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

Trevor M. Kitson

Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand

Received August 5, 1999

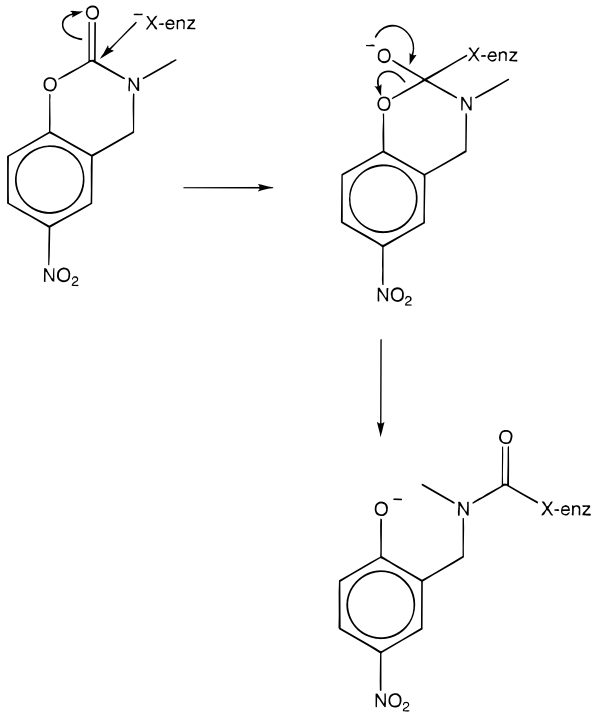
3,4-Dihydro-3-(2-hydroxyethyl)-6-nitro-2*H*-1,3-benzothiazin-2-thione (**II-HE**), a cyclic dithiocarbamate, has the potential to react with an esterase to furnish it with a *p*-nitrobenzenethiolate "reporter group." However, unlike a closely similar cyclic carbamate, **II-HE** is totally without reaction 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.

0045-2068/00 $35.00



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

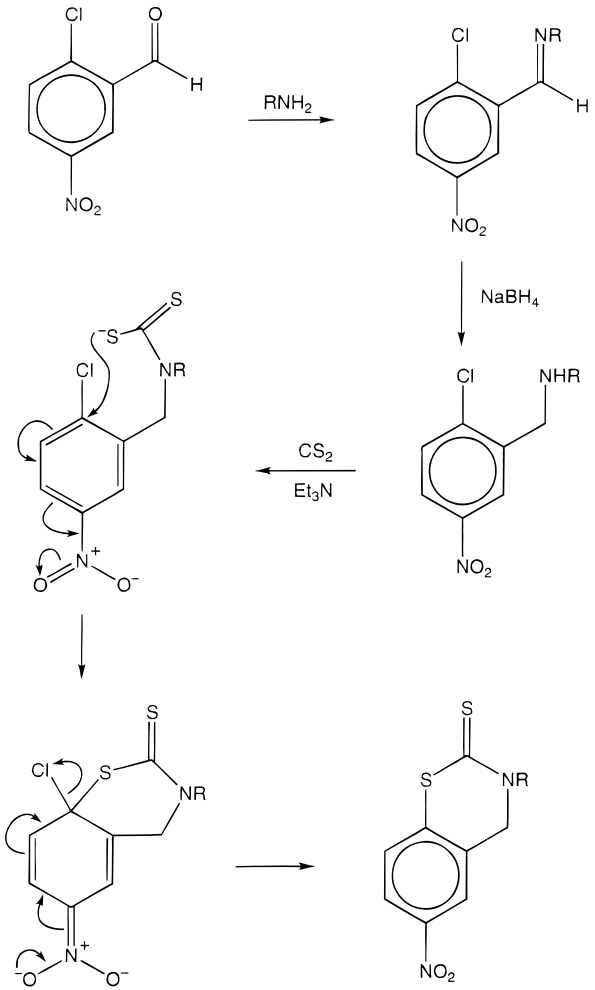
methyl-6-nitro-2*H*-1,3-benzothiazin-2-thione (**II-Me**) and 3,4-dihydro-3-(2-hydroxyethyl)-6-nitro-2*H*-1,3-benzothiazin-2-thione (**II-HE**); the route is shown in Scheme 2. The advantage of this synthesis over that of **I** (*1*) is that it is shorter and does not involve the dangerously toxic phosgene.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

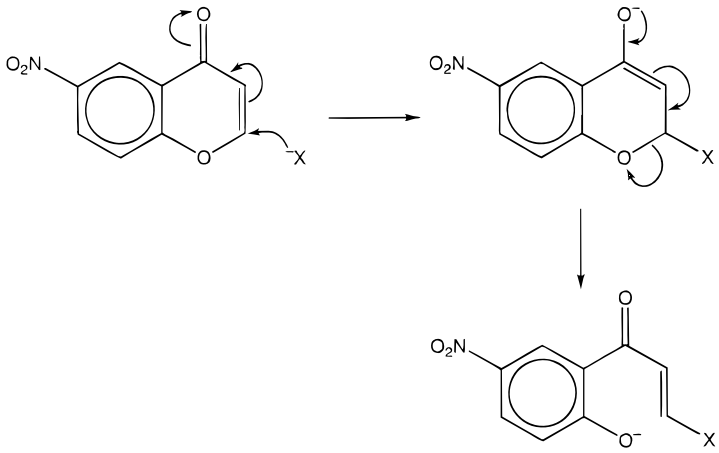
*Preparation of **II-HE** (See Scheme 2)*

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

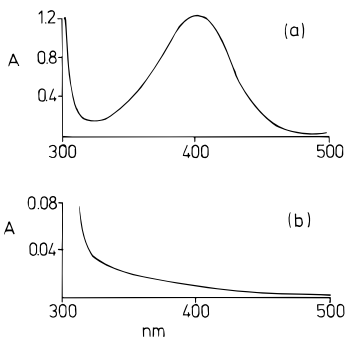
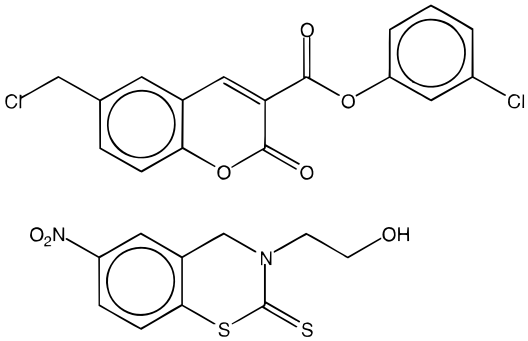


FIG. 1. (a) The UV/visible spectrum of the reporter group (at alkaline pH) supplied to chymotrypsin by **I.** (b) The equivalent result obtained when chymotrypsin was treated with **II-HE** under exactly the same conditions as used with **I** in (a).



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).

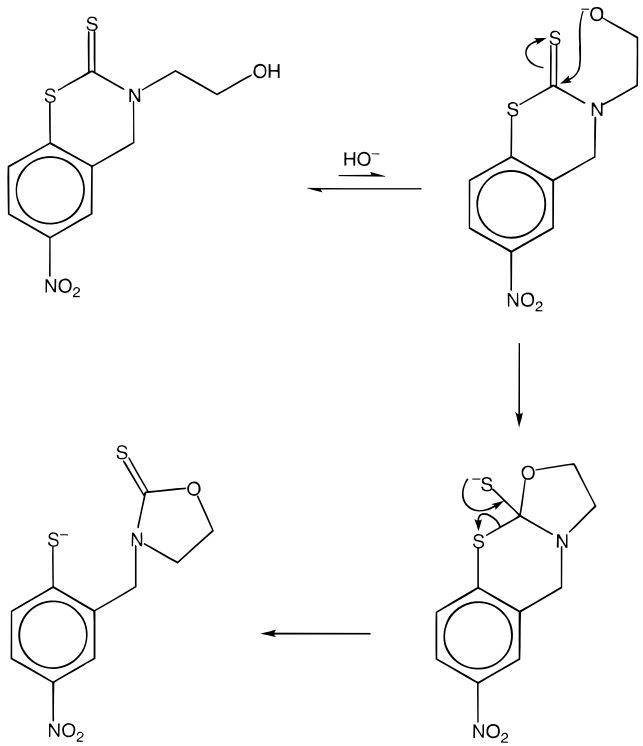
An observation that is relevant to the present results is that chymotrypsin does not measurably catalyze the hydrolysis of thionoesters (substrate analogues with a thiocarbonyl group), although thioloesters (in which the singly bonded oxygen of an ester group is replaced by sulfur) are good substrates (*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.**