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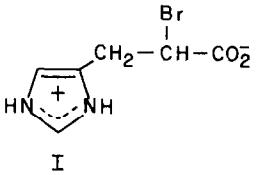
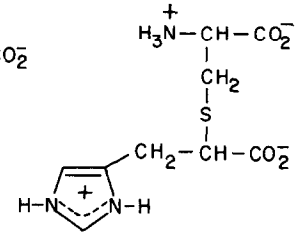
COMMUNICATIONS

A Sulfhydryl Reagent Containing an Imidazole Ring: D,L-*Alpha*-Bromo-*Beta*-(5-Imidazolyl)-Propionic Acid[1]

Haloacetic acids have long been employed in the alkylation of sulfhydryl groups and other amino acid side chains (1–3). More recently substituted haloacetic acids have been used to explore the stercochemical sensitivity of enzyme active sites to changes in reagent structure (4, 5). In the course of examining the behavior of a number of reagents designed to introduce imidazole groups into proteins we have prepared the substituted haloacetic acid, D,L-*alpha*-bromo-*beta*-(5-imidazolyl)-propionic acid (I). The purpose of this communication is to describe the preparation of this reagent and its behavior with cysteine and the sulfhydryl enzyme papain.



I



II

Compound I was prepared as follows: D,L-histidine (free base, 15.2 g) was dissolved in 220 ml of 48% HBr. The solution was chilled to −5°. Aqueous sodium nitrite (20 g in 40 ml water) was added dropwise over a period of 75 min during which time the temperature of the mixture was not permitted to rise above 5°. After stirring for an additional 60 min at 0°, the dark solution was concentrated at reduced pressure *without exceeding* 58° to produce a yellow oil containing a white precipitate. The concentrate was extracted with acetone and the salt residue was discarded. The acetone extract was concentrated to an oil under a stream of nitrogen and additional salt was removed by centrifugation. The oil (crude hydrobromide of I was dissolved in water and again concentrated to an oil *to insure removal of excess HBr*. Water (80 ml) was added to the concentrate and the pH was adjusted to 4.6 with cold 2 N NH_4OH. The solution was decolorized with Norit and evaporated to dryness at 50°. The residue was dissolved in 6 parts water (v/w) at 60° and then stored at 4°. The product, which crystallized in the form of hemispheres, was dried over P_2O_5 under vacuum (25 μ Hg). Yield: 10.5 g (46%) of I as the monohydrate, mp 108–111°. λ_{max}^{KCl} 6.20, 7.22 μ (carboxylate anion). The product migrated as a single Pauly-positive component (R_f, 0.53) in paper chromatography (2). *Anal.* Calcd for $C_6H_7N_2O_2Br \cdot H_2O$: C, 30.40; H, 3.83; N, 11.82; Br, 33.71. Found: C, 30.32; H, 3.92; N, 11.69; Br, 33.74. The structure of I was verified by ammonolysis to histidine (51%) and hydrogenolysis (Raney nickel) to *beta*-(5-imidazolyl)-propionic acid (50%).

Solutions of standard amino acids were incubated with 0.1 M I at pH 8.0, 25° for periods of up to 4 hr. Paper chromatography revealed an amino acid derivative only in the case of cysteine and it gave a ninhydrin- and Pauly-positive product (R_f 0.16)[2]. The progress of reaction of L-cysteine (0.1 M) with I (0.1 M) under nitrogen at pH 8.0 (25°) was determined with 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (6). A plot of $1/C_t$–$1/C_0$ against time was linear to 90 min and gave an apparent second order rate constant of 3.6×10^{-3} M^{-1} sec^{-1} (C_t and C_0 are the sulfhydryl titres of the reaction mixture at time t and time zero, respectively). A control solution of cysteine maintained under the same conditions retained its original sulfhydryl titre over a 90 min period. Amino acid analysis of the reaction mixture after 60 min showed it to consist of cysteine, cystine and a pair of new amino acids. In a standard 21-hr, single-column analysis on the Technicon analyzer the peaks of the new amino acid doublet emerge 16 and 25 ml after norleucine as compared to 18 and 29 ml for tyrosine and phenylalanine, respectively (Fig. 1). The diastereoisomeric new amino acids

[1] This research was supported by Grants GB-4731 and GB-17985 from the National Science Foundation.

[2] R_f values for paper- and thin-layer chromatography (cellulose) were obtained using butanol: acetic acid:water (12:3:5, v/v) as developer.

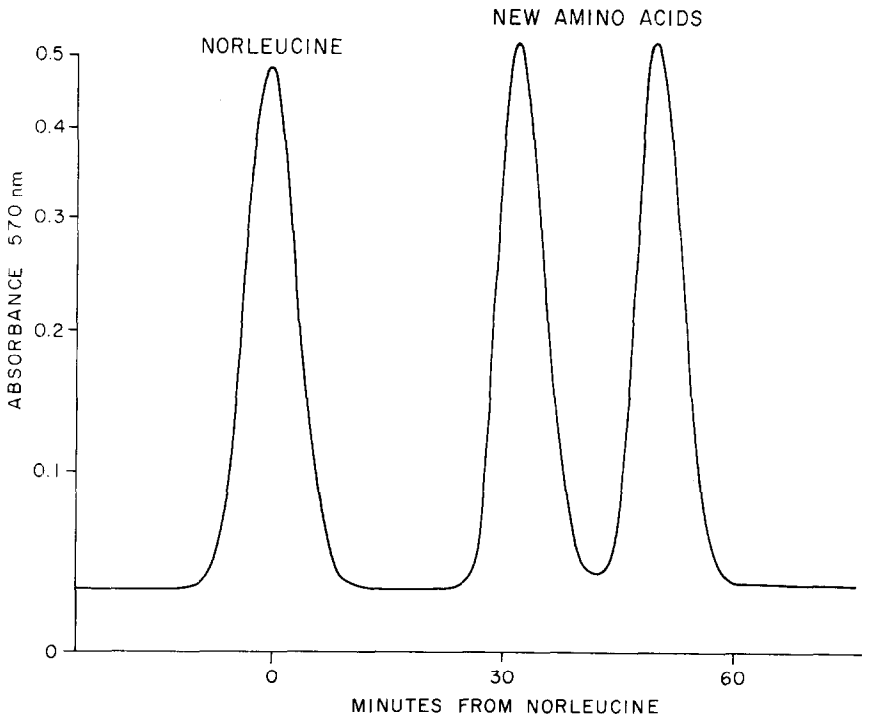


FIG. 1. Chromatographic behavior of diastereoisomeric new amino acids. Reaction of I with L-cysteine was carried out at pH 8.0 as described for the kinetic study. After 60 min an aliquot was quenched (pH 2.2) with HCl and diluted for analysis with standard pH 2.2 citrate buffer with added norleucine. The sample was chromatographed using a Technicon Amino Acid analyzer (21 hr separation). The new amino acids have an average ninhydrin constant of 0.79 times that of norleucine.

expected from a simple displacement reaction of L-cysteine with I are D- and L-*alpha*-(S-L-cysteinyl)-*beta*-(5-imidazolyl)-propionic acids (II). A sample of II which had been purified by gel filtration was desulfurized with Raney nickel to produce alanine (55% by amino acid analysis) and imidazolyl propionic acid (identified by thin-layer chromatography).

Papain (Sigma Chemical Company) was activated with mercaptoethanol (7). Enzymatic activity was determined with benzyloxycarbonyl-glycine *p*-nitrophenyl ester (8) using split-compartment cells (9) to initiate hydrolysis. Results were verified by titrimetric assay with *alpha*-N-benzoyl-L-arginine ethyl ester (10). Treatment of papain (7.0×10^{-4} M) with a 50-fold molar excess of I was performed at pH 8.0, 37° in the wide range buffer of Gerwin (11). Under these conditions papain was inactivated with a half-time of 6.5 min. After 30 min reaction, protein was separated from excess reagents by Sephadex G-25 chromatography using 0.1 M ammonium acetate as eluent. The modified inactive protein had lost 0.56 mole of sulfhydryl group per mole of protein[3] as titrated by *p*-mercuribenzoate (12). Performic acid oxidation (13) of the control and modified samples of papain showed 6.5 and 5.6 moles of cysteic acid per mole of protein, respectively. Treatment of papain with radiolabeled I (specific activity 1.54×10^{-2} mCi/mmole) prepared from [2-^{14}C] D,L-histidine (New England Nuclear) resulted in 0.59 to 0.61 mole of reagent incorporated per mole of papain. In the light of the model studies it can be concluded that an imidazolyl propionate group has been introduced at cysteine-25 of the active site.

While the results described here suggest that I

[3] At a time when this study was essentially complete, methods for isolating papain containing 1 mole of sulfhydryl group per mole of protein (15, 16) appeared. The titre of reactive SH in papain prepared without these techniques is typically 0.5–0.6 mole per mole of enzyme (15). The present results are in accord with the earlier studies.

behaves as a typical "sulfhydryl reagent", it should be recognized that special structural features present in proteins may enhance the reactivity of *alpha*-bromo-*beta*-(5-imidazolyl)-propionic acid toward other amino acid side chains. The classical suflhydryl reagent, iodoacetate, for example, in specific proteins, will alkylate histidine, methionine, lysine (2) or even glutamic acid (14). In addition to its potential for exploration of reactivity profiles of sulfhydryl groups in proteins, the present reagent may prove useful in affinity labeling studies of enzymes of histidine metabolism and other proteins which bind histidine or imidazoles as ligands.

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