The Chemistry of a Reporter Group: 2-Hydroxy-5-nitrobenzyl Bromide*

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SUMMARY

The chemistry of the reaction of 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) with tryptophan ethyl ester to form monosubstitution adducts has been elucidated. The initial compounds are formed by addition at position 3 of the indole ring to form two diastereomeric indolenines. These compounds can then cyclize and rearrange in a number of ways. The absolute stereochemistries of the products have been determined. The 2-hydroxy-5-nitrobenzyl moiety has been shown to form complexes with amino acid esters and the spectral shifts of the complexes can be correlated with the "hydrophobicity" of the amino acid side chains. The specificity of HNB-Br for tryptophan in proteins can be explained by a combination of a hydrophobic attraction and a specific charge-transfer complex of the quinone methide form of the reagent. The hydrophobic affinity of the HNB group, the specificity of HNB-Br for tryptophan, and the number of potential reaction products of HNB-Br lead to a number of possible modes of reaction of HNB-Br with tryptophan in proteins which enhance the value of the reagent as an environmental probe.

Suggestions have been made in regard to the reasons for the high reactivity of the reagent (2) and the spectral changes which it undergoes (22). Nevertheless, the ultimate utility of the reagent depends on a thorough understanding of the nature of its chemical reactivity and of its products in the reaction with tryptophan in proteins. The present work on the reactivity and spectral properties of HNB-Br clarifies these processes.

EXPERIMENTAL PROCEDURE

Preparation of 2-Hydroxy-5-nitrobenzyltryptophan Ethyl Ester Derivatives—Tryptophan ethyl ester hydrochloride (Compound 1), 500 mg (1.9 mM), was allowed to react with 500 mg (2.2 mM) of HNB-Br (Compound 2), which was added dropwise in 5 ml of anhydrous acetone or dioxane. A pH of 4.7 was maintained by addition of methanolic sodium acetate. A complex mixture of at least seven components resulted. Unreacted Compound 1 and salts were removed by washing a CHCl₃ suspension of the reaction mixture with water.

The remaining components of the reaction mixture were separated most effectively by preparative thin layer chromatography with a 0.5-mm thickness of Silica Gel G on plates, 8 x 8 inch. The reaction mixture, isolated by evaporation of the MgSO₄-dried CHCl₃ solution, was applied to each plate in 50- to 90-μg segments, and the plates were eluted successively with 6 volume % ethanol in chloroform four times. After each elution, the plates were allowed to dry before commencing the next elution. The various compounds were isolated by repeated extraction of the silica gel bands with absolute ethanol and concentration of the resulting ethanol solutions to dryness. Column chromatography was less satisfactory. The order of elution of components of the reaction mixture was: solvolysis products of HNB-Br (HNB-OCH₃, HNB-OH), disubstitution products (5 and 6), and monosubstitution products (3 and 4, in that order). Subsequent work was carried out on the monosubstitution products.

Compound 3 had a melting point of 189-190°, and Compound 4 had a melting point of 198-199°. Mass spectral analysis of Compound 3 (probe 160°, source 230°, at 70 eV, 100 μA) revealed a molecular ion at m/e = 383 (Compound 3 - HCl), and peaks at M - 73 (M - CO₂H₂O) and m - 151 (M - HNB). Compounds 3 and 4 had identical infrared spectra (N-H, -OH stretch, ester carbonyl) and identical electronic spectra: λmax (95% ethanol) 240 μm (ε = 11,800), 310 μm (8,900); λmax (2 N NaOH) = 422 μm (19,800).

Elemental analyses gave the following result. For Compound 3,

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1 The abbreviations used are: HNB, 2-hydroxy-5-nitrobenzyl; NMR, nuclear magnetic resonance.
For Compound 4

\[
\text{C}_{9}\text{H}_{12}\text{N}_{2}\text{O}_{2}\text{HCl}
\]

Calculated: C 57.10, H 5.24, N 10.00
Found: C 58.86, H 5.72, N 10.36

Because of the importance of the value of the extinction coefficient of these derivatives at 410 rnp in established analytical procedure (3), this number was determined to be \(\varepsilon (410 \text{ rnp}, 2 \text{ cm}) = 18,450\)

\textbf{Acid-catalyzed Rearrangement of Compounds 3 and 4}—Compounds 3 and 4 were each converted to the same diacetyl derivative, 7-Acz, of a rearrangement product, 7, in the following manner. Either Compound 3 or 4 (50 to 70 mg), or a mixture of both, was dissolved in 5 ml of absolute ethanol-concentrated NaOH (1:1, v/v) and heated at reflux for 15 min. The mixture was cooled to room temperature, brought to a pH meter reading of 5 with 1 M aqueous sodium acetate, and concentrated to dryness under reduced pressure. The amorphous residues, 7, after treatment of Compound 3 or 4 or both were chromatographically identical. The residue was shaken for 10 min with 10 ml of freshly distilled dry acetic anhydride and set aside at room temperature for 24 hours. After removal of acetic anhydride by evaporation, the residue was dissolved in CHCl3-saturated aqueous NaHCO3 (1:1, v/v) and the mixture was stirred. The CHCl3 layer was removed, washed extensively with water, and concentrated to dryness under reduced pressure. The residue was chromatographed (silica gel, 1.5 volume % ethanol in chloroform) to yield the same compound, 7-Acz, in each case. This amorphous compound was pure by thin layer chromatography and nuclear magnetic resonance (see below), but it had an aberrant elemental analysis, possibly because of coprecipitation with solvent.

\textbf{Difference Spectra of HNB-OH and Amino Acid Ethyl Esters}—In a typical experiment, a stock solution of 0.13 M HNB-OH in dioxane-water (~75% v/v) was formed by permitting the same amino acid ester stock solution, approximately 10⁻⁴ M. Into Compartment 1 of each of two 9-mm tandem cuvettes was pipetted 1.0 ml of the 10⁻⁴ M HNB-OH solution, and into Compartment 2 of these cuvettes was placed 1.0 ml of a 0.5 M amino acid ester stock solution, appropriately diluted. Difference spectra were run with the 0 to 0.1 slide wire of the Cary model 14 ultraviolet spectrophotometer with the cell compartment thermostatted at 25°C. The concentration range of amino acid esters used was typically 0.02 to 0.20 M. The reciprocal of the change in absorbance after mixing was plotted against the reciprocal of the concentration of the amino acid ester in the sample cell after mixing, and the association constants and saturation absorbance values were calculated from the slope and intercept of the straight line obtained.

The evidence that the resulting difference spectra were not due to bulk dielectric effects is 2-fold. First, the position of the maximum in the difference spectrum did not change with the concentration of the amino acid ester. Second, a control was run in which HNB-OH was brought to identical concentrations in dioxane-water (10:90, v/v) and 100% dioxane, respectively. The HNB-OH-dioxane solution was placed in the reference cuvette, and the HNB-OH-water-dioxane solution was placed in the sample cuvette. The difference spectrum was run, and it was found that a positive difference spectrum was obtained at the higher wave length. If one assumes that dioxane should exert a dielectric effect on the spectra that is in the same direction as that produced by the amino acid esters, then this result may be used to show that the amino acid ester-HNB-OH difference spectra are not due to bulk dielectric effects.

\textbf{RESULTS AND DISCUSSION} Substitution Products Formed when HNB-Br Reacts with Tryptophan—When equal amounts of HNB-Br and L-tryptophan ethyl hydrochloride were allowed to react under conditions closely simulating a protein modification experiment, two monosubstitution products, 3 and 4, were obtained, with Compound 3 predominating over 4 in the ratio of about 6:4 (quantitative thin layer chromatography). The identification of Compounds 3 and 4 as monosubstitution products rests on data from proton magnetic resonance spectra (see below), ultraviolet extinction coefficients of the p-nitrophenolate chromophore, elemental analysis, and mass spectra (see "Experimental Procedure"). Compounds 3 and 4 comprised about 40 to 50 weight % of the over-all reaction mixture. The two remaining addition compounds, 5 and 6, were shown to result from the addition of 2 eq of HNB-Br to 1 of tryptophan ethyl ester. Compounds 3 and 4 could be converted to 5 and 6, respectively, by reaction with additional HNB-Br, and Compounds 5 and 6 did not react further with additional HNB-Br.

When either Compound 3 or 4, or a mixture of both, was subjected to treatment with ethanolic HCl followed by acetylation, the same N₁,Ο-diacetyl derivative, 7-Acz, was obtained in each case.

Alkyl halides are known to form indolines with indoles by addition at position 3 of the indole nucleus (23-26). That the indole position 3 should be the most reactive position to electrophiles is also confirmed by theory (27). The close similarity of the infrared and ultraviolet spectra and similarity in yields of the nonidentical Compounds 3 and 4 could be accounted for if both resulted from the addition of the hydroxynitrobenzyl group at the indole position 3 of L-tryptophan ethyl ester. Because the starting material was pure L isomer of the amino acid ester, two diastereomeric compounds would be expected as a result of the new asymmetrical center created at position 3 (cf. Equation 1).
FIG. 1. NMR spectra, 220 MHz, of tryptophan ethyl ester-HNB-Br adducts. All spectra were taken in CDCl₃. a, spectrum of Isomer 3, sweep width 2500 Hz. The assignment of the 7.4 δ signal to residual CHCl₃ was checked by rerunning the spectrum in CD₂OD. b, spectrum of Isomer 4, sweep width 2500 Hz. c, low field portion of the spectrum of Isomer 4, sweep width 500 Hz. The corresponding spectrum of Isomer 3 was essentially identical. Integration of all bands and observation of splitting characteristics were carried out on expanded spectra of this type for all compounds studied. d, spectrum of Compound 7-AC, sweep width 2500 Hz. Internal Standard is tetramethylsilane (TMS).
cal center and yields a single compound from either 3 or 4. If the ability to support positive charge is the phenomenological criterion for the effectiveness of a migrating group (28, 29), then the hydroxynitrobenzyl group (which can revert to a quinone methide type of structure merely by the loss of a proton) would be expected to rearrange exclusively with a rate that is very rapid relative to both rearrangement of the amino acid ester side chain and the polymerization that is known to accompany the acid treatment of indolines (25).

NMR Evidence for Assigned Structures—The 220 MHz proton NMR spectra of Compounds 3 and 4, shown in Fig. 1, a and b, respectively, provide evidence in support of the postulated structures. Particularly noteworthy are the following: the AB quartet resonances at 7.9 6 for the aromatic ring protons in Compounds 3 and 4, shown in Fig. 1, a and b. These resonances result from aromatic ring protons in the plane of the hydroxynitrobenzyl group, and the polymerization that is known to accompany the acid treatment of indolines (25).

Other features of the spectra, however, are not in accord with the interpretation of Compounds 3 and 4 as simple indolines. Fig. 1 shows the expanded aromatic region of Compound 4 (that of Compound 3 is essentially identical). There is no resonance that can be attributed to an indolene-2-carboxylic acid, which would be expected to fall in the 7.5 to 8.5 6 range. However, the minor function of indolines easily undergoes nucleophilic attack (19, 21), and two nucleophiles are readily available in this case in the same molecule, namely, the phenolic oxygen (which has been shown to be the cyclizing nucleophile in the NNB-skatole adducts (10) (Equation 3)) and the side chain amino group in its free base form which might cyclize as shown in Equation 4. The singlet resonance at 1140 Hz (5.2 6) in both Compounds 3 and 4 is that expected of a diaminomethine or aminooxymethine proton resulting, respectively, from these types of cyclizations (30, 31).

Since this signal does not distinguish unambiguously the two possible modes of cyclization, the following experiment was executed. The NMR spectra of Compounds 3 and 4 were rerun in neutral methanol-d4. There is a solvent effect on the signal of the methine proton in question (probably due to solvent shielding of the methine proton from the electrical effects of nearby charged groups), and this signal appears at 4.9 6 in CD3OD. The ultraviolet spectra of Compounds 3 and 4 in methanol indicated that the phenolic moiety was un-ionized. Enough concentrated NaOD-D2O was then added so that the phenolic group was ionized, a fact determined from the presence of the p-nitrophenolate chromophore in the visible spectrum. Clearly, an ionized phenolic hydroxyl group cannot be involved in cyclization.

The NMR spectrum was rerun, and the 4.9 6 resonance was still present in the same relative intensity, unshifted. Had the hydroxyl group been involved in cyclization before addition of base, the ring opening attendant upon ionization would have resulted in a dramatic chemical shift for the methine proton, since an indolene would have resulted. Even the attack of a different nucleophile on the indolene under such basic conditions should lead to a substantial (although less dramatic) chemical shift. Since absolutely no shift was observed in the methine proton resonance upon addition of base, it is therefore concluded that the side chain amino group rather than the phenolic oxygen is the nucleophile which has attacked the initially formed indolone. The rather substantial downfield methine proton shift found in CDCl3 is perhaps somewhat greater than one might expect for a diaminomethine proton (30, 31); however, it can be accounted for by electrical effects resulting from protonation of the adjacent secondary amino group in the HCl salt.

The 220 MHz NMR spectrum of Compound 7-Ac5, shown in Fig. 1d, confirms the hypothesis of an acid-catalyzed indolene rearrangement of Compounds 3 and 4. (The cyclized structure for 3 and 4 would be quite labile in acid, and, after opening to the indolene, the rearrangement would be able to proceed normally.) The 5.2 6 singlet is absent in this compound, and a new, broad singlet resonance, whose position was found to be somewhat concentration-dependent, is evident at 8.1 6; this signal is expected of an indole-1-proton. The signal attributed to the hydroxynitrobenzyl methylene protons, observed as an AB quartet at 3.2 6 in Compounds 3 and 4, is a sharp singlet which is found further downfield at 890 Hz (4.0 6) in 7-Ac5, and the methylene protons of the amino acid side chain are found at 740 Hz (3.4 6), a downfield shift relative to the position of this signal in Compounds 3 and 4 (2.9 6). These results indicate the formation of an indole ring with two substituted, or Structure 7-Ac5.

Absolute Configurations of Compounds 3 and 4—The absolute configurations of 3 and 4 can be assigned on the basis of the data.
High Reactivity of HNB-Br—The extremely high reactivity of HNB-Br has already been considered (2) and the contribution of a quinone methide type structure in the reactions of HNB-Br appears to explain the rapid rates of reaction of this species. However, the reasons for the specificity of the reagent for tryptophan and cysteine are not clear. The relative reactivity of tryptophan to water with HNB-Br has been estimated to be at least 10,000.

Further confirmation of the proposed absolute stereochemistry is provided by the relative yields of Compounds 3 and 4. If one envisions a transition state for the reaction of L-tryptophan ethyl ester and HNB-Br in which the two aromatic rings are parallel and the indole position 3 and the HNB-Br methylene carbon are directly opposed, somewhat less hindrance is expected from the transition state in which the HNB group and H$_2$ are syn (leading to Compound 3) than when the HNB group and the somewhat more bulky carboxy group are syn (leading to Compound 4). This expectation accounts for the higher relative yield of 3 over 4 by an amount which is typical of asymmetrical induction. Thus, the absolute configuration of Compound 3 may be described in the Cahn-Ingold-Prelog scheme (32) (reading clockwise from the top of the structures in Fig. 2) as R,S,S and that of 4 as S,S,R.

Further examination of models, it is clear that the time-averaged environment of the $\alpha$-carbon of the amino acid side chain in Compounds 3 and 4 will contain a substantial contribution from a rotamer in which the face of the aromatic ring of the hydroxynitrobenzyl group is positioned directly opposite this carbon. This consideration predicts that, when the hydroxynitrobenzyl ring is syn to the proton on this $\alpha$-carbon (H$_3$ in Fig. 1), H$_4$ occupies space within the shielding cone of this ring, and should be shifted upfield. When the aromatic ring is anti to H$_3$, the methylene protons of the ethyl group (H$_4$) will be shielded and should display the upfield shift.

The NMR spectra show that, in Isomer 3, the resonances of H$_3$ and H$_1$ are separated by 82 Hz, with H$_4$ in the upfield position. In Isomer 4, however, an upfield shift of the H$_1$ signal and a downfield shift of the H$_3$ signal, relative to the positions of these signals in 3, have caused these signals to converge. In fact, the chemical shift difference of H$_3$ and H$_1$ changes by over 100 Hz between Compounds 3 and 4. These facts, taken with the knowledge of the absolute configuration of the starting L-tryptophan ethyl ester, define the absolute stereochemistry of Compounds 3 and 4, as shown in Fig. 2, a and b. The cyclization, of course, creates a third asymmetrical center, but it is postulated to yield exclusively a cis-ring juncture in both cases on thermodynamic grounds.

Unfortunately, relative rates of reaction of indoles compared to other nucleophiles with alkyl halides are not readily available, but information available suggests that indole should not be particularly nucleophilic. For example, the relatively low basicity of indole toward protonation at position 3 is reflected in a pK$_a$ of -2.70 (33), compared with a value of -1.8 for water. The pK$_a$ of skatole, possibly a better model for tryptophan, is even lower, being in the range of -3 to -4 (35). Considered theoretically as well, indole and skatole should be poor nucleophiles, since reaction of an electrophile at position 3 disrupts a 4n + 2 $\pi$-electron aromatic system with about 12 kcal of resonance energy (34). Enzymes, however, derive much of their rate accelerations from a binding equilibrium in which substrates are bound in the proper orientation for catalysis. It is conceivable that a similar equilibrium could account for the high relative reactivity of indoles with HNB-Br. Indoles are known to be donors in various complexes that exhibit the characteristics expected of charge-transfer interaction (35-39). If the indole of tryptophan were to form a complex with 2-hydroxy-5-nitrobenzyl bromide in such a way that the HNB-Br methylene group and the indole position 3 were juxtaposed, the increased reactivity resulting from such a complex might be quite substantial. Accordingly, we attempted to detect spectrophotometrically complex formation of the hydroxynitrobenzyl group with tryptophan ethyl ester.

Evidence for such an interaction could be detected by difference spectra of tryptophan ethyl ester and HNB-OH. A maximum difference spectrum at 365 mp was observed and the position of this maximum did not vary with the concentration of the amino acid. The interaction could be described by a linear double reciprocal binding curve which yielded an association constant of $K_a = 3.0$ for the tryptophan ethyl ester-HNB-...
OH—complex.” This curve is shown in Fig. 3. Tyrosine ethyl ester showed similar behavior ($K_e = 2.0$), and leucine ethyl ester joined in complex with HNB OH also ($K_e = 1.0$). In fact, a large number of “hydrophobic” amino acid esters, both nonaromatic and aromatic, gave difference spectra with HNB-OH whose wave length of maximum difference spectrum varied with the identity of the amino acid. It appeared that the wave length of the maximum of the difference spectrum, $\lambda_{max}$, seemed to correspond to the “hydrophobicity” of the amino acid side chain.

When the “amino acid hydrophobicity” as determined by Tanford’s $\Delta F^p$ of transfer of the various amino acids from ethanol to water (40) is plotted against the positions of the maxima in the difference spectra, the results of Fig. 4 are obtained. The fact that we used ethyl esters, although the $\Delta F^p$ values were obtained for the free acids, does not alter the correlation, since the presence of an ethyl ester group merely adds a constant increment to the $\Delta F^p$ values (41).

The excellent correlation of nonaromatic amino acids and aromatic amino acids suggests that hydrophobic interactions are involved. That the aromatic acids tyrosine and phenylalanine fit the same curve implies that no additional interactions (e.g. charge-transfer forces) contribute significantly (or else charge-transfer effects complicate Tanford’s $\Delta F^p$). No $\Delta F^p$ for tryptophan is given, but its $\lambda_{max}$ implies that it is somewhat more hydrophobic than tyrosine. This conclusion is also reached by Tanford (40), and it suggests that tryptophan as well falls on the correlation of Fig. 4. Evidently, charge-transfer complex formation of tryptophan (or other indoles) to HNB-OH—and presumably, HNB-Br—is not important, and hydrophobic interactions determined here are sufficient to explain a complex. This would not require any special orientation of reagent and tryptophan.

However, it has already been suggested that a quinone methide type species is involved in the attack on the indole moiety. From a valence bond standpoint, this species should be a much more effective charge transfer acceptor than HNB-Br or HNB-OH, since donor electrons can be delocalized into the carbonyl as well as the nitro group. Donation of charge at the exocyclic methylene group by an appropriate donor, $D$, would yield a species which has a resonance structure in which charge is donated into the carbonyl group and the ring has aromatic character:

$$\text{O}_2 \text{N} \quad \text{CH}_2 \text{D} \quad \text{etc.}$$

The frontier electron density of the indole nucleus has been calculated (27), and these calculations as well as the calculations of “superdelocalizability” (42) suggest that the indole position 3 could be involved in a “localized” charge-transfer complex with appropriate acceptors. Therefore, theoretical considerations indicate that the indole position 3 and the methylene group of the quinone methide could interact in a charge-transfer complex. But these are precisely the loci which react to form an HNB-modified indole. Hence, in order to achieve reaction (at low pH) between the quinone methide and an indole in such a complex, no dramatic alteration of nuclear positions need take place. Only a shift of electrons and the net transfer of a proton (from the indole nitrogen to the phenolic oxygen) must occur, and these two processes can be envisioned to occur quite rapidly. Since formation of the complex could also be fast, because relatively weak intermolecular forces are involved, the over-all modification of indoles would therefore be facile. These theoretical considerations indicate a possible explanation for the high reactivity of indoles toward HNB-Br despite a relatively low nucleophilicity toward ordinary alkyl halides.

The reactivity of thiols and thioalkyl groups of sulfur-containing amino acids and the ionized phenolic hydroxyl group of tyrosine with HNB-Br has been established (2). The extent to which these residues might form a complex with a quinone methide is more difficult to assess, but complexes are not needed to explain the rates of reaction of these residues relative to the rate of reaction of hydroxide ion and water with HNB-Br; the rates are fully rationalized by the relative nucleophilicities of the species involved. Phenoxide, for example, is 4 powers of 10 more nucleophilic than water, and only slightly less nucleophilic than hydroxide ion (49). This fact implies that phenoxide will be competitive with a huge molar excess of water and a 10-fold smaller concentration of hydroxide ion toward reaction with HNB-Br at high pH. This statement explains the substantial modification of tyrosine by HNB-Br that is observed at pH = 11.5. Un-ionized tyrosine, however, is less nucleophilic by a factor of 10$^4$ than its ionized counterpart, and should not be modified when it is in competition with tryptophan and large concentrations of water. Thus, tyrosine at low pH is not modified by HNB-Br, nor is any other nucleophilic residue containing hydroxyl groups.

Relative nucleophilicities of thiol groups are difficult to establish with precision because of the scarcity of data, but through the use of $n_f$ values (44, 45) and other nucleophilic data (43) one finds that phenoxide and thiophenol are equally nucleophilic. The thiol or thiomethyl groups of cysteine and methionine, respectively, should be even more nucleophilic than their thiophenyl counterparts since there is no phenyl ring to decrease...
FIG. 5. Possible modes of reaction of HNB-Br with tryptophan in proteins. The left and right sides of the figure represent the two respective diastereomeric modes of addition. The shaded areas represent the protein molecule to which the side chains are attached. Reactions A, A' through C, C' represent different modes of nucleophilic attack on the initially formed indolenine compound 8a and compound 8b. Path A, A' is the attack of the phenolic hydroxyl group; Path B, B' is the reaction of the side chain amido nitrogen; and Path C, C' is the attack of other nucleophilic groups, X, of the protein. Reactions D, D' represent the conversion of any of these derivatives to a modified tryptophan, compound 12, via an indolenine rearrangement catalyzed by protons donated by solvent or acidic groups on the protein, or both.

The sulfur electron density by delocalization. Since tyrosine in the phenoxide form readily competes with tryptophan, water, and hydroxide ion for modification by HNB-Br, it then follows that the equal or greater S_z2 reactivity of thiol or thioalkyl-containing amino acids should result in modification of these residues as well. This statement is borne out experimentally at low and high pH. It has already been pointed out that the methionine modification is rapid but no final modification is observed because the sulfonium adduct is unstable (2).

**Conclusions Affecting Protein Modification Experiments**—The specificity of HNB-Br for various amino acids can be fully explained on the basis of existing physical organic theory. Furthermore, the chemistry of the tryptophan modification by HNB-Br to form monosubstitution products has been elucidated. The results suggest some intriguing possibilities in a protein modification experiment (Fig. 5). The apparent affinity of HNB-Br for hydrophobic residues will be useful in guiding the reagent into hydrophobic regions where its specificity for tryptophan will then result in a modification of that amino acid. One expects that this modification in proteins will yield one or both of the two possible diastereomeric indolenine adducts, Compounds 8a and 8b, depending on the accessibility of the tryptophan to the reagent. This adduct may then suffer a number of fates. The side chain amide nitrogen may close on the imino carbon to yield a cyclic adduct analogous to that found in this work (Compounds 9a and 9b). Preliminary results from this laboratory and the group of Witkop (19) indicate that amidation of the a-amino group of tryptophan (as in a protein backbone) does not prevent cyclization in this manner. However, if the nitrogen is sterically blocked, or if cyclization by this mode induces undue strain in the protein, the work of Witkop's (19) and Schellenberg's (7) groups shows that the phenolic oxygen can cyclize (Compounds 10a and 10b). Of course, there is no reason that other nucleophiles X, elsewhere on the protein chain (e.g. serine hydroxyls, lysine amino groups, tyrosine hydroxyls, etc.), cannot attack the indolenine imino group if they are suitably juxtaposed (Compounds 11a and 11b). Finally, adjacent carboxylic acid or other acidic residues could protonate the indolenine or any of these other adducts in an intramolecular, general acid-catalyzed process which could initiate an indolenine rearrangement such as the one leading to 7; the result would be a different isomer of modified tryptophan, Compound 12. Thus, HNB-Br has a potential as an environmental probe not only through its use as a reporter group (46) in the study of binding, conformational changes, etc., but also by determination of its products in reactions with particular tryptophan residues in specific proteins.

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