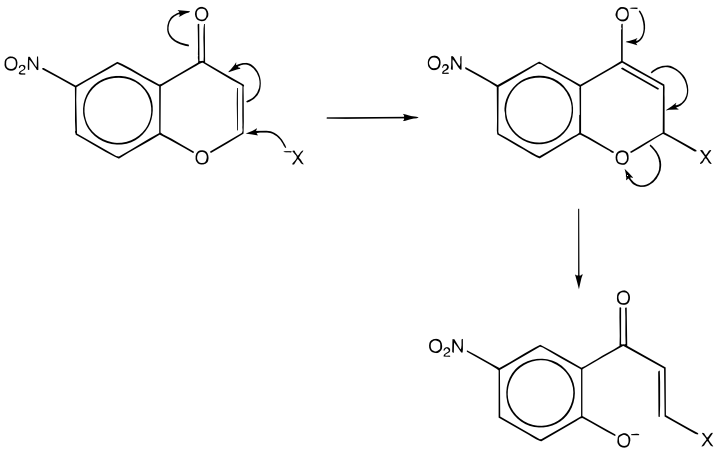
*Preparation of **II-HE** (See Scheme 2)*

2-Chloro-5-nitrobenzaldehyde (4.0 g) was dissolved in ethanol (40 ml) and an equimolar amount of 2-aminoethanol was added; the mixture was left overnight at room temperature. The ethanol was evaporated and the residue was dissolved in chloroform; this solution was then washed with water (to remove any unreacted aminoethanol), dried



SCHEME 2. Synthetic route leading to cyclic dithiocarbamates. **II-Me,** R = CH_3; **II-HE,** R = CH_2CH_2OH.

over $MgSO_4$, and evaporated to give a viscous yellow oil. The oil was dissolved in ethanol (50 ml) and cooled in ice. Sodium borohydride (0.8 g) was added slowly in portions and the resulting orange solution was left overnight at room temperature. The ethanol was evaporated and the residue partitioned between chloroform and water. The chloroform layer was dried over $MgSO_4$ and evaporated to dryness. The resulting oil crystallized to a pale orange solid. The yield of *N*-(2-hydroxyethyl)-2-chloro-5-nitrobenzylamine was 4.07 g (82% over the two steps). δ ($CDCl_3$): 8.36 (d, 1H), 8.10 (d of d, 1H), 7.53 (d, 1H), 3.99 (s, 2H), 3.74 (t, 2H), 2.85 (t, 2H), 2.02 (s, 2H, NH and OH). This material was dissolved in ethanol (40 ml) and equimolar amounts of triethylamine and carbon disulfide were added; the mixture was left overnight at room temperature. After removal of solvent, the residue was extracted into chloroform and



SCHEME 3. Nucleophilic attack on **6-NC.** X^- = HO^- or represents an enzymic nucleophilic group.

water; the chloroform layer was dried over $MgSO_4$ and evaporated to dryness. The resulting red solid was dissolved in a small amount of warm chloroform and applied to a column of silica gel, eluting with chloroform. The major fraction that eluted from the column was evaporated to give a yellow solid. The yield of 3,4-dihydro-3-(2-hydroxyethyl)-6-nitro-2*H*-1,3-benzothiazin-2-thione was 1.4 g (29%); the NMR spectrum showed the compound to be pure. δ ($CDCl_3$): 8.17 (d, 1H), 8.22 (d of d, 1H), 7.41 (d, 1H), 4.83 (s, 2H), 4.32 (t, 2H), 4.05 (t, 2H), 1.9 (s, 1H).

*Preparation of **II-Me***

This compound was prepared by essentially the same method as described above for **II-HE,** except methylamine (as a 40% aqueous solution) was used instead of 2-aminoethanol. δ ($CDCl_3$): 8.17 (d, 1H), 8.21 (d of d, 1H), 7.38 (d, 1H), 4.74 (s, 2H), 3.66 (s, 3H). *m/z* = 240.002239 (calcd for $C_9H_8N_2O_2S_2$: 240.002721).

*Preparation of **6-NC***

Chromone was nitrated by a published method (*5*); the m.p. and NMR spectrum of the product were the same as in the literature.

Spectrophotometry

All UV/visible spectra were recorded using a Varian Cary 1 instrument. The activity of chymotrypsin was monitored using *p*-nitrophenyl acetate (0.1 or 0.25 mM) as substrate in Tris (50 mM, pH 8.5) or phosphate buffer (50 mM, pH 8.0) at 25°C. Stopped-flow experiments were carried out with a Hi-Tech Scientific instrument using *p*-nitrophenyl acetate (0.25 mM) as substrate in 50 mM phosphate buffer, pH 8.0, at 25°C.

Modification of Chymotrypsin

II-HE or **I** in acetonitrile (0.2 ml) was added to 0.2 M phosphate buffer (pH 8.0, 2.8 ml) containing chymotrypsin (α-chymotrypsin, type II from bovine pancreas,

Aldrich); the resulting concentrations of enzyme and modifier were 0.22 and 0.5 mM, respectively. The mixture was protected from light and left at 29°C for 45 h. Samples were taken at intervals for enzyme assay. The mixture was then passed down a gel filtration column (Biogel P-6, 25 × 0.8 cm), eluting with 10 mM phosphate buffer, pH 7.4, and after making the solution alkaline the protein fraction was scanned spectrophotometrically. **6-NC** in acetonitrile (0.2 ml) was added to 50 mM phosphate buffer (pH 8.0, 4.8 ml) containing chymotrypsin; the resulting concentrations of enzyme and modifier were 0.25 and 0.5 mM, respectively. The mixture was left at room temperature overnight and then subjected to gel filtration using 10 mM phosphate (pH 6.0) as eluant. Samples (0.5 ml) of the protein fraction were added to 2 ml of various more concentrated buffers; the resulting pH was measured and the solutions were scanned spectrophotometrically.

*Hydrolysis Product of **II-HE***

II-HE (5.76 mg) was dissolved in acetonitrile (2 ml) and added to 0.1 M NaOH (200 ml) at room temperature. After 5 min, the yellow solution was acidified by adding 1.9 ml of concentrated hydrochloric acid (specific gravity 1.18) and then extracted with chloroform (25 ml). The chloroform solution was dried over $MgSO_4$ and evaporated to dryness under high vacuum. The residue was dissolved in $CDCl_3$ for NMR spectrometry and also subjected to mass spectrometry. *m/z* = 270.011548 (calcd for $C_{10}H_{10}N_2O_3S_2$: 270.013286), 272.002052 (calcd for $C_{10}H_9N_2O_3S^{35}Cl$: 272.002242), 274.000076 (calcd for $C_{10}H_9N_2O_3S^{37}Cl$: 273.999292).

*Treatment of **II-HE** with Perchloric Acid*

II-HE (20 mg) was dissolved in chloroform and perchloric acid (70%, 8.0 μl) was added. The mixture was left overnight at room temperature and then evaporated to dryness. The residue was dissolved in d_6-DMSO for NMR spectrometry.

RESULTS AND DISCUSSION

Synthesis of Cyclic Dithiocarbamates

The compounds **II-Me** and **II-HE** were successfully prepared by the route shown in Scheme 2, albeit in low yield for the cyclisation step. No doubt an extensive exploration of the reaction conditions would allow the yield to be optimized, but this was not considered necessary for the present work as only very small amounts of the reagents are required for enzyme experiments. The route involves a nucleophilic aromatic substitution reaction that occurs under unusually mild conditions; the dithiocarbamate ion is ideally positioned for intramolecular attack, and the nitro group provides an electron sink to stabilise the intermediate. Using the nitro group in this way could be described as “chemical parsimony,” since the nitro group already has the essential functions (in the reaction for which these reagents are designed) of enhancing the leaving group ability of the benzenethiolate ion and of rendering it colored.

***II-HE** and Chymotrypsin*

Unfortunately **II-Me** (the simple dithio analogue of **I**) was found to be too insoluble in water to use as a potential enzyme-modifying reagent. This was the reason why

the hydroxyethyl derivative (**II-HE**) was also synthesized, and as expected its solubility is much higher.

When chymotrypsin (0.22 mM) was incubated at 29°C and pH 8.0 with **I** (0.50 mM), there was substantial loss of enzyme activity (60% inactivation in 25 h). However, under the same conditions, the activity of the enzyme in the presence of **II-HE** was 128% of the control incubation without reagent. Upon isolation of the **I**-modified enzyme by gel filtration (and after making the solution alkaline with a small volume of NaOH solution), the UV/visible spectrum shown in Fig. 1a was obtained. It can be calculated from the magnitude of the absorbance that approximately 61% of the enzyme is covalently labeled by **I.** (These results are similar to those of previous work; *1*) When treated in exactly the same way, the solution of enzyme that had been incubated with **II-HE** gave the spectrum shown in Fig. 1b; clearly there is no trace at all of any reaction between **II-HE** and chymotrypsin. (Presumably the higher level of enzyme activity after incubation with **II-HE** is due to the compound binding noncovalently in the active site and protecting the enzyme somewhat from the appreciable spontaneous denaturation or autolysis that occurs over a long time period.)

It seems conceivable but unlikely that the different behavior of chymotrypsin toward **II-HE** and **I** is due to a steric effect of replacing **I**'s methyl group by the only slightly larger and conformationally flexible hydroxyethyl sidechain. This conclusion is strongly supported by the fact that chymotrypsin reacts avidly at the ring carbonyl group of the compound shown (along with **II-HE** for structural comparison) in Scheme 4 (*6*). Rather, the different outcomes may reflect a major difference in chemical reactivity of a dithiocarbamate compared to a carbamate (and evidence on this point is presented in the next section). Dithiocarbamates are reported to be fairly unreactive (*7*); for example, treatment of $Me_2NCS\text{-}SCH_2COOMe$ with ammonia at 100°C gives $Me_2NCS\text{-}SCH_2\text{-}CONH_2$, in which the dithiocarbamate group is unchanged. However, the simple dithiocarbamate $Et_2NCS\text{-}SMe$ is by no means totally inert under mild conditions; it is oxidized *in vivo* first to $Et_2NCO\text{-}SMe$ and then to $Et_2NCO\text{-}SOMe$ (*8*). Moreover, tetramethylthiuram monosulfide ($Me_2NCS\text{-}S\text{-}CSNMe_2$) reacts with a variety of nucleophiles (RO^-, RS^-, amines; *9*) in just the manner in which it was envisaged

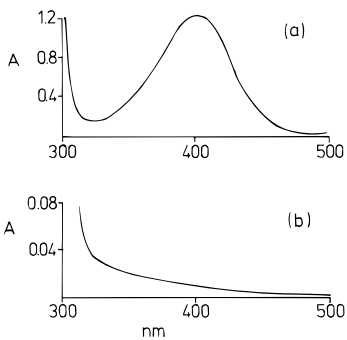
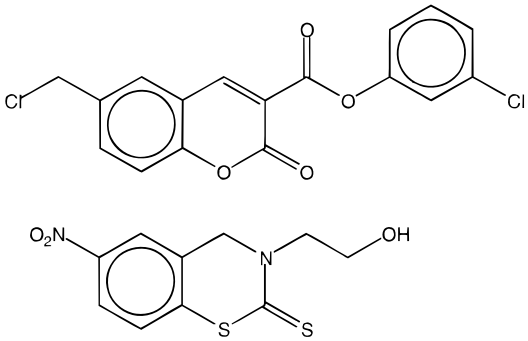


FIG. 1. (a) The UV/visible spectrum of the reporter group (at alkaline pH) supplied to chymotrypsin by **I.** (b) The equivalent result obtained when chymotrypsin was treated with **II-HE** under exactly the same conditions as used with **I** in (a).



SCHEME 4. Comparison of the structure of a known inactivator of chymotrypsin (*6*) with that of **II-HE.**

that **II-HE** might react with an enzyme (in this case the leaving group being the dimethyldithiocarbamate ion rather than a *p*-nitrobenzenethiolate ion).

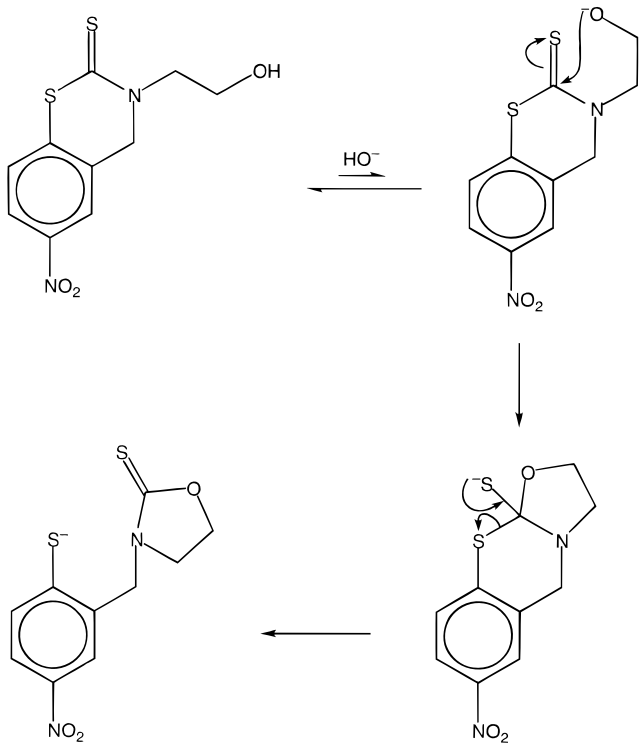
An observation that is relevant to the present results is that chymotrypsin does not measurably catalyze the hydrolysis of thionoesters (substrate analogues with a thiocarbonyl group), although thioloesters (in which the singly bonded oxygen of an ester group is replaced by sulfur) are good substrates (*10,11*). It was suggested from this that the enzyme's "oxyanion hole" is very specific for oxygen and cannot stabilize the tetrahedral transition state when sulfur is involved. Interestingly, cysteine proteases such as papain do act on thionoesters (*12*). Thus **II-HE** may prove to be a useful reporter group reagent for some enzymes (other than serine proteases); in particular, it seems likely to react with aldehyde dehydrogenase (which has been extensively studied using **I:** *2,3*) since this enzyme is known to be inactivated by a noncyclic dithiocarbamate analogue of **II-HE,** *p*-nitrophenyl dimethyldithiocarbamate (*13*).

*Alkaline Hydrolysis of **I**, **II-Me**, and **II-HE***

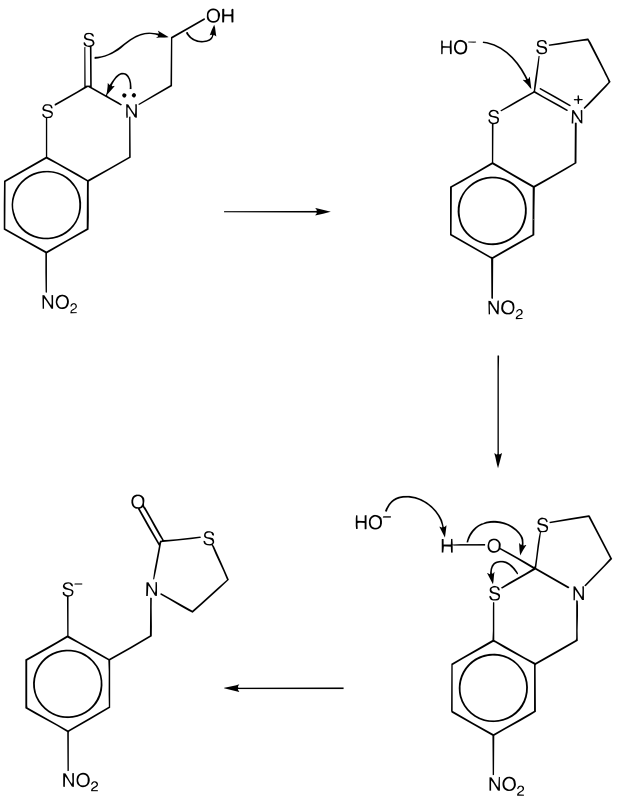
The difference in reactivity of a carbamate and a dithiocarbamate is clearly demonstrated by adding solutions of **I** and **II-Me** (in acetonitrile) to 0.1 M NaOH. In the case of the former, hydrolysis occurs rapidly giving a yellow solution with λ_{max} = 411 nm and ε = 18,200, as expected. However, with **II-Me** the initial absorbance at 320–330 nm slowly declines (losing about half its intensity in 20–30 min), but with no development of any absorbance in the visible region whatsoever. This is evidently due to the very poorly soluble **II-Me** slowly being deposited from the originally 50 μM solution, but without undergoing any hydrolysis. (This phenomenon happens in any aqueous solution, not just 0.1 M NaOH.) Oxygen and sulfur differ in terms of size, polarizability, stability of their multiple bonds, etc., but perhaps the simplest way of rationalizing the observed difference in reactivity of the carbamate and the dithiocarbamate is to note that in the former the carbon atom is connected to two highly electronegative oxygen atoms and is therefore more electrophilic than in the latter. In interesting contrast to **II-Me,** the hydroxyethyl analogue **II-HE** quickly gives a yellow solution when added to 0.1 NaOH; the λ_{max} is coincidentally the same as that of the hydrolysis product of **I**

(411 nm), but the absorbance peak is somewhat broader and has a lower molar absorptivity (ε = 12,400).

It is quite clear from the observations just described that the neighboring hydroxyl group of **II-HE** provides anchimeric assistance in some way to the alkaline cleavage of the dithiocarbamate group. The most obvious mechanism by which this might occur is shown in Scheme 5. Here it is envisaged that although the dithiocarbamate group is not productively attacked by hydroxide ion intermolecularly (as shown by the inertness of **II-Me**), it is perfectly capable of being cleaved if the attacking nucleophile is held in close proximity to it. However, there is an alternative mechanism for the hydrolysis of **II-HE,** as shown in Scheme 6, in which the dithiocarbamate acts as the nucleophilic rather than the electrophilic component. This route involves the initial displacement of hydroxide ion, a poor leaving group, but again the close proximity of the neighboring (dithiocarbamate) group may render the process favourable. Certainly a very similar reaction occurs with a better leaving group, as in $Me_2NCS\text{-}SCH_2CH_2Cl$, and there is some evidence that it can happen in $Me_2NCS\text{-}SCH_2CH_2OH$ as well (*13*). In Scheme 6, the resulting dithioiminium ion is then attacked by hydroxide ion as shown leading to the release of the nitrobenzenethiolate moiety. The key steps in Schemes 5 and 6 are defined as 5-exo-trig and 5-exo-tet, respectively, and thus are both allowed by Baldwin's rules. The products shown in the schemes both possess a cyclic thiocarbamate group, but



SCHEME 5. A proposed mechanism for the alkaline cleavage of **II-HE.**



SCHEME 6. An alternative proposed mechanism for the alkaline cleavage of **II-HE.**

differ as to whether the oxygen or the sulfur is in the ring or outside it. It was decided to isolate the hydrolysis product of **II-HE** and characterise it by NMR spectroscopy in order to elucidate which of Schemes 5 and 6 actually occurs.

II-HE was subjected to alkaline hydrolysis and, after acidification of the reaction mixture with hydrochloric acid, the product was extracted into chloroform and its NMR spectrum was determined; see Fig. 2. The large peak at 7.27 ppm is due to chloroform. The small peaks at 4.83, 4.32, and 4.05 ppm are due to residual unhydrolyzed **II-HE.** The spectrum clearly shows the presence of two main compounds of similar structure (and integration suggests they are present in a ratio of 57 : 43). For example, regardless of whether Scheme 5 or 6 operates, we would expect the product to show one singlet (the benzyl CH_2), but there are two (near 5 ppm). We would expect one doublet from the aromatic proton *ortho* to the sulfur, but again there are two doublets (at 7.83 and 7.48 ppm). (The other aromatic signals are poorly resolved at about 8 to 8.2 ppm.) We would expect two triplets from the adjacent CH_2 groups, but there are two close together at about 3.7 ppm and two more appear to be overlaid at about 4.6 ppm. The latter resonances must be due to CH_2O groups in both components of the mixture (and the peaks near 3.7 due to CH_2N groups); critically there is no sign of any resonance in the region expected for a CH_2S group. For instance, the CH_2 group in $Me_2N\text{-}CO\text{-}SCH_2CH_3$ has a

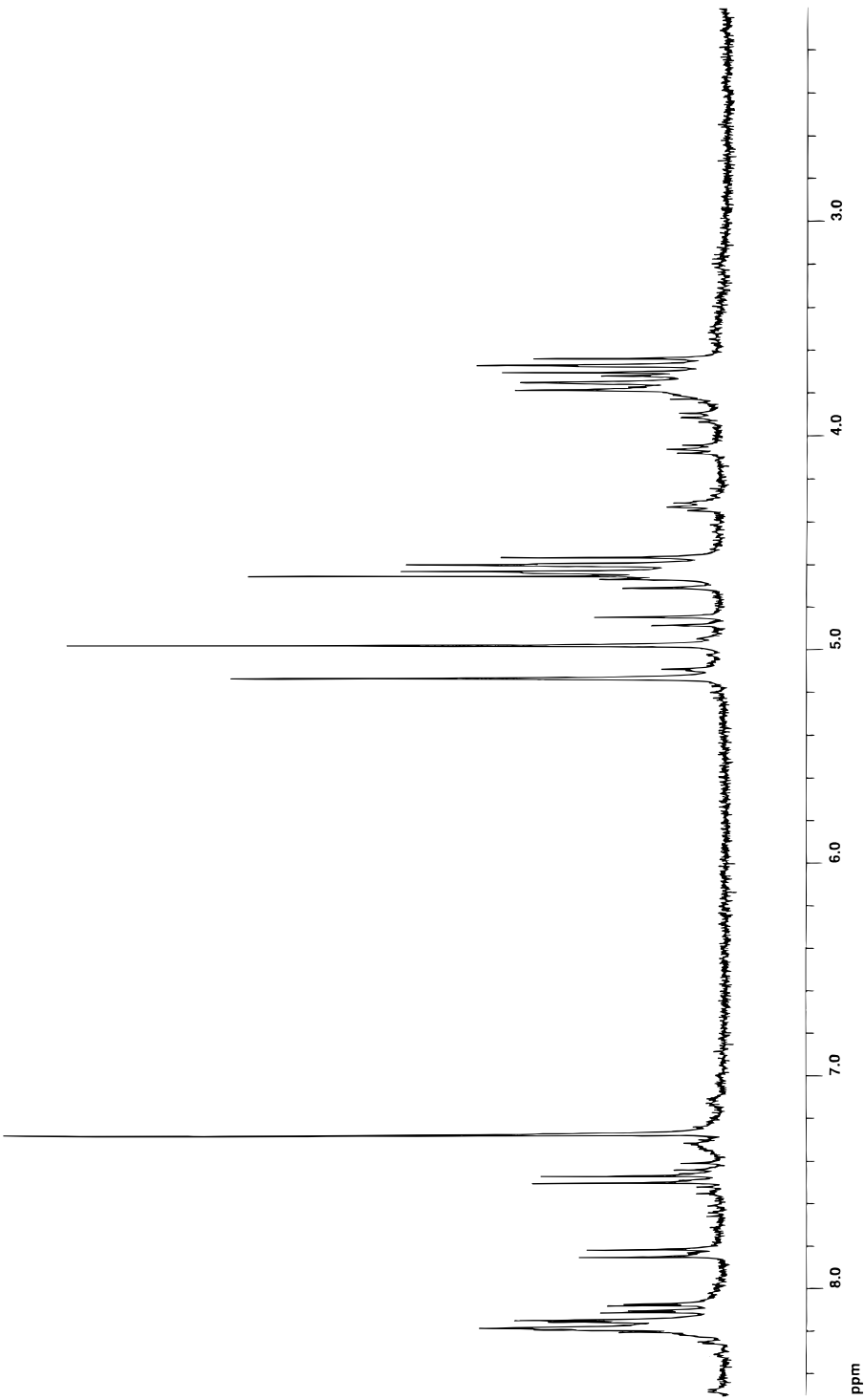


FIG. 2. NMR spectrum of the product obtained upon subjecting **II-HE** to alkaline hydrolysis followed by acidification with HCl.

signal at 2.87 ppm (*15*), whereas that in Me_2N-CS-OCH_2CH_3 appears at 4.40 ppm (*16*). The cyclic analogue of the latter compound, 3-methyl-1,3-oxazolidin-2-thione (which is a good model for the product of Scheme 5), shows triplets at 3.90 and 4.52 corresponding to the methylene groups next to nitrogen and oxygen, respectively (*17*). Thus we can conclude with confidence that Scheme 6 does not apply, and that the major component of the reaction mixture is that shown in Scheme 5 (after protonation). We know it is the major component since the larger of the two doublets in the region from 7 to 8 ppm has a chemical shift that is more compatible with a proton *ortho* to a neutral sulfur substituent; **II-HE** itself for instance has a doublet at virtually the same position, 7.41 ppm. (The other doublet is discussed below.)

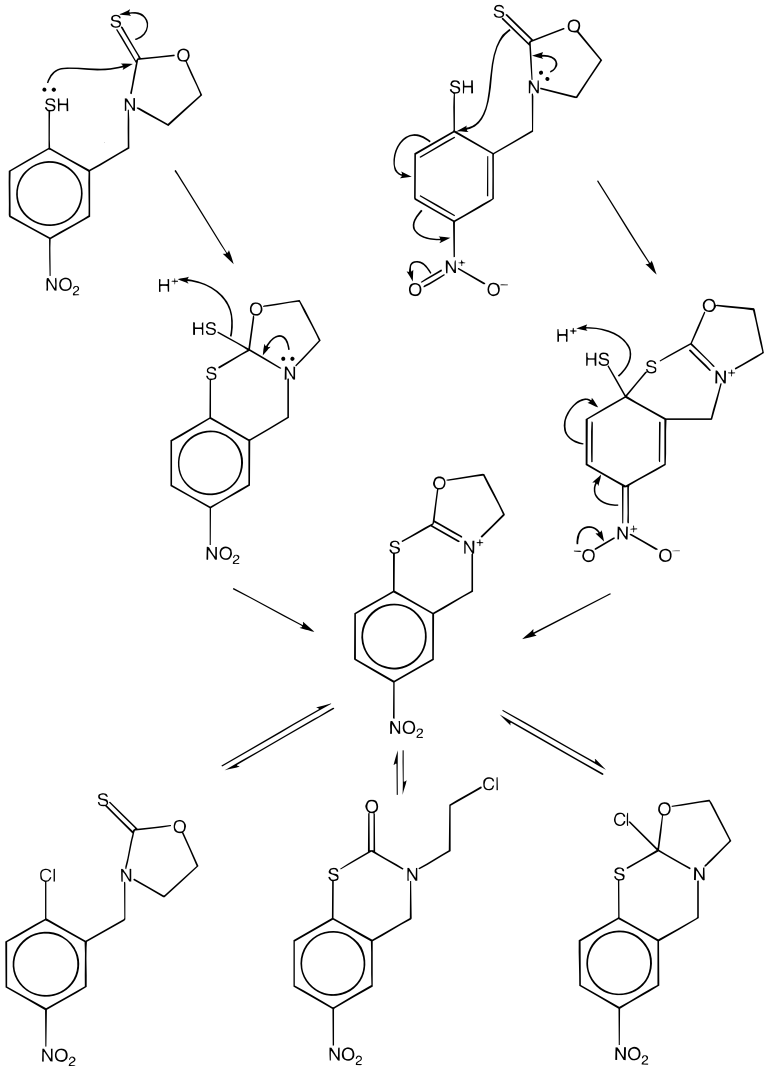
To gain further information about the identity of the minor product, the mixture was analyzed by mass spectrometry. A molecular ion at 270 was observed, confirming the presence of the product shown in Scheme 5 (after protonation). Large peaks were also seen at 272 and 274 in a ratio of approximately 3:1, strongly suggestive of the presence of chlorine. High resolution mass spectrometry confirmed the formula $C_{10}H_9N_2O_3SCl$. Thus the minor product is related to the major by loss of SH and gain of Cl; two plausible mechanisms for how this might happen when the initial hydrolysis solution is acidified with HCl are shown in Scheme 7. It is difficult to say which of these is the more likely; the one on the left is, in part, effectively the reverse of Scheme 5, whereas the one on the right is similar to the mechanism that must oocur in the synthesis of **II-HE** (Scheme 2). The two routes converge on a common oxythioiminium ion, which could be in equilibrium with a variety of compounds containing covalently-linked chlorine, as shown. One or other of these must be responsible for the 272/274 peaks in the mass spectrum, but none of them is as nicely compatible with the chemical shifts in the NMR spectrum as the oxythioiminium ion itself. (We can immediately rule out the structure without a CH_2O group, for instance.) As mentioned above, the minor product has a doublet at 7.83 ppm, and this is practically identical with that shown by the dithioiminium ion (7.81 ppm) prepared in unequivocal manner by treatment of **II-HE** with perchloric acid, as shown in Scheme 8. (It is rather too high a chemical shift to belong to a proton *ortho* to chlorine; for example in *N*-(2-hydroxyethyl)-2-chloro-5-nitrobenzylamine, the precursor to **II-HE,** this is at 7.53 ppm.) Thus it appears that the minor product exists predominantly in the ionic form in solution, even in a solvent as nonpolar as chloroform.

Conclusion

The fact that **II-HE** is rapidly hydrolyzed (by the mechanism of Scheme 5) makes it more remarkable that the compound shows no reactivity at all toward chymotrypsin. If it were to bind in the enzyme's active site, one could envisage the dithiocarbamate group being held in close proximity to the nucleophilic Ser-195 residue, just as it finds itself near to the neighboring ethoxy group in Scheme 5. Evidently, however, this scenario fails to lead to reaction, supporting the idea mentioned above that the oxyanion hole's inability to interact properly with a sulfur atom is the key factor.

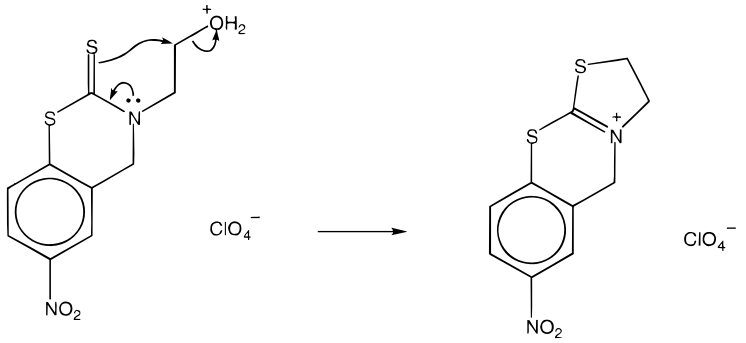
*Hydrolysis of **6-NC***

As a test of the likelihood that **6-NC** would react with an enzymic nucleophile as suggested in Scheme 3, its reaction with dilute hydroxide ion was first examined. It



SCHEME 7. Mechanistic proposals for how the product of alkaline hydrolysis of **II-HE** can lead, upon acidification with HCl, to a compound of formula $C_{10}H_9ClN_2O_3S$.

was found that a solution of **6-NC** does indeed rapidly turn yellow at pH 10.2, as expected if a *p*-nitrophenoxide ion is being produced. The λ_{max} is 404 nm, but this changes to a value of 355 nm when the pH is lowered; the observed pK_a of the *p*-nitrophenol group involved is 7.6. It is assumed that attack of hydroxide ion will give rise to an enol as this is likely to be more stable than the tautomeric ketoaldehyde because of conjugation and intramolecular hydrogen-bonding as shown in Scheme 9. This structure also suggests that similar hydrogen-bonding stabilises the undissociated phenol group, explaining its rather high pK_a value compared to that of *p*-nitrophenol itself



SCHEME 8. Formation of a dithioiminium ion from **II-HE** and perchloric acid.

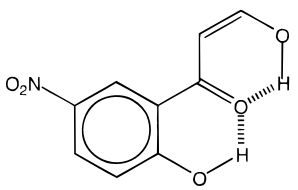
(7.1). (Under more extreme conditions, alkaline hydrolysis of **6-NC** involves partial degradation leading to 2′-hydroxy-5′-nitroacetophenone; *5*)

The high pH absorbance at 404 nm is stable, but the low pH peak (at pH 6, for example) slowly declines in size, by about 50% in an hour at 25°C. This is consistent with the recyclization of the hydrolysis product as suggested in Scheme 10 (where the conformation shown—poised for intramolecular 1,4-addition—would be in equilibrium with the putative hydrogen-bonded form of Scheme 9), giving back the original **6-NC** (which has a λ_{max} of 300 nm but does not absorb at higher wavelengths). At high pH the phenoxide ion is of course greatly stabilized by delocalization of the negative charge over the nitro group, but evidently the compound with the undissociated phenol group is not as stable as the chromone.

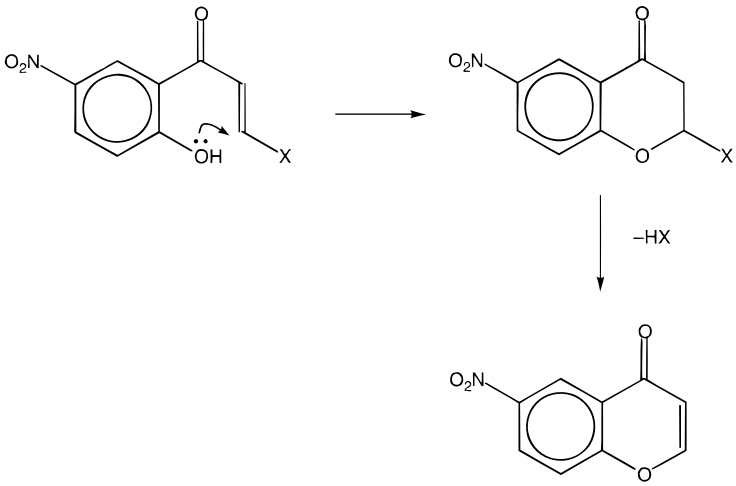
The hydrolysis product of **6-NC** was added to chymotrypsin at pH 8 and then the enzyme was passed down a gel filtration column: Fig. 3 shows there is no color associated with the protein fraction. Clearly the enzyme and the hydrolysis product do not have any particularly strong affinity for each other, confirming that in the results discussed below we are dealing with covalently modified enzyme.

*Reaction of **6-NC** with Chymotrypsin*

Incubation of chymotrypsin with **6-NC** at pH 8 over a period of several hours led to the development of a much more intensely yellow solution than that formed by spontaneous hydrolysis of **6-NC** in the absence of enzyme. After gel filtration of the reaction mixture, the protein fraction was found to be still yellow at high pH, with λ_{max}



SCHEME 9. Proposed stable conformation of the hydrolysis product of **6-NC** (at low pH).



SCHEME 10. Proposed cyclisation of certain *p*-nitrophenol derivatives giving rise to **6-NC.** Loss of HX signifies dehydration or it can represent the elimination of a covalently-linked enzymic group.

of 405 nm, and to absorb at 361 nm at low pH (see Fig. 4); the pK_a associated with the change in maximal wavelength is 7.9 as shown in Fig. 5. The magnitude of the absorbance of the covalently-linked *p*-nitrophenoxide ion indicated that about 66% of the enzyme was modified. (It seems from a variety of experiments that the chymotrypsin used in this work is at the most 70–77% pure active enzyme; *18.*) These observations are entirely consistent with the reaction proposed in Scheme 3.

Following modification of chymotrypsin by **6-NC,** the enzyme activity (toward *p*-nitrophenyl acetate) was measured; three independent experiments gave values of 93, 86, and 97% of the control activity of untreated enzyme. In stopped-flow experiments with the same substrate, the modified enzyme showed a burst of *p*-nitrophenoxide ion production that had 86% of the magnitude of the control and a burst rate constant that was 93% of the control. It is concluded with certainty therefore that **6-NC** does not react with the catalytically essential nucleophilic group of chymotrypsin (Ser-195) as this of course would result in complete inactivation. It is also clear that the reporter group must be in an "ordinary" environment since its pK_a and λ_{max} values are insignificantly different from those of the free hydrolysis product of **6-NC;** again this suggests

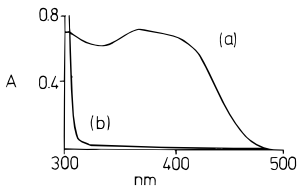


FIG. 3. The UV/visible absorbance of the hydrolysis product of **6-NC** at pH 8.0 is shown in (a). Spectrum (b) is that of the protein fraction after a mixture of chymotrypsin and the hydrolysis product of **6-NC** at pH 8.0 had been passed down a gel filtration column.

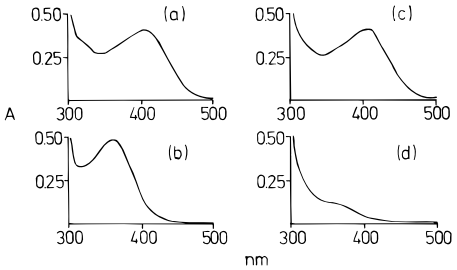


FIG. 4. Spectra (a) and (b) show the UV/visible absorbance of the reporter group supplied to chymotrypsin by **6-NC** at pH 10.22 and pH 6.02, respectively. Spectra (c) and (d) are the equivalent results obtained after the solutions had stood overnight at room temperature.

that the reagent does not react at chymotrypsin's active site. With **I,** on the other hand, the more extensive loss of activity and the grossly perturbed pK_a value are consistent with the idea that the label is in the enzyme's hydrophobic substrate-binding pocket, attached to Ser-195(*1*). As stated before, **I** and **6-NC** are of very similar size and shape, and both are nucleophilically attacked at position 2. However, with **I,** the transition state leading to the tetrahedral intermediate en route to acylation of the enzyme is exactly analogous to that of a substrate such as *p*-nitrophenyl acetate, but with **6-NC** the negative charge develops on a different part of the molecule (see Schemes 1 and 3) and evidently cannot be effectively stabilized by the enzyme's oxyanion hole. Thus it appears that the same factor explains the lack of reaction of both **II-HE** and **6-NC** at Ser-195. It is significant that a wide variety of recently developed active-site-directed inactivators of chymotrypsin all involve the attack of Ser-195 directly on a carbonyl group (*6, 19–21*); one exception involves an epoxide instead (*22*).

As observed with the hydrolysis product of **6-NC,** the chymotrypsin-linked reporter group is stable in the anionic form but not in the neutral form; representative spectra are shown in Fig. 4. It is concluded therefore that the label detaches from the protein at low pH by reversal of the modification reaction, as shown in Scheme 10. This was confirmed by incubating labelled enzyme overnight at room temperature and pH 8.0 (where the reporter group is approximately 50% ionized) and then subjecting it again to gel filtration at the same pH; taking into account the resulting dilution of the solution, the UV/visible absorbance showed that 40% of the label had been lost from the

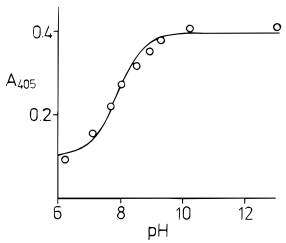


FIG. 5. Ionisation curve for the *p*-nitrophenol reporter group supplied to chymotrypsin by **6-NC.** The figure shows the best-fit curve calculated by Enzfitter (*23*); the pK_a is 7.9.

enzyme under these conditions. If this is released in the form of **6-NC** as suggested in Scheme 10, it would be expected then to undergo slow spontaneous hydrolysis, and indeed the small molecule fraction of the eluate in the experiment just referred to was observed to be yellow.

Conclusion

6-NC does react as envisaged with chymotrypsin to provide a conveniently monitorable reporter group, but it involves a nonessential enzymic nucleophile and probably occurs on the outer surface of the enzyme molecule. The label is chemically stable only above its pK_a (7.9) and therefore identifying the amino acid residue that is modified may prove to be problematic.

ACKNOWLEDGMENTS

I am grateful to Mr G. H. Freeman for the nitration of chromone and to Dr P. J. B. Edwards for recording NMR spectra.

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