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Synthesis and Properties of a Bis-Imidazole*

Charles N. C. Drey and Joseph S. Fruton

ABSTRACT: 4,4′(5,5′)Bis-imidazolylmethane (BIM) was synthesized from histidine methyl ester by reduction with NaHg in the presence of KSCN, followed by desulfurization of the resulting 2-mercapto compound with $FeCl_3$ or HNO_3. BIM has apparent ionization constants of 5.3 and 7.0. It exhibits strong intermolecular hydrogen bonding in dimethyl sulfoxide and no evidence was found for intramolecular hydrogen bonding.

Examination of the catalytic effect of BIM on the hydrolysis of *p*-nitrophenylacetate showed that, on an equimolar basis, BIM was slightly less effective than imidazole itself. BIM had no catalytic effect on the hydrolysis of uridine-2′,3′-phosphate.

In the catalytic action of a variety of enzymes, the participation of one or more imidazolyl groups, strategically located in the catalytic protein, has been repeatedly postulated. A valuable summary of the earlier literature on this subject has been prepared by Barnard and Stein (1958). The possible catalytic role of the imidazolyl group was given considerable support by the studies of Bender and Turnquest (1957) and of Bruice and Schmir (1957) on the catalysis of the hydrolysis of *p*-nitrophenylacetate by imidazole and its derivatives (for a review, see Bender, 1960).

Recent studies on the structural basis of the catalytic action of ribonuclease (RNAase) have suggested the involvement of two histidine residues (Nos. 12 and 119) in the "active site" of the enzyme. This view has emerged from several lines of experimental approach. The studies of Herries *et al.* (1962) on the variation of the K_m of RNAase with *p*H led these workers to suggest that the observed apparent *pK* values of 5.2 ± 0.2 and 6.8 ± 0.2 for the dissociation of ionizing groups at the "active site" might be assigned to imidazolyl groups. More direct evidence has come from the studies of Crestfield *et al.* (1963) on the reaction of RNAase with iodoacetate. These investigators showed that near *p*H 5.5, treatment of RNAase with iodoacetate produces two monosubstituted derivatives, 1-carboxymethylhistidine(119)-RNAase and 3-carboxymethylhistidine(12)-RNAase. Although neither of these products is enzymically active, when they are allowed to form dimers activity is regained, and Crestfield *et al.* (1963) have proposed that each of the inactive RNAase derivatives has contributed an unsubstituted histidine residue (12 or 119) to the regeneration of the "active site." This hypothesis is based in part on the earlier work of Richards (1958) on the cleavage of RNAase by subtilisin to yield the amino-terminal 20-amino acid peptide (S-peptide) and the residual 104-amino acid fragment (S-protein), each of which was inactive separately, but which readily recombined near *p*H 7 to regenerate the original enzymic activity. For a recent discussion of the mechanism of RNAase action see Witzel (1963), who has suggested that the imidazolyl groups of histidines 12 and 119 are hydrogen bonded in the active enzyme.

Furthermore, recent studies on the amino acid sequence of trypsinogen and chymotrypsinogen have shown that in both enzymes derived from these zymogens there are two histidine residues in the sequences His-Phe-Cys and Ala-His-Cys, the two half-cystine residues being joined by a disulfide bridge, and it has been suggested that these two histidine residues may participate in the "active site" of trypsin or chymotrypsin (Walsh *et al.*, 1964). The participation of histi-

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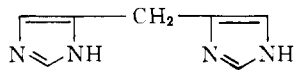
dine residues in the activity of these two enzymes has been strongly indicated by the work of Schoellman and Shaw (1963) and of Ong *et al.* (1964). The latter investigators have shown that, in chymotrypsin, the histidine residue adjacent to the half-cystine residue is specifically altered by the inhibitor L-1-tosylamido-2-phenylethyl chloromethyl ketone.

Because of the increased interest in the possible cooperative catalytic effects of two imidazolyl groups with defined mutual spatial orientation, it seemed desirable to examine the properties of model bis-imidazole compounds of known structure. In this communication are described the synthesis and some properties of 4,4′(5,5′)bis-imidazolylmethane (compound I), to be abbreviated in what follows as BIM.[1]



(I)

This compound does not appear to have been described in the literature, although the isomeric 1,1′-bis-imidazolylmethane was prepared by Schütze and Schubert (1959) and tested by them for its effect on the catalase and peroxidase activity of hemin.

For the synthesis of BIM, the route selected was based on the general method of the synthesis of imidazoles via the 2-mercapto derivative, by reaction of an α-amino aldehyde with thiocyanate, followed by removal of the sulfur by mild oxidation with $FeCl_3$ or dilute HNO_3. A synthesis of the 2-mercapto compound from L-histidine methyl ester hydrochloride has been described by Bullerwell and Lawson (1951), and this procedure was adopted in modified form (for details, see Experimental). In particular, the conditions used by Adams (1955) for the preparation of L-histidinal were employed, with the difference that potassium thiocyanate was present during the reduction of histidine methyl ester by sodium amalgam. The desulfurization of the resulting 2-mercapto compound was readily effected by oxidation either with $FeCl_3$ or dilute HNO_3, and the dihydrochloride of BIM was prepared via the dipicrate. The free base was most readily prepared by passage through a Dowex 1 column in the OH^- form, but could also be obtained by sublimation after neutralization of the dihydrochloride.

Titration of BIM dihydrochloride with KOH gave two pK' values of 5.32 ± 0.04 and 6.98 ± 0.13 (30°). Under these conditions titration of imidazole hydrochloride gave a pK' of 6.82 ± 0.03. Clearly, the magnitude of pK_1' of BIM reflects the effect of one protonated imidazolyl group on the dissociation of the other. Similar effects of positively charged groups located in proximity to an imidazolium group have been reported previously (for a recent summary, see Schneider, 1963). For example, whereas the imidazolyl group of N^{α}-acetylhistidine has a pK' of 7.1, for histidine the corresponding value is 6.1, and for histidinamide (or methyl ester) it is 5.4. Similarly, histidylhistidine has been reported to have pK' values of 5.4 and 6.8, the first of which may be assigned to the amino-terminal residue. In the case of BIM, the value of pK_2' is similar to that of imidazole itself, but somewhat lower than that (7.25) reported for 4(5)-methyl imidazole. Apparently, separation of two imidazolyl groups by a $-CH_2SCH_2-$ bridge (linking the 4(5) positions) reduces markedly their interaction, since Schneider (1963) reported pK' values of 6.6 and 6.9 for bis(imidazolyl-4-methyl) sulfide. This compound was obtained by treatment of *S*-(imidazolyl-4-methyl)isothiouronium dihydrochloride with alkali.

The question of the presence of intramolecular hydrogen bonding in BIM was examined by means of infrared spectroscopy (Beckman IR-5 spectrophotometer, 0.1-mm NaCl cells). Otting (1956) reported that, in $CHCl_3$ solution, imidazole exhibits strong absorption at 3475 cm^{-1} (assigned to unbonded NH stretching) and at 2600–3200 cm^{-1}, and Zimmermann (1959) showed that the latter absorption was attributable to hydrogen-bonded NH stretching in polymers of imidazole. A detailed investigation by Anderson *et al.* (1961) showed that, in CCl_4 solution, the intensity of the hydrogen-bonded NH absorption (in the region 2000–3400 cm^{-1}) increased with increasing imidazole concentration, with a concomitant decrease in the unbonded NH absorption at 3485 cm^{-1}. This concentration-dependent hydrogen bonding is clearly associated with the well-known tendency of imidazole to form linear polymers in nonpolar solvents. Lukton (1961) has reported that N^{α}-acetylhistamine and methyl dihydrourocanate in $CHCl_3$ exhibit strong bands at 2957 cm^{-1} and 2986 cm^{-1}, respectively, indicating a shift from the value of 3048 cm^{-1} associated with the intermolecular hydrogen bonding of imidazole, and suggesting intramolecular bonding. Because of the sparing solubility of BIM in either $CHCl_3$ or CCl_4, dimethyl sulfoxide was used as the solvent. Dimethyl sulfoxide has a high dielectric constant (49.0), whose effect on hydrogen bonding may be appreciable (Allerhand and Schleyer, 1963). Therefore a study was made of the infrared spectra of imidazole in this solvent as a function of concentration. In concentrated solution (0.65 M), bands were seen at 3440 (m), 3178 (s), 3030 (m-s), 2930 (m), 2840 (m), 2690 (w), and 2579 (w) cm^{-1}. Upon progressive dilution of the solution to 0.076 M, a decrease in the band at 3178 cm^{-1} and a parallel increase in the band at 3440 cm^{-1} were observed. It may be concluded, therefore, that intermolecular hydrogen bonding of imidazole is demonstrable in dimethyl sulfoxide solution. A concentrated solution of BIM (free base) in dimethyl sulfoxide (0.27 M) exhibited bands at 3440 (m-s), 3165 (s), 2940 (m-sh), 2858 (m), 2700 (w-sh), and 2581 (w) cm^{-1}. Upon dilution to 0.016 M, the band at 3440 cm^{-1} increased in intensity with a concomitant decrease in the intensity of the band at 3165 cm^{-1} and of the other bands associated with hydrogen-bonded NH absorption. A plot of the ratio of absorbance at 3440 and 3178 cm^{-1} for imidazole and at 3440 and

[1] Abbreviations used in this work: BIM, 4,4′(5,5′)bis-imidazolylmethane; IM, imidazole.

TABLE I: Hydrolysis of *p*-Nitrophenylacetate in Presence of 4,4′(5,5′)Bis-Imidazolylmethane (BIM) or of Imidazole (IM).[a]

*p*H and Buffer[b]	Catalyst (M × 10^4)	$k_1 \times 10^4$ BIM (min^{-1})	$k_1 \times 10^4$ IM (min^{-1})	$k_w \times 10^4$ (min^{-1})	k_2' BIM ($mole^{-1} \times min^{-1}$)	k_2' IM ($mole^{-1} \times min^{-1}$)
7.1 (P)	2	42.9	54	12.2	15.3[c]	20.9
7.1 (T)	2	31.8	44.3	8.1	11.9[c]	18.1
8.1 (P)	1	84	84	43.4	40.6	40.8
8.1 (P)	2	119	134	49.2	34.8	42.7
8.05 (T)	2	114	122	45.9	34.1	43.1
8.1 (P)	5	220	250	43.2	35.3	41.4
8.1 (P)	7	269	303	40.0	32.6	37.6
8.1 (P)	10	382		48.9	33.1	
8.9 (T)	2	297	315	239	29.0	38.0

[a] For experimental conditions and procedure, see Experimental section. [b] P = phosphate buffer; T = Tris buffer. [c] The difference in k_2' for the two buffers appears to lie outside experimental error. Although the possibility of a cooperative effect of BIM and buffer may be considered, further work is needed to clarify this question.

3165 cm^{-1} for BIM as a function of concentration (in equivalents of imidazolyl groups) gave curves that were nearly superimposable. The available data, therefore, give no evidence for intramolecular hydrogen bonding of BIM in dimethyl sulfoxide solution under conditions where intermolecular bonding is possible, and suggest that the favored conformation of the molecule is one in which the two imidazolyl groups are farthest apart.

Data on the effect of BIM on the rate of hydrolysis of *p*-nitrophenyl acetate in the *p*H range 7.1–8.9 are given in Table I. It will be noted that, on an equimolar basis, the bis-imidazole compound has a catalytic effect slightly less than that of imidazole itself, and that there is no rate enhancement that might be attributed to a cooperative effect of the two imidazolyl groups. This finding is consistent with the observations of Bruice and Schmir (1958) and of Schneider (1963) on the effect of pK' on the catalytic activity of 4(5)-substituted imidazoles.

Overberger *et al.* (1963) have reported that the product of the polymerization of 4(5)-vinylimidazole (Overberger and Vorchheimer, 1963) is somewhat more effective in the hydrolysis of *p*-nitrophenylacetate than is imidazole itself at *p*H values above 8. On the other hand, the most effective synthetic catalyst for the hydrolysis of *p*-nitrophenylacetate thus far reported is the pentapeptide Thr-Ala-Ser-His-Asp (Cruickshank and Sheehan, 1964). The available data on the action of synthetic imidazole derivatives do not provide clear-cut evidence, therefore, for the cooperative action of two imidazolyl groups in the hydrolysis of *p*-nitrophenylacetate.

As will be seen in the Experimental section, 0.01 M BIM exerted no measurable effect on the hydrolysis of uridine-2′,3′-phosphate under conditions where 5×10^{-6} M ribonuclease caused rapid hydrolysis.

Experimental

Preparation of 4,4′(5,5′)Bis-Imidazolylmethane. To a solution of 2.42 g (0.01 mole) of L-histidine methyl ester hydrochloride in 25 ml ice-cold water was added 2.13 g (0.22 mole) of KSCN. The solution was stirred and chilled to 0–5° during the addition of 60 g of 2.5% sodium amalgam in small portions, and the *p*H was kept at 1.5–3.5 by the simultaneous addition of 5 N hydrochloric acid. After the addition of the sodium amalgam the reaction mixture was stirred for 2 hours, the supernatant fluid was decanted, and the mercury was washed with water. The combined fluids were filtered and heated under reflux for 1 hour, the *p*H was adjusted to 6 with NaOH, and the solution was concentrated to dryness under reduced pressure. The residue was extracted with five 25-ml portions of hot ethanol, and evaporation of the ethanol yielded 2.4 g of the thiocyanate salt of the 2-mercapto derivative of BIM. After recrystallization from water and from ethanol–ethyl acetate, it melted at 208–210°; λ_{max} 257 mμ in water (ϵ = 14,600). Bullerwell and Lawson (1951) reported a melting point of 207° and Heath *et al.* (1951) reported λ_{max} 258 mμ (ϵ = 14,600) for 2-mercapto-4(5)-methylimidazole.

Anal. Calcd for $C_7H_8N_4S \cdot CHNS$ (239.3): C, 40.2; H 3.8; N, 29.3; S, 26.8. Found: C, 40.4; H, 4.0; N, 29.0; S, 26.1.

The 2-mercapto compound (0.6 g) was dissolved in 20 ml of hot water, and a solution of 5 g of $FeCl_3$ in 10 ml H_2O was added. The mixture was heated in the steam bath for 45 minutes and neutralized with 10% Na_2CO_3, and the precipitated ferric hydroxide was removed by filtration. The precipitate was extracted thoroughly with hot water, and the combined filtrate and washings were brought to boiling temperature. Picric acid (0.25 g in 15 ml water) was added, and the

solution was boiled for 10 minutes, treated with 50 mg of Norit, and filtered. Upon cooling, the dipicrate of the desired product was obtained. After one recrystallization from methanol, the product melted at 254–255°.

Anal. Calcd for $C_7H_8N_4 \cdot C_{12}H_6N_6O_{14}$ (606.4): C, 37.7; H, 2.3; N, 23.1. Found: C, 38.0; H, 2.4; N, 23.1.

Desulfurization of the 2-mercapto compound was also effected by treatment with dilute HNO_3. A solution of 0.65 g of this compound in a mixture of 20 ml methanol and 1 ml 5 N HCl was evaporated to dryness, and the residue was dissolved in 13 ml 1 N HNO_3. The solution was heated carefully so as to concentrate it to 1–2 ml within an hour, and was neutralized with Na_2CO_3. Picric acid (1 g in 30 ml hot water) was added, and the dipicrate was isolated as before. After recrystallization from acetone–ethyl acetate, the product (0.91 g) melted at 254°. The yield from the HNO_3 oxidation tended to be somewhat higher (*ca.* 60% of the theory) than in the $FeCl_3$ oxidation procedure, and was therefore used routinely. Another possible procedure for desulfurization of the 2-mercapto compound is the use of Raney nickel, but this was not tested.

The dipicrate (0.75 g) was decomposed by warming with 3.5 ml of *ca.* 3 N HCl. The picric acid was removed by repeated extraction with benzene to yield a colorless aqueous solution which, upon concentration *in vacuo*, yielded the desired dihydrochloride. After recrystallization from methanol–ethyl acetate, it melted at 268–269°; yield, 70% of theory.

Anal. Calcd for $C_7H_8N_4 \cdot 2$ HCl (221.1): C, 38.0; H, 4.6; N, 25.4. Found: C, 37.9; H, 4.5; N, 25.3.

Paper chromatography (Whatman No. 1 paper) in 1-butanol–acetic acid–pyridine–water (15:3:10:12, v/v) gave R_F 0.46 for BIM dihydrochloride under conditions where imidazole had R_F 0.55.

The conversion of the dihydrochloride (0.32 g) to the free base was effected by passage through a column of Dowex 1 (OH^- form) and elution with distilled water.[2] The Pauly-positive fractions were collected and concentrated to dryness under reduced pressure to yield 0.14 g of the product, melting at 229–232°. Infrared bands (Nujol mull) at 3080, 2730, 2630, 2355, 1818, 1653, 1564, 1518, 1492, 1365, 1265, 1241, 1220, 1170, 1086, 994, 939, 889, 826, 767, 752, 718, 674, and 652 cm^{-1}.

Anal. Calcd for $C_7H_8N_4$ (148.2): C, 56.7; H, 5.5; N, 37.8. Found: C, 57.1; H, 5.6; N, 37.4.

Determination of the Ionization Constants of BIM. Titration of BIM dihydrochloride was conducted with 0.1 M KOH at 30°, using a Radiometer TTT1 assembly and following the procedure of Albert and Serjeant (1962). The data were treated by the method of Noyes (see Albert and Serjeant, 1962) for the determination of the *pK'* values of two ionizing groups separated by less than 2.7 units of *pK*.

Catalysis of Hydrolysis of p-Nitrophenylacetate. In all cases, the concentration of *p*-nitrophenylacetate was 2×10^{-4} M, the buffer (phosphate or Tris) concentration was 0.67 M, the ionic strength was brought to 0.2 with KCl, and 10% ethanol (v/v) was present, in a total volume of 10 ml. The reaction was followed at 30° ± 0.05° by measurement of the absorbance at 400 mμ in a Beckman DU spectrophotometer, with 1-cm cuvets and at a slit width of 0.13 mm. The rate of the hydrolysis was calculated from the equation $k_2' = (k_1 - k_w)I_o$, where k_2' is the apparent second-order constant, k_1 is the observed first-order constant, k_w is the observed constant in the absence of added catalyst (in the presence of the appropriate buffer and KCl to bring the ionic strength to 0.2), and I_o is the molar concentration of catalyst. BIM was used in the form of its dihydrochloride, and imidazole was a recrystallized sample (mp 89.5°) of the free base. *p*-Nitrophenylacetate was a recrystallized sample melting at 79–80°.

Experiments with Uridine-2',3'-phosphate. The spectrophotometric method of Richards (1955) was used and the change in absorbance at 275 mμ was determined by means of a Beckman DU spectrophotometer (slit width 1.12 mm). Uridine-2',3'-phosphate barium salt (Schwartz BioResearch) was present at a concentration of 2.5×10^{-4} M, the *p*H was controlled by means of sodium malonate buffer (*p*H 6.25) and Tris buffers (*p*H 7.0 and 8.65), the ionic strength was adjusted to 0.2 with KCl, and the temperature was 30°. Under these conditions the following first-order rate constants were obtained in the presence of $5.0–5.5 \times 10^{-6}$ M pancreatic ribonuclease (Seravac Labs. lot 144A): *p*H 6.25, 0.13 min^{-1}; *p*H 7.0, 0.066 min^{-1}; *p*H 8.65, 0.0033 min^{-1}. In the presence of 0.01 M BIM or 0.01 M imidazole, no hydrolysis of uridine-2',3'-phosphate was observed over a period of 24 hours.

Added in Proof

In connection with the measurement of metal ion–BIM stability constants (to be reported in a subsequent paper), the ionization constants of BIM were determined at $\gamma/2 = 0.16$ and 30°, and found to be $pK_1' = 5.61 \pm 0.02$ and $pK_2' = 7.39 \pm 0.03$. Under these conditions, imidazole gave a pK' of 6.95 ± 0.02.

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[2] We are indebted to Mr. A. Aboderin for the preparation of the free base of BIM by this method.

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Countercurrent-Distribution Studies with Bence-Jones Protein in a Dissociating System*

Norbert Hilschmann and Lyman C. Craig

ABSTRACT: The Bence-Jones protein isolated from the urine of a selected patient has been subjected to fractionation experiments with the objective of preparing starting material of sufficient purity for reliable sequence studies. Of the fractionation methods tried, countercurrent distribution in a dissociating system revealed the most heterogeneity. A major fraction could be isolated by countercurrent distribution from the crude protein, which appeared to have adequate purity by the criteria of countercurrent distribution, gel filtration, starch-gel electrophoresis, and quantitative amino acid analysis.

Bence-Jones proteins are excreted in the urine by patients with multiple myeloma (Jones, 1847, 1848). While these proteins obtained from different patients differ in their physical, chemical, and antigenic properties (Putnam, 1957, 1960, 1962; Putnam *et al.*, 1962; Mannik and Kunkel, 1963), a Bence-Jones protein from one patient has been widely assumed to be homogeneous (Putnam, 1960).

Bence-Jones proteins have recently been shown (Edelman and Gally, 1962) to be very similar to if not identical with the L-polypeptide chain of the myeloma globulin from the same patient. The problem of their nature is therefore part of the much larger and more important problem of the nature of the γ-globulins. In the latter case the problem of preparing a sufficient amount of a sample of pure protein for structural study, to say nothing of the proof of purity, is a formidable one. Perhaps it is even beyond the capabilities of existing methods.

On the other hand, gram quantities of Bence-Jones protein can be recovered rather easily from a single patient. On the basis of these considerations the study of Bence-Jones proteins has been taken up in a number of laboratories. There remains, however, a fundamental question of their purity, which has been considered (Van Eijk *et al.*, 1963; Bernier and Putnam, 1964) but not completely solved.

Even those preparations considered most homogeneous show a considerable heterogeneity when investigated by starch-gel electrophoresis. However, earlier studies with the ultracentrifuge indicated heterogeneity caused by aggregation. Fractions with an $s_{20,w}$ of 3.5 S appeared to dissociate into smaller units at an acidic *p*H (Putnam and Stelos, 1953). Such behavior seemed to explain the values for the molecular weights of Bence-Jones proteins from different patients which range from 20,000 to 45,000 and even higher (Putnam, 1960). In addition to the ultracentrifuge investigations purity studies by gel filtration and ion-exchange chromatography have been made (Van Eijk *et al.*, 1963; Bernier and Putnam, 1964).

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