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Synthesis of Some Histidine Analogs and Their Effect on the Growth of a Histidine-Requiring Mutant of *Leuconostoc mesenteroides*†

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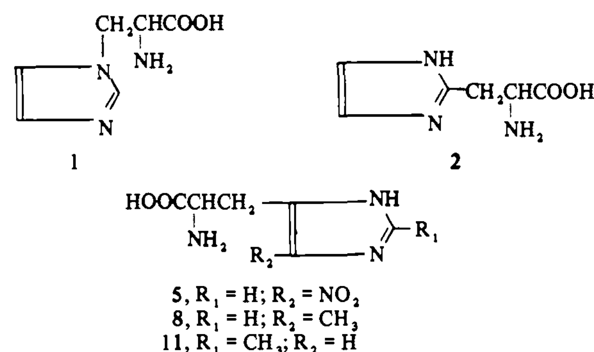
A number of histidine analogs and isomers have been synthesized and examined for biological activity by their ability to support the growth of a histidine-requiring bacterial mutant. None of the compounds investigated were capable of replacing histidine in the system selected even when present in large concentrations. 2-Isohistidine, although incapable of supporting growth of the organism, was shown to stimulate the growth produced by limiting amounts of the natural amino acid. No antimetabolic action of the analogs was detected.

Despite the importance of histidine, relatively little is known of the structural essentials for biological function. Studies in which the alanyl side chain of histidine has been modified, as in α-methylhistidine[1] and α-hydrazinoimidazolepropionic acid,[2] or in which the imidazole ring has been replaced, as in 2-pyridylalanine,[3] 2-thiazolealanine,[3] and 1,2,-4-triazolealanine,[4] have been reported. The biological action of histidine where substituents have been added to the imidazole ring or where the position of the alanyl side chain has been modified has received scant attention, although disturbances in the structure of alkaline phosphatase from *Escherichia coli* grown on media containing 2-methylhistidine[5] have been described. The action of histidine decarboxylase on a range of derivatives and analogs of histidine has also been reported.[6,7]

The present study is an attempt to determine the importance of various sites on the imidazole nucleus of histidine in influencing subsequent biological activity. The structural modifications selected were (1) transfer of the alanyl side chain from the 4(5) position to the 1 and 2 positions of the ring and (2) substitution of a methyl or nitro group on the imidazole ring. Biological activity was evaluated by an examination of the growth of a histidine-requiring mutant of *Leuconostoc mesenteroides* in a histidine assay medium[8] supplemented with either the analog alone or with the analog and limiting amounts of L-histidine.

Chemistry. The synthesis of α-amino-β-(1-imidazolyl)-propionic acid (1-isohistidine) by Michael condensation of methyl 2-acetamidoacrylate with imidazole and hydrolysis of the product has been briefly described[9] but the free amino acid was not isolated. An essentially identical method was initially employed in the present study and extended to obtain both the hydrochloride and the free base in crystalline form. A more convenient synthesis was subsequently developed by condensing diethyl α-acetamido-α-dimethylaminomethylmalonate methiodide[10] with sodium imidazole in liquid NH_3. Attempts to use EtOH as solvent were not successful though some 1-isohistidine was detected by tlc.



The remaining analogs in the present series were prepared by a standard route in which the corresponding hydroxymethyl derivative was converted to the chloromethyl compound, condensed with sodium diethyl acetamidomalonate, and hydrolyzed. 4-Nitro-5-chloromethylimidazole for the preparation of 4-nitrohistidine was prepared by direct nitration of 5-chloromethylimidazole hydrochloride and isolated by precipitation on dilution with ice water. The synthesis of 2-methylhistidine[6,11] by the general route required 2-methyl-4-hydroxymethylimidazole prepared in the present investigation by LAH reduction of ester obtained from 2-methylimidazole-4,5-dicarboxylic acid.[12] This approach minimized the risk of contamination of the final product with traces of histidine as is possible in alternative methods.[11]

The amino acids were used for biological study as racemates though some investigation of their resolution was undertaken. In exploratory experiments the standard enzymatic procedure utilizing the *N*-acetyl derivative[13] was extremely slow and in some cases the enzyme appeared to be subject to end-product inhibition. It did not appear to be practicable to effect resolution by this route.

Metabolic Activity. The metabolic activity of the com-

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pounds was assessed with a mutant of *L. mesenteroides* which required exogenous histidine for growth.[8] In the range 0-0.012 μmoles/ml of L-histidine, a linear growth response was obtained as measured by increasing turbidity of the solution. Replacement of the natural amino acid by one of the histidine analogs permitted some measure to be made of the compounds' ability to mimic histidine in supporting growth of the organism. On the other hand, a diminished growth in a medium supplemented by an analog plus a limiting amount of histidine would be an indication of antimetabolic activity. The use of analogs in their racemic form was justified by the observation that DL-histidine gives the same response as the L-histidine when used in double the concentration specified for the L-amino acid.[8] In the present investigation the effect of the analog was compared with that of L-histidine only after utilizing double quantities of the DL form.

Results

(a) Action of Analogs as Histidine Substitutes. The isomeric compounds **1** and **2** were unable to replace histidine in the test system even in amounts up to 2.5 μmoles/ml. The latter value represents a thousandfold excess over the minimum level of histidine required for measurable growth. Substitution of the imidazole ring in either the 4(5) or the 2 position with a methyl group (**8** and **11**) or in the 4(5) position with a nitro group (**5**) yielded a derivative incapable of supporting growth of the organism in the absence of histidine. Compounds **5**, **8**, and **11** were tested in concentrations up to 2.5 μmoles/ml of DL form.

(b) Action of Analogs in the Presence of Histidine. The analogs **1**, **5**, **8**, and **11** were all without effect on the growth of the organism in the presence of limiting amounts of histidine when tested at concentrations up to 2.5 μmoles/ml and appeared to be inert in the present assay. The addition of **2** to the medium resulted in a 10-15% increase in the growth produced by histidine alone.

Discussion

Numerous examples of the incorporation of modified amino acids into proteins have been recorded.[14] Even when incorporation is not achieved, an analog can frequently mimic other functions of the natural amino acid such as feed-back inhibition.[15] Histidine, as a consequence of the reactive imidazole ring, has been implicated as an important amino acid at the active site of several enzymes.[16] It is generally accepted that part at least of the catalytic properties of histidine arise as a consequence of the basic nitrogen of the imidazole ring. It has been suggested that the amino and carboxyl groups, together with a site on the imidazole ring, are utilized for the binding of at least one enzyme involved in histidine metabolism.[17] It is therefore of significance to consider the spacial relationship between the alanyl side chain normally attached at the 4(5) position of the imidazole ring and the various other atoms of the ring as found in the isomeric compounds **1** and **2**. The lack of either metabolic or antimetabolic activity in either of these analogs indicates the critical relationship of the position of the side chain relative to the ring. The displacement of the side chain from C-4 to C-2 eliminates completely any direct metabolic activity, although the stimulation of growth by **2** in the presence of histidine was noted. As **2** alone showed no growth-promoting action, the increased growth of between 10 and 15% may be due to inhibition of an enzyme necessary for the catabolism of histidine resulting in higher effective histidine levels. Alternatively, it may stimulate the production or activity of some system required for the incorporation of histidine into protein. At present, no information is available on the mechanism of the effect.

By analogy with the methylimidazoles, substitution of a methyl group at positions 4(5) or 2 of histidine may be expected to have a relatively small effect on the pK of the basic ring nitrogen. In the present investigation, complete loss of biological activity was noted for both 2-methylhistidine and 4-methylhistidine. If the small changes in ring basicity may be discounted as an explanation of the dramatic loss of biological activity in the present compounds, the influence of the methyl groups may be due either to a blocking of essential sites not previously implicated in binding or to a steric effect in which adequate contact with the relevant site in an enzyme system is prevented. In support of the latter possibility, it should be noted that the growth of a histidine auxotroph of *E. coli* in a medium in which histidine was replaced by 2-methylhistidine resulted in the accumulation of inactive alkaline phosphatase subunits which were not able to form the active enzyme.[5] Further, in the nonbiological catalytic hydrolysis of *p*-nitrophenyl acetate by imidazole derivatives, reaction takes place at a similar rate in the case of imidazole and 4-methylimidazole but at a significantly slower rate with 2-methylimidazole despite the closeness of the pK values.[18] A steric factor was suggested as explanation.

The substitution of a nitro group at position 4(5) resulted in a histidine derivative with no biological action. The nitro group greatly decreases the basicity of the ring but, in the catalytic hydrolysis of *p*-nitrophenyl acetate already discussed, 4-nitroimidazole is a potent catalyst.[18] From the present findings it would appear that the biological activity of histidine is related in a more complex manner to the nature of the ring than basicity alone.

Experimental Section

Melting points were determined with a Büchi melting point apparatus and are corrected. The ir spectra were taken as Nujol mulls with a Perkin-Elmer Model 700 infrared spectrophotometer but the findings are not reported as they were only confirmatory and as expected. Carboxyl C was determined by the Van Slyke manometric procedure. Elemental analyses were by Dr. Fuhr, Department of Chemistry, University of Cape Town, and in part by Dr. Pascher, Microanalytical Laboratory, Bonn, Germany. Chromatography was on either silica gel G plates (Merck) or cellulose plates (Merck) using the following solvent systems: (A) n-BuOH-AcOH-H_2O (3:1:1); (B) *tert*-BuOH-EtMeCO-Me_2CO-MeOH-H_2O-NH_4OH (40:20:20:1:14:5).[19] Visualization was with ninhydrin or Pauly's reagent.

α-Amino-β-(1-imidazolyl)propionic Acid (1-Isohistidine) (1). A solution of imidazole (4.08 g, 0.06 mole) in anhydrous NH_3 (200 ml) maintained at approx −35° was treated with Na (1.38 g, 0.06 g-atom). The addition of a slight excess of Na gave an intense blue color which was discharged by a trace of imidazole. Diethyl acetamido-α-dimethylaminomethylmalonate methiodide[10] (12.06 g, 0.03 mole) was added, and the solution stirred 2-3 hr. The NH_3 evaporated overnight and the oily residue was dissolved in water, dried *in vacuo* on a rotary evaporator, redissolved in water, and adjusted to pH 7 with concd HCl. It was again dried *in vacuo* and extracted with dry MeOH, filtered, and evaporated *(in vacuo)* to leave a yellow oil which was hydrolyzed overnight with concd HCl. Excess acid was removed by repeated evaporations of an aqueous solution in the rotary evaporator. The free base was prepared by one of the following procedures. (i) The residue was dissolved in a minimum of water and adjusted to pH 7.2 with LiOH (6 *N*), and the base precipitated by addition of EtOH, or (ii) an aqueous solution of the hydrochloride was passed through a column of Amberlite IR-4B (OH), and the effluent concentrated and precipitated with EtOH. The base was recrystallized from aqueous EtOH: yield 2.7 g (58%); mp 211.5° dec. *Anal.* ($C_6H_9N_3O_2$) H, N; C: calcd, 46.4; found, 45.6.

Tlc on cellulose plates in solvent B gave a single spot, R_f 0.16 (ninhydrin). The ir spectrum of 1 was identical with that of a sample

prepared from imidazole and methyl 2-phenacetamidoacrylate[10] and converted to the free base as described above. A sample of the hydrochloride was submitted for analysis. *Anal.* ($C_6H_{11}Cl_2N_3O_2$) C, H, N.

α-Amino-β-(2-imidazolyl)propionic Acid (2-Isohistidine) (2). This compound was prepared as described:[20] mp 254–256° dec; lit. 254–255° dec. Tlc in solvent B gave a single spot, R_f 0.27.

5-Chloromethyl-4-nitroimidazole (3). 5-Chloromethylimidazole hydrochloride[21] (10 g) was added in portions to 40 ml of concd H_2SO_4-HNO_3 (3:1) previously cooled to −10°. After the initial reaction had moderated, the mixture was heated on the steam bath for 2 hr, cooled, and poured onto ice. Filtration and recrystallization from abs EtOH gave 6.0 g (57%); mp 180–181° dec. *Anal.* ($C_4H_4ClN_3O_2$) C, H.

5-(2-Carbethoxy-2-acetamido)-4-nitroimidazole Propionate (4). A solution of 3 (3.24 g, 0.02 mole) in abs EtOH (100 ml) was added to diethyl acetamidomalonate (4.77 g, 0.022 mole) in abs EtOH in which Na (1.01 g, 0.022 g-atom) had previously been dissolved, and the mixture was stirred 2 hr at room temperature. The dark solution was evaporated to dryness *in vacuo*, and the residue dissolved in 6 *N* HCl and extracted with $CHCl_3$ (3 × 5 ml). Solid Na_2CO_3 was added until alkaline when, on cooling and scratching, the product crystallized: recrystallized from water, yield 2.2 g (39%); mp 181–183° dec. *Anal.* ($C_{13}H_{18}N_4O_4$) C, H.

4-Nitrohistidine Hydrochloride Monohydrate (5). A solution of 4 (5 g) in concd HCl was refluxed for 16 hr. Excess acid was removed by repeated evaporation of an aqueous solution and the residue recrystallized from aqueous EtOH–Et_2O: yield 1.9 g (51%); mp could not be obtained. *Anal.* ($C_6H_{11}ClN_4O_5$) C, H, N.

The compound was homogeneous on tlc (silica gel) in solvent A (R_f 0.9) and solvent B (R_f 0.30).

5-Hydroxymethyl-4-methylimidazole (6). 4-Methylimidazole-5-carboxylic acid ethyl ester[22] (7.2 g, 0.05 mole) was reduced with LAH (THF) and the product isolated by standard procedures.[23] The crude hydroxymethyl derivative was obtained as a yellow oil which solidified on standing: yield 5.2 g (93%). It was used without further purification. A picrate was prepared and recrystallized from 50% EtOH: mp 180–182°; lit.[24] 181–182°.

5-Chloromethyl-4-methylimidazole Hydrochloride (7). Thionyl chloride (25 ml) was added dropwise to 8.0 g of 6. A vigorous reaction commenced but the solid failed to dissolve. Any lumps were crushed with a glass rod, and the mixture was refluxed for 15 min. Dry Et_2O was added to the mixture, and the residue was filtered, washed with Et_2O, and dried *in vacuo*: yield 9.7 g (81%). It was used without further purification.

4-Methylhistidine (8) was prepared from 7 as described.[21] The intermediate 5-(2-carbethoxy-2-acetamido)-4-methylimidazole propionate was extracted into EtOAc, the solvent removed, and the residue hydrolyzed with 6 *N* HCl overnight. The free base was obtained by either of the methods outlined above, recrystallized from MeOH–Et_2O, yield 49%. *Anal.* ($C_7H_{11}N_3O_2 \cdot 1.5H_2O$) C, H, N, carboxyl C. Tlc in solvent B gave a single spot of R_f 0.24.

2-Methylimidazole-4(5)-carboxylic Acid Ethyl Ester (9). A mixture of 2,7-dimethyl-5*H*,10*H*-diimidazo[1,5-*a*:1′,5′-*d*]pyrazine[25] and abs EtOH previously saturated with dry HCl was refluxed for 6 hr. The EtOH was removed, and the residue dissolved in water and made alkaline with K_2CO_3. A dark precipitate formed and was filtered, dried, and recrystallized (charcoal) from EtOAc: yield 51%; mp 152–153°; lit.[26] 156°.

4-Hydroxymethyl-2-methylimidazole (10) was prepared by LAH (THF) reduction of 9. A picrate was prepared for analysis: mp 171–172; lit.[6,11] 171–172°; 187–189°. *Anal.* ($C_{11}H_{11}N_5O_8$) C, H, N.

2-Methylhistidine dihydrochloride (11) was prepared from 10 by described methods:[11,21] mp 254° dec; lit. 247°,[11] 257–259°.[6] *Anal.* ($C_7H_{13}Cl_2N_3O_2$) C, H. Tlc in solvent B gave a single spot, R_f 0.14.

Microbiological Assay.[8] A strain of *Leuconostoc mesenteroides* P60‡ was subcultured on lactobacilli broth (Difco) for 24 hr at 37° and stored as stab cultures on lactobacilli agar at 5° after growing 48 hr at 37°. Difco histidine assay medium (5 ml) supplemented with either the analog under test, histidine, or both materials (0.0025–2.5 μmoles/ml) in a total of 10 ml was inoculated with 1 drop of a saline suspension of washed cells. After incubation at 37° for 20 hr, the turbidity was measured at 660 nm in a spectrophotometer.

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