Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium Salts

WATER-SOLUBLE ENVIRONMENTALLY SENSITIVE PROTEIN REAGENTS*

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SUMMARY

Water-soluble reagents were prepared by converting 2-hydroxy-5-nitrobenzyl halides into corresponding dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium salts. The resulting salts were found to selectively modify tryptophan and cysteine when added to amino acids in aqueous solutions. N-Acetyltryptophan, glycyltryptophan, and tryptophanylglycine appeared equal to tryptophan in reactivity with the sulfonium chloride; comparable reactivity was observed with chymotrypsin. Although the sulfonium salts are rapidly hydrolyzed in neutral and alkaline solutions, aqueous solutions at pH 3 are relatively stable. Thus, in contrast to 2-hydroxy-5-nitrobenzyl bromide, an aqueous solution of dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride can be used to modify proteins.

It has been observed that 2-hydroxy-5-nitrobenzyl bromide reacts rapidly and selectively with tryptophanyl residues to introduce the environmentally sensitive p-nitrophenolic grouping into protein and peptide structures (1, 2). Consequently, the reagent provides one means of assessing the role of tryptophan moieties in the functional activities of proteins, and, under denaturing conditions, it has also found application in the quantitative analysis of this amino acid residue (3). The fact that HNB° bromide can react with proteins under mild conditions at low temperatures and over a broad pH range (2) has made it an excellent reagent for a wide variety of proteins including chymotrypsin, phosphoglucuronase (4), pepsin and pepsinogen (5), trypsin inhibitor (6), glycoprotein (7), botulinum toxin (8), and antibodies.

Accordingly, to circumvent the difficulties experienced as a result of such solubility problems, it seemed highly desirable to synthesize a water-soluble reagent which, like HNB-bromide, could react selectively with tryptophanyl residues to attach the p-nitrophenolic chromophore to proteins, but in the absence of organic solvent. The finding that methionine reacts with HNB-bromide to produce an unstable sulfonium salt which rapidly decomposes in the presence of water and tryptophan (2) led to the investigation of sulfonium salts of the 2-hydroxy-5-nitrobenzyl alkylating agent as likely candidates for water-soluble tryptophan reagents.

EXPERIMENTAL PROCEDURE

Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium Chloride—A solution of 2.28 g (12.1 mmoles) of 2-hydroxy-5-nitrobenzyl chloride (9) in 20 ml of dimethyl sulfide was stirred at room temperature for 6 hours. During this time a considerable amount of precipitate formed. The remainder of the product was precipitated by addition of 400 ml of ether. The solid was collected, washed well with ether, and dried. The melting occurred with evolution of dimethyl sulfide. The sulfonium salt was dissolved in warm methanol and reprecipitated with ether, m.p. 152-153° (decomposition).

Calculated: C 43.68, H 4.85, N 5.61

Found: C 43.80, H 4.74, N 5.69

Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium Bromide—In a similar manner, the bromide sulfonium salt was prepared from 2-hydroxy-5-nitrobenzyl bromide (4) and dimethyl sulfide. The...
reaction in this case appeared considerably faster, and the product which was isolated (87% yield) melted with decomposition at 172° to 173°.

\[ C_6H_5NO_2SBr \]

**Calculated:** C 36.78, H 4.12, N 4.75  
**Found:** C 36.64, H 4.02, N 4.75

**2-Hydroxy-5-nitrobenzyl Methyl Sulfide**—To an ice-cold solution of 27.8 g (0.2 mole) of \( p \)-nitrophenol in 21.4 g (0.22 mole) of chloromethyl methyl sulfide was added 13.4 g (0.1 mole) of authydrous aluminum chloride, and the mixture was stirred at room temperature for 30 min. It was then heated to 55° for 3 hours with continuous stirring. The viscous material was poured onto 120 g of crushed ice, allowed to stand at room temperature overnight, and then extracted twice with 80-ml portions of chloroform. The combined chloroform extract was washed several times with water and the solvent was removed by flash evaporation yielding 28 g of crude product, m.p. 112° to 116° (70% yield). Twice recrystallizing from ethanol-water (1:2) afforded 19 g of white needles, m.p. 126° to 127°. The nuclear magnetic resonance spectrum was consistent with that expected.

\[ C_6H_5NO_3S \]

**Calculated:** C 48.23, H 4.55, N 7.03  
**Found:** C 48.02, H 4.52, N 6.90

**Dimethyl(2-hydroxy-5-nitrobenzyl)sulphonium Fluoroborate**—A cold solution of 2-hydroxy-5-nitrobenzyl methyl sulfide (3.0 g, 15 mmoles) in 40 ml of nitromethane was stirred for 2 hours with 2.25 g (15 mmoles) of trimethylxonium fluoroborate (10) and allowed to stand overnight at room temperature. The solution was poured into 400 ml of dry ether, and the solid product was filtered and washed thoroughly with ether (91% yield). The salt was recrystallized as fine needles from a mixture of acetone and chloroform, m.p. 169° to 171°.

\[ C_6H_5NO_2SBr_3 \]

**Calculated:** C 35.89, H 4.02, N 6.95  
**Found:** C 35.71, H 3.96, N 6.83

**Other Materials**—Free amino acids and \( N \)-acetyl-L-tryptophan were obtained from Calbiochem; glycyl-L-tryptophan, L-tryptophan, glycyl-L-methionine, and glycyl-L-valine were purchased from Sigma Chemical Company; and \( \alpha \)-chymotrypsin and carboxy-peptidase A were obtained from Worthington Biochemical Corporation.

**RESULTS**

**Reactivity with Tryptophan**—Aqueous solutions of amino acids common to proteins were treated with varied ratios of dimethyl (2-hydroxy-5-nitrobenzyl)sulphonium bromide at acidic, neutral, and basic \( pH \), and at various concentrations of common electro-lytes and buffer salts. At appropriate intervals, aliquots were removed for amino acid analyses using a modification of the automatic apparatus described by Spackman, Stein, and Moore (11). At \( pH \) 6.0 the sulphonium bromide (final concentration of 4.8 \times 10^{-2} M), added as a solid to an aqueous solution \( 10^{-5} M \) in each amino acid with continuous stirring (\( pH \) maintained with 1 N NaOH delivered from a \( pH \)-stat), selectively reacted with L-tryptophan to result in its complete modification. Increasing the concentration of the sulphonium reagent to 4.0 \times 10^{-2} M again resulted in total modification of tryptophan without detectable modification of any of the other amino acids present. Essentially identical results were obtained at \( pH \) 3.3, 5.6, 7.0, and 8.8, in the presence or absence of 0.2 M NaCl.

Moreover, it was found that the sulphonium bromide reagent could be dissolved in water prior to its addition to the amino acid solution. Complete modification of tryptophan occurred when a solution 10^{-3} M in each amino acid was treated with an aqueous solution \( (4 \times 10^{-3} M) \) of dimethyl(2-hydroxy-5-nitrobenzyl)sulphonium bromide to produce a final reagent concentration of 1 \times 10^{-3} M. In this experiment the sulphonium reagent had been dissolved in water 30 min before its addition to the amino acids. By contrast, even 20 sec of exposure of dissolved HNB-bromide to water prior to addition of amino acids resulted in no detectable modification of tryptophan when each was present at these same concentrations (2).

The same selectivity in amino acid modification was exhibited by the chloride and fluoroborate salts of the dimethyl(2-hydroxy-5-nitrobenzyl)sulphonium species as by the bromide. Consequently, most of our subsequent studies of reactivity with amino acids, dipeptides, and proteins have been made with the chloride form of the reagent.

Further examination of the rate of hydrolysis of the HNB-sulphonium reagent provided the results given in Table I. Even after 3 days in aqueous solution at room temperature, considerable reactivity toward tryptophan remains. The extent of reaction with tryptophan in phosphate buffers (\( pH \) 7.0, 8.0) and in Tris-chloride buffer (\( pH \) 7.6) could not be distinguished from that in imidazole buffer. However, in contrast to these findings, dissolving the HNB-sulphonium chloride in any of these buffers instead of water, prior to combination with tryptophan, resulted in considerable hydrolysis. For example, treating tryptophan with a 1:1 ratio of dimethyl(2-hydroxy-5-nitrobenzyl)sulphonium chloride which had been dissolved 60 sec previously in 0.02 M imidazole buffer, \( pH \) 8.23, under conditions otherwise identical to those given in Table I, resulted in recovery of 0.211 pmole of unmodified tryptophan.

Measurements of \( pH \) revealed that the limited hydrolysis which occurs when the sulphonium salt is dissolved in water is accompanied by proton release, as expected. In a nonbuffered system at \( pH \) 7.0, addition of HNB-(CH_3)SOCl is accompanied by a rapid decrease in \( pH \) to 3.5 (10 sec) followed by a slower continued decline to a fairly constant value of 3.1 (30 min). Maintenance of a nonbuffered solution of the reagent at \( pH \) 7.0 by means of a \( pH \)-stat produces the same loss of tryptophan reactivity as is observed in \( pH \) 7.0-buffered solutions. Reagent hydrolysis is correspondingly slower at intermediate \( pH \) values (e.g. \( pH \) 5.0) than at \( pH \) 7.0.

**Reactivity with Other Amino Acids**—Because of the problems which arise in quantitating cysteine in the presence of cystine and the other common amino acids, separate experiments were conducted to access the cysteine-modifying activity of dimethyl (2-hydroxy-5-nitrobenzyl)sulphonium chloride. Mixtures of glycine and L-cysteine \( (10^{-4} M) \) each) were treated under nitrogen with various concentrations of the sulphonium reagent, and the degree of alkylation was determined by (a) direct analysis of HNB-cysteine on the short column of the amino acid analyzer (2), (b) recovery of cysteic acid following performic acid oxidation by the procedure of Moore (12), and (c) recovery of carboxymethylcysteine following treatment with excess sodium iodoacetate.
The reactivity of HNB-(CH$_3$)$_2$S with $l$-tryptophan after various periods of solution in water

- The conditions were: $1.0 \times 10^{-3}$ M $l$-tryptophan in 0.01 M imidazole buffer, 0.1 M NaCl, pH 8.23, treated with aqueous solutions of the sulfonium chloride ($4.0 \times 10^{-3}$ M) which had been prepared at designated intervals.

<table>
<thead>
<tr>
<th>Ratio of total reagent to tryptophan</th>
<th>Age of aqueous reagent solution</th>
<th>Tryptophan recovered</th>
<th>HNB groups reacted with tryptophan</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:1 (control)</td>
<td></td>
<td>0.227</td>
<td>%</td>
</tr>
<tr>
<td>1:1</td>
<td>1 min</td>
<td>0.080</td>
<td>65</td>
</tr>
<tr>
<td>1:1</td>
<td>8 min</td>
<td>0.094</td>
<td>50</td>
</tr>
<tr>
<td>1:1</td>
<td>30 min</td>
<td>0.055</td>
<td>58</td>
</tr>
<tr>
<td>1:2</td>
<td>1 min</td>
<td>0.028</td>
<td>44*</td>
</tr>
<tr>
<td>1:2</td>
<td>3 days</td>
<td>0.063</td>
<td>36*</td>
</tr>
</tbody>
</table>

* Theoretical maximum = 50% assuming a 1:1 reaction.

The rate at pH 9 (13). In the case of the iodocetate-treated samples, total recoveries of cysteine moieties (carboxymethylcysteine + HNB-cysteine + half-cystine) could be checked; these ranged from 97.7 to 100.3% (based on glycine recoveries).

When the solid sulfonium reagent was added to $10^{-3}$ M cysteine at pH 7.6 in equimolar concentration, 41% of the amino acid became alkylated and could be recovered as HNB-cysteine. When the reagent was added in a 2:1 molar ratio, 61.8% of the cysteine became modified. An indication of the rate of alkylation of cysteine at pH 7.6 was obtained by treating $10^{-3}$ M cysteine with a 1:1 ratio of dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride in the form of a $10^{-3}$ M aqueous solution. After various intervals, excess iodocetate was added to each reaction mixture, and the degree of hydroxynitrobenzylation of cysteine was then determined. Within the first 60 sec, 30% of the cysteine had been modified by the aqueous solution of the sulfonium reagent; within 10 min, 34%; and within 60 min (or longer), 39%. Although the reactivity of L-cysteine was less than half that of $l$-tryptophan with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride, it was found that the cysteine-modifying activities of aqueous solutions of the reagent paralleled their tryptophan-modifying activities.

Earlier investigation had established that, even though methionine residues are not modified following treatment with 2-hydroxy-5-nitrobenzyl bromide, methionine does react with that reagent to produce a short-lived product (2). Moreover, some modification of histidine could be observed when amino acids were treated with 2-hydroxy-3,5-dinitrobenzyl chloride (14). Thus, even though amino acid analyses revealed that only tryptophan and cysteine are modified by treatment with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride, it was of interest to examine the effects of these other amino acids on the rate of reaction of the sulfonium reagent in aqueous solutions using pH-stat titrations.

Because of the rapid rate of hydrolysis which occurred in neutral or alkaline solutions, rate measurements with the pH-stat could be made only in acidic media. Results are shown in Fig. 1. The reactivity of tryptophan and tryptophan-containing peptides with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride was evident in terms of the rate of base uptake. The presence of $5 \times 10^{-2}$ M methionine, tyrosine, or alanine did not affect the observed rate. However, the rate of base uptake in the presence of $4 \times 10^{-2}$ M histidine or serine appeared slightly elevated over that of the controls.

Reactivity with Peptides—Solutions of N-acetyl-$l$-tryptophan, $l$-tryptophan, and $l$-tryptophanylglycine were each found to react with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride at rates indistinguishable from that of $l$-tryptophan itself (see Fig. 1). Treatment of $10^{-3}$ M glycyl-$l$-tryptophan with a 1:1 molar ratio of the $10^{-3}$ M aqueous reagent (dissolved 1 min earlier) led to recovery of 38.2% of the unmodified dipeptide. Thus, 61.8% of the tryptophan-containing peptide had reacted with the sulfonium salt, a reactivity apparently equal to that of free tryptophan (cf. Table I). No modification of glycyl-$l$-methionine was detected following similar treatment.

Reactivity with Proteins—Solutions of $\alpha$-chymotrypsin and of carboxypeptidase A were treated with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride under a variety of conditions. Each of these enzymes can be modified through such reaction without detectable denaturation by selection of appropriate conditions.

Chymotrypsin has been treated with molar ratios of the sulfonium salt varying from 18:1 to 125:1, at pH values ranging from 3.3 to 7.5. In all instances, even when labeling occurred in the presence of 10 M urea, introduction of the hydroxynitrobenzyl group occurred without detectably altering the recoveries of amino acids (excluding tryptophan) from acid hydrolysates of the treated protein. Evidence for tryptophan modification was obtained from amino acid analyses performed on alkaline hydrolysates (15) and on acid hydrolysates containing 1.2% thioglycolic acid (16). However, even though the recovery of tryptophan from HNB-labeled enzyme was significantly lower than that from unmodified chymotrypsin, neither of these hydrolytic methods has provided consistent, quantitative tryptophan recoveries. Thus, it is difficult to make accurate assessments of unmodified tryptophan. Modifications of these methods of hydrolysis are receiving continued study.
nitrobenzyl)sulfonium chloride at pH 3.5 in an aqueous solution 4 × 10^{-4} \text{M} in chymotrypsin was monitored with the pH-stat. The initial rate of sodium hydroxide delivery corresponded to reaction of 1.2% of the total reagent per min. By comparison, in a solution 10^{-3} \text{M} in glycyl-L-tryptophan, the initial reaction rate was 2.6% per min; similarly, a rate of 2.5% per min was recorded with 10^{-3} \text{M} L-tryptophan.

As expected, the degree of labeling of chymotrypsin, determined by the method of Berman and Koshland (3), was found to depend on the concentration of added reagent and on the pH of the reaction mixture. As shown in Table II, the greater the reagent concentration, the greater the incorporation of HNB groups into protein. Less labeling occurred at pH 7.2 than at pH 4.0. At pH 3.5, the protein became more highly labeled in the presence of 10 \text{mM} urea than in its absence.

Treatment of carboxypeptidase A in 3 \text{mM} sodium chloride at pH 7.7 with an 80:1 molar ratio of dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride resulted in a degree of labeling of 1.2 moles of HNB groups bound per mole enzyme. Such labeling resulted in a doubling of esterase activity toward L-tryptophan. As shown in Table II, the greater the degree of labeling, the greater the recovery of tyrosine or the other amino acids (excluding tryptophan) in the presence of 10 \text{mM} urea.

**Discussion**

Several features of dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium salts as protein modification reagents are worthy of note. First is the environmental sensitivity of the nitrophenolic chromophore, a feature shared by the analogous substituted benzyl halides (2, 14) and the bromoacetamidinonitrophenols (17, 18). In addition to the characteristic ultraviolet-visible absorption spectral shifts which can be obtained for proteins to which the nitrophenolic group is bound, we have observed circular dichroic evidence for dissymmetry in the region of HNB absorbance in labeled carboxypeptidase A. Such a signal is not evident in mixtures of unlabeled carboxypeptidase and p-nitrophenol. Thus, it will be of interest to further examine not only the difference spectra generated by these conformational probes to proteins, but also their circular dichroism and optical rotatory dispersion.

Although these salts possess amino acid-modifying selectivity similar to that previously reported for 2-hydroxy-5-nitrobenzyl bromide and the corresponding chloride, a distinguishing feature is their water solubility. Whereas all attempts to introduce the HNB chromophore into the structure of carboxypeptidase through reaction with HNB-bromide have led to inactivation and precipitation of the enzyme, good yields of labeled carboxypeptidase have been obtained through treatment with the water-soluble reagent. Thus, the utility of the labeling technique can now be extended to cases in which the protein of interest becomes inactivated, changes its degree of polymerization upon exposure to organic solvent, or coprecipitates with the alkyl halides. It should also be noted that the sulfonium salts, although rapidly hydrolyzed at neutral or alkaline pH, are relatively stable in acidic aqueous solutions. Consequently, it may be convenient to dissolve an appropriate excess of the reagent in water (or salt solution) prior to its addition to a buffered solution of the protein. An alternative is simply to add the reagent as a solid. We have successfully employed both techniques in labeling chymotrypsin and carboxypeptidase.

Because of the rapidity of hydrolysis, it has not been possible to examine the effects of various amino acids on the rate of HBr release from 2-hydroxy-5-nitrobenzyl bromide by pH-stat measurements. However, in the case of the sulfonium reagent, such measurements are possible in acidic media. Interestingly, methionine had no effect on the rate of base uptake either at pH 4.0 (Fig. 1) or pH 3.1 (not shown). On the other hand, histidine and serine each produced a perceptible increase in rate, both at pH 4.0 and pH 3.1. Neither of these amino acids is modified by treatment with the sulfonium reagent in acidic, neutral, or basic solution as evidenced by their complete recoveries on column chromatography. Similarly, no losses of histidine or serine were detected in acid hydrolysates of chymotrypsin or carboxypeptidase which had been treated with the reagent in comparison with untreated controls.

In terms of amino acid or protein modification, then, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium salts appear to be completely selective for tryptophan and cysteine.

One other feature of the sulfonium reagents deserves mention. It has become clear that the phenolic hydroxyl group plays an important role in the reactivity and selectivity of the 2-hydroxy-5-nitrobenzyl halides, as evidenced by the properties of the 2-methoxy analogue (19) and the 2-acetoxo analogue (14). In the case of 2-acetoxy-5-nitrobenzyl chloride, it was possible to modify the hydroxyl group in such a manner as to generate the 2-hydroxynitrobenzyl reagent in situ through reaction with the active site of chymotrypsin (14). Since similar approaches may be useful for modifying tryptophanyl residues near the active sites of other enzymes, the synthesis of other “blocked” reagents could be of interest. Because of the reactivity of the HNB halides, and the ease with which their \( \alpha \)-halogen atoms can be displaced, there are limited numbers of reactions which can be used to successfully modify the 2-hydroxy group. Accordingly, the method of synthesis of the sulfonium fluoroborate reported here is of particular interest. The desired analogue for a given enzyme, containing the modified 2-hydroxyl grouping, can be synthesized first as the unreactive methyl sulfide. The methyl sulfide can then be converted to the blocked dimethyl sulfonium salts.
salt suitable for generating the 2-hydroxy-5-nitrobenzyl reagent upon reaction with the enzyme.

REFERENCES
Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium Salts: WATER-SOLUBLE ENVIRONMENTALLY SENSITIVE PROTEIN REAGENTS
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