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THE REACTION OF KOSHLAND'S PROTEIN REAGENT WITH TRYPTOPHAN

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A valuable reagent for labelling the tryptophan residue in proteins has been described by Koshland and co-workers.[2,3] The reagent, 2-hydroxy-5-nitrobenzyl bromide, HNBB,[4] is both highly selective for the tryptophan residue under mild reaction conditions and introduces the environmentally sensitive p-nitrophenol chromophore (λ_{max} (acidic) 320 mμ; λ_{max} (basic) 409 mμ) into the protein. It was shown that the reagent exhibited similar affinity for tryptophan in a mixture of amino acids[2,3] and a spectrophotometric method for determination of unreacted tryptophan in HNB-protein, based on the absorptivity of HNB-tryptophan, was developed.[5] However, other than UV spectral data, the properties and structure of HNB-tryptophan have not been reported. We now wish to record our studies leading to the structural determination of HNB-tryptophan, a mixture of two isomers. Since our initial report of these investigations,[6] a study of the reaction of HNBB with other tryptophan and indole derivatives[7] and the detection of several HNB-tryptophan products, resulting from varying experimental conditions,[8] have been reported.

For preparative formation of the product, we have employed a one molar excess of the reagent, HNBB, over tryptophan. A dry acetone solution (4M) of HNBB was added to a solution (2×10^{-2}M) of tryptophan in water. The resulting reaction mixture was extracted repeatedly by ether to remove the HNB-alcohol formed. The remaining solution was evaporated to a small volume and neutralized to pH 4, whereupon, the mixture of reaction products precipitated. The products were collected and washed by centrifugation and dried (70% yield, decomposition point: 210-212°). The products thus obtained had electrophoretic mobilities different from HNB-alcohol and tryptophan, and consisted of a major and a minor component. By preparative paper electrophoresis and UV spectrophotometry, the ratio of major and minor products was found to be 96 to 4. The major product exhibited the UV absorption maxima of the p-nitrophenolic chromophore (417 mμ at pH ~ 8.9, ε: 20,600*; 320 mμ at pH 4, ε: 9,750). The minor product had maxima at 409 and 320 mμ, respectively.

Potentiometric titration of the reaction product showed that its molecular weight was from 350 to 400, or a 1 : 1 combination of the reagent and tryptophan, with pK_a values of 6.9 and 8.8 (pK_a of HNB-alcohol, 7.1; of tryptophan, 9.4). The molecular formula was found to be $C_{18}H_{17}O_5N_3$ $2H_2O$ (anal. calcd.: C, 55.24; H, 5.41; N, 10.74; found: C, 55.49; H, 4.98; N, 10.54).

The integrated 100MHz NMR spectrum of HNB-tryptophan (trifluoroacetic acid + deuterium oxide) exhibited five aliphatic protons (3.4 to 4.8 ppm) and eight protons in the aromatic region (7.0 to 8.2 ppm), in agreement with the 1 : 1 ratio of HNB- to tryptophan moieties obtained from potentiometric titration. Addition of benzene-d_6 to a trifluoroacetic acid solution of HNB-tryptophan (63% C_6D_6-TFA) allowed assignment of both the HNB- and tryptophanyl portions of the 100MHz NMR spectrum, as shown in Figure 1.

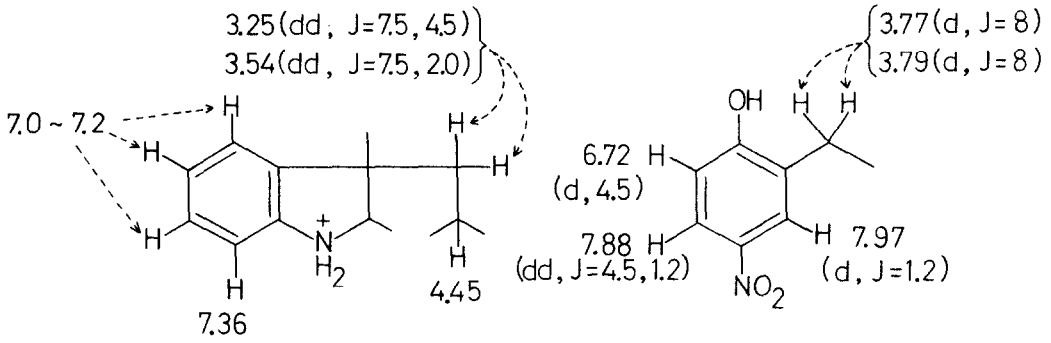
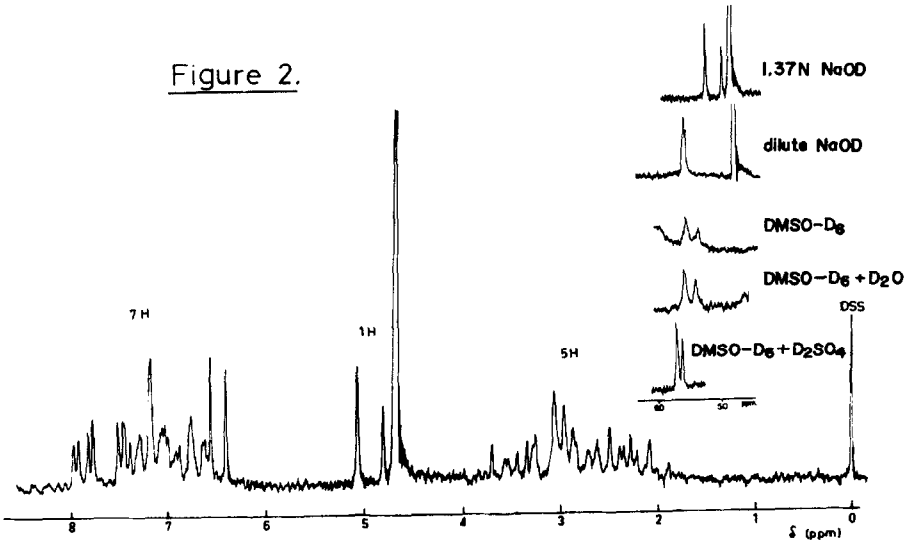
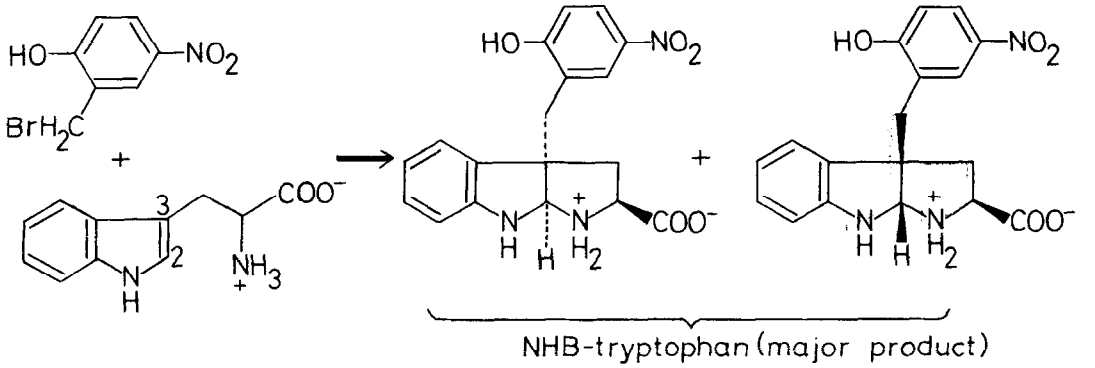


Figure 1 Chemical shifts in ppm, I in H

In less acidic solvents, HNB-tryptophan exhibits two signals, of unequal size around 5 ppm, totaling one proton, separated from the aliphatic (five protons) and aromatic (seven protons) signals. The chemical shifts of these signals were extremely pH dependent, as illustrated in Figure 2. The pH dependence of this isolated signal, the marked decrease in basic pK_a of HNB-tryptophan, compared to tryptophan, and the NMR spectral analysis (Figure 1.) are in agreement with the structure of HNB-tryptophan shown in Figure 3. This product results from initial attack of 2-hydroxy-5-nitrobenzyl cation at the indole 3-position. Assuming that the product is formed almost exclusively with the cis ring juncture, there are two possible isomers, in which the carboxyl and HNB- groups bear either a cis or trans relationship, as indicated in Figure 3. Thus, a singlet for each isomer is observed for the isolated proton at the indole 2-position.

Figure 2.





+ NHB-[tryptophan]$_2$ (minor product)

Figure 3.

Further evidence for two isomers was obtained from the 100MHz NMR spectrum (70% DMSO + $CDCl_3$). By decoupling measurement, it was possible to separately assign the chemical shifts and coupling constants of the aromatic HNB- protons for both isomers in this solvent system.[9]

We have also investigated the reaction of two moles of HNBB with one mole of N-acetyltryptophan, as a tryptophan peptide model. The two major components of the reaction product were separated by DEAE cellulose chromatography and exhibited characteristic p-nitrophenolic UV absorption ($\lambda_{max\ (basic)}$, product 1 : λ420 mμ, product 2 : (UV) 410 mμ). The decomposition of the product on removal of solvent and in some solutions precluded accurate NMR analysis. However, initial NMR data indicate a 1 : 1 combination of HNBB and tryptophan in the preponderant product. It is conceivable that the carbonium ion at C_2 of the N-acetyltryptophan residue has been neutralized by the nucleophilic attack of a water molecule or a carboxylate group. In actual labelling experiments of tryptophan residue(s) in proteins, possibilities for other nucleophilic attacks exist, and further studies are required before drawing a conclusion.

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* At pH's $>$ pK_a + 2, slight enhancement of p-nitrophenolate absorption was observed. This effect seems to be due to the disruption of electrostatic interactions between the phenolate ion and the ammonium ion site in a molecule on the deprotonation at higher pH.

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9) Minute amounts of the two isomers could be separated by DEAE-cellulose column chromatography. However, the very strong absorption due to the p-nitrophenol chromophore on one hand, and the weak rotatory power of the chiral centers on the other hand precluded measurements of RD curves.