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The Chemistry of the Reaction of 2-Hydroxy-5-nitrobenzyl Bromide with His-32 of α-Lactalbumin

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His-32 of bovine or human α-lactalbumin reacts with the tryptophan reagent 2-hydroxy-5-nitrobenzyl bromide at pH 7. The reaction depends on the native conformation of the α-lactalbumin molecule and it is restricted to position 1 of the imidazole nucleus. The synthesis and characterization of 1-(2-hydroxy-5-nitrobenzyl)-histidine, 3-(2-hydroxy-5-nitrobenzyl)-histidine and 1,3-bis(2-hydroxy-5-nitrobenzyl)-histidine are described.

In previous work from this laboratory, it was tentatively concluded that when bovine α-lactalbumin is modified with 2-hydroxy-5-nitrobenzyl bromide, His-32 is modified in addition to Trp-26, Trp-104 and Trp-118 [1]. The elucidation of the chemistry of this reaction could lead not only towards a greater understanding of α-lactalbumin, the specifier protein of lactose synthetase [2], but also towards further knowledge of the chemistry of the widely used tryptophan reagent 2-hydroxy-5-nitrobenzyl bromide [3]. The purpose of this work, therefore, was first to confirm that when α-lactalbumin is treated with 2-hydroxy-5-nitrobenzyl bromide His-32 is modified, second to characterize the modified histidine and third to determine the conditions required for His-32 modification.

MATERIALS AND METHODS

Materials

Bovine and human α-lactalbumins were prepared as described previously [4]. N^{α}-Acetyl-L-histidine, 1-methyl-L-histidine and 3-methyl-L-histidine were bought from Sigma, methanesulphonic acid and *N*-ethylmorpholine (sequanal grade) from Pierce Chemical Corporation (Rockford) and aminopeptidase M from Röhm and Haas (Darmstadt). All other products were purified as described previously [1] or obtained from commercial sources and used without further purification.

Abbreviations. HONBzl-, 2-hydroxy-5-nitrobenzyl group; HONBzl-Br, 2-hydroxy-5-nitrobenzyl bromide; HONBzl-OH, 2-hydroxy-5-nitrobenzyl alcohol.

Enzymes. Lactose synthetase (EC 2.4.1.22); aminopeptidase M (EC 3.4.11.2).

Reaction of α-Lactalbumin with HONBzl-Br at pH 7

Bovine or human α-lactalbumin was treated with HONBzl-Br by a modification of the method of Bell *et al.* [5]. Typically, 10 μmol of α-lactalbumin was dissolved in 100 ml 0.1 M NaCl. The pH was adjusted to 7 (0.1 M NaOH) and the well-stirred solution treated by the dropwise addition of 400 μmol HONBzl-Br in 5 ml dry acetone. The pH of the reaction mixture was maintained at 7 on an automatic titrator and after 15 min subjected to gel filtration on a column (1000 ml) of Sephadex G-25 (coarse) in 1 mM NH_4OH (pH about 8). This desalting step took about 6 min. The protein fraction was immediately frozen and the protein recovered by freeze-drying. The distribution of the HONBzl group in the modified protein was determined as described previously [1].

Analytical Methods

Several of these have already been described [1]. The purity of peptides and HONBzl derivatives of histidine was ascertained by thin-layer chromatography in solvent A = propan-1-ol/pyridine/H_2O (5/1/2, v/v/v) or solvent B = butanone/formic acid/water (24/1/6, v/v/v) and by thin-layer electrophoresis at pH 1.8 (2 M acetic acid, 0.8 M formic acid) or pH 6.2 (0.8 M pyridine adjusted with acetic acid) on precoated cellulose glass plates obtained from E. Merck (Darmstadt).

Synthesis of HONBzl Derivatives of Histidine

The N-1 and/or N-3 derivatives of histidine were prepared for characterization and reference purposes by treating N^{α}-acetyl-L-histidine with HONBzl-Br in

dimethylformamide. The HONBzl-substituted N^{α}-acetyl-L-histidines were separated by gel filtration and deacetylated by acid hydrolysis.

N^{α}-Acetyl-histidine (37 mg or 188 μmol) was dissolved in dimethylformamide (8 ml). HONBzl-Br (17.9 mg or 77 μmol) in dimethylformamide (0.4 ml) was added dropwise to the well-stirred N^{α}-acetyl-histidine solution. After 1 h the reaction was stopped by the addition of H_2O (10 ml). The solvents were removed on a rotary evaporator and the residue dissolved in 50 mM NH_4HCO_3 (26 ml). This solution was chromatographed on a column of Sephadex G-25 (superfine) and in this way four peaks absorbing at 410 nm were obtained (Fig. 1). The materials in the two fastest moving peaks were further purified by gel filtration.

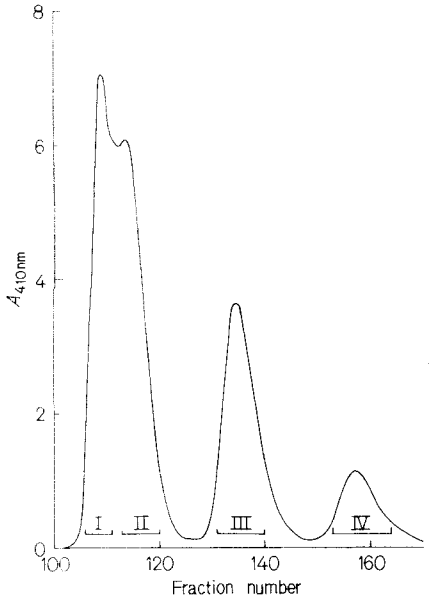

Fig. 1. *Separation of the components of a reaction mixture of HONBzl-Br and* N^{α}*-acetyl-histidine.* The reaction mixture (77 μmol in HONBzl-Br) was applied to a column (500 mm × 50 mm) of Sephadex G-25 (superfine) equilibrated with 0.05 M NH_4HCO_3 (pH 8). The column was developed with the same buffer at a flow rate of 60 ml h^{-1}. Fractions (10 ml) were collected and analyzed at 410 nm and pooled as indicated by the horizontal bars. For other details see the text

The yields (based on HONBzl-Br) of materials in peaks I and II were each 25%, in peak III 29%, in peak IV 5%. The four products were recovered by freeze-drying. The HONBzl derivatives of histidine were prepared by hydrolyzing their corresponding N^{α}-acetyl derivatives in sealed, evacuated tubes in 6 M HCl at 118 – 120 °C for 24 h. The HCl was removed on a rotary evaporator and the residue (in 10 ml 50 mM NH_4HCO_3) subjected to gel filtration (Fig. 1 and Table 1).

Chemical Degradation Studies

For studies involving the cleavage of the HONBzl-His bond a procedure based on that of Edmondson *et al.* [6] was followed. Each HONBzl-substituted N^{α}-acetyl-histidine (4 – 5 μmol, based on the HONBzl group) was methylated in 0.3 ml dimethylformamide containing 0.1 ml methyl iodide. The mixtures were kept for 96 h in the dark, the excess solvent and methyl iodide removed *in vacuo*, and the methylated products purified by gel filtration (Fig. 1 and Table 1). The HONBzl-His bond was cleaved by hydrolysis, in sealed evacuated tubes, in 6 M HCl (1 ml) at 184 °C for 96 h. Histidine and its methyl derivatives were identified and estimated on the amino acid analyzer.

RESULTS

The Modification of His-32 by HONBzl-Br

His-32 is the *N*-terminal amino acid of a chymotryptic fragment (T5Ch4) common to bovine and human α-lactalbumins: His-Thr-Ser-Gly-Tyr [2]. It has already been tentatively concluded that when bovine α-lactalbumin is treated with HONBzl-Br at pH 6, His-32 is modified [1]. Thus a chymotryptic peptide was isolated whose amino acid composition showed that it could be assigned unambiguously to residues 33 – 36. Since this fragment (HONBzl-T5Ch4) contained the HONBzl group and since residue 31 is an aromatic amino acid (phenylalanine), we concluded that modification of His-32 had occurred. In this work, the same peptide has been isolated from bovine and human α-

Table 1. *Properties of HONBzl-substituted histidines*
For experimental details of the gel filtration see Fig. 1. Ac-His(1HONBzl) (peak I, Fig. 1) was eluted in 1100 ml; Ac-His(3HONBzl) (peak II) in 1160 ml; Ac-His[1,3(HONBzl)$_2$] (peak III) in 1340 ml; Ac-His(1HONBzl,3Me) in 870 ml and Ac-His(1Me,3HONBzl) in 920 ml

Compound	Elution volume by gel filtration	R_F value in solvent		Electrophoretic mobility at		λ_{max} (in 0.1 M NaOH)
		A	B	pH 6.2	pH 1.8	
	ml					nm
His(1HONBzl)	1500	0.43	0.55	1	1	406
His(3HONBzl)	1680	0.53	0.61	0.86	1	406
His[1,3(HONBzl)$_2$]	1760	0.55	0.74	0.68	0.73	399
HONBzl-OH	1700	1	1	0.65	0.10	408

Table 2. *N-terminal analysis of HONBzl-T5Ch4*
0.15 µmol of HONBzl-T5Ch4 was analyzed by the procedure of Stark [9]. The resulting hydantoin was freed from ninhydrin-positive materials and salts by gel filtration and investigated by thin-layer chromatography and electrophoresis. His-32(HONBzl) was prepared by digesting HONBzl-T5Ch4 exhaustively with aminopeptidase M (see text)

Fragment	Relative mobility towards cathode on electrophoresis at pH 1.8	R_F in solvent B	Ninhydrin colour (0.25% w/v in acetone)
His-32(HONBzl)	1.00	0.55	grey
His-32(HONBzl) hydantoin	0.75	0.47	none
HONBzl-T5Ch4	0.83	0.50	purple
HONBzl-OH	0.10	1.00	none

lactalbumins treated with HONBzl-Br at pH 7 (also see [7]).

In 0.1 M NaOH the λ_{max} of the HONBzl group of HONBzl-T5Ch4 is 406 nm ($\varepsilon = 18\,800\ M^{-1}\ cm^{-1}$, based on amino acid content). The λ_{max} of HONBzl-Trp and HONBzl-Trp-containing peptides is 422 nm [1,3]. This difference in λ_{max} is a convenient way of differentiating between peptides containing HONBzl-Trp and HONBzl-His.

Previous work showed that acid or alkaline hydrolysates of HONBzl-T5Ch4 contained HONBzl-substituted fragments which behaved identically on paper electrophoresis (pH 1.8) and which could not be assigned to either HONBzl-OH or HONBzl-Trp. Neither hydrolysate contained free histidine [1]. In this work, the HONBzl-substituted fragment in an acid hydrolysate (6 M HCl, 114 °C, 20 h) of bovine HONBzl-T5Ch4 was purified by gel filtration in the conditions described in the legend to Fig. 1. Only one HONBzl-substituted fragment was obtained (yield of HONBzl group 71%, elution volume 1500 ml) which was pure by thin-layer chromatography (solvents A and B) and electrophoresis (pH 1.8 and 6.2) and which reacted with ninhydrin but not with the Pauly reagent. When HONBzl-T5Ch4 was digested with aminopeptidase M [8], the same HONBzl-substituted fragment was released (thin-layer chromatography and electrophoresis). The hydrolysis of HONBzl-T5Ch4 was slow and I was unable to determine the order of release of amino acids.

The amino-terminal residue of HONBzl-T5Ch4 was examined by the cyanate method of Stark [9] and the results obtained are summarized in Table 2. The hydantoin of the HONBzl-substituted fragment released when HONBzl-T5Ch4 was digested exhausively (48 h) with aminopeptidase M was also prepared and purified (gel filtration) and from its properties was judged to be identical to the hydantoin prepared directly from HONBzl-T5Ch4. Both hydantoins yielded the same HONBzl-substituted amino acid when subjected to hydrolysis in 6 M HCl (110 °C for 96 h). These results show that it is the amino-terminal amino acid of HONBzl-T5Ch4 which is labelled with the HONBzl group. Finally, when the HONBzl-substituted fragment was hydrolyzed in 6 M HCl at 184 °C, histidine was released (56% based on the HONBzl content before hydrolysis).

Table 3. *Cleavage of the HONBzl-His bond in HONBzl derivatives of N^{α}-acetyl-histidine*
The HONBzl derivatives of N^{α}-acetyl-histidine (4–6 µmol of each, Fig. 1) were hydrolyzed at 184 °C directly or after methylation. In measuring the percentage recovery of histidines, the HONBzl content before hydrolysis was taken as 100%

Peak	Recovery following cleavage of HONBzl-His bond of		
	histidine	1-methyl histidine	3-methyl histidine
I	51	>0.2	>0.2
II	33	>0.2	>0.2
III	24	>0.2	>0.2
I (methylated)	>0.2	≈1	49
II (methylated)	>0.2	6	≈1.5
III (methylated)	7	>0.2	>0.2

It is concluded that when α-lactalbumin is treated with HONBzl-Br at pH 7, His-32 is modified.

Characterization of Synthetic HONBzl Derivatives of Histidine

When N^{α}-acetyl-histidine was treated with a low molar excess of HONBzl-Br, four products were obtained (Fig. 1, peaks I, II, III and IV). At a high molar excess of HONBzl-Br, peaks I and II decreased in size whereas peak III increased. The material in each of these peaks was pure by thin-layer chromatography and electrophoresis. The materials in the four peaks were subjected to acid hydrolysis (deacetylation) and investigation of the products obtained. From its chromatographic and electrophoretic properties and lack of reaction with ninhydrin, it was concluded that peak IV is due to HONBzl-OH. The three other products were pure by thin-layer chromatography and electrophoresis and they all reacted with ninhydrin but not with the Pauly reagent. Each released histidine upon acid hydrolysis at 184 °C (Table 3). It is tentatively concluded that peaks I and II are due to N^{α}-acetylhistidines

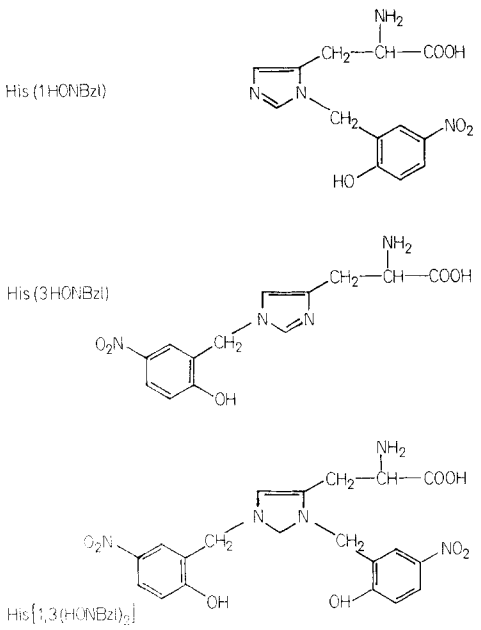

Fig. 2. *The chemical structures of HONBzl-substituted histidines*

substituted with the HONBzl group on either of the imidazole nitrogens, namely Ac-His(1HONBzl) and Ac-His(3HONBzl), and that peak III is due to substitution on both imidazole nitrogens, *i.e.* AcHis-[1,3(HONBzl)$_2$].

An unambiguous procedure for distinguishing between N^1 and N^3-substituted isomers of histidine is to methylate the free imidazole nitrogen, cleave the product and then to identify the resulting 3-methyl or 1-methyl histidine [6]. This procedure was applied to the materials in peaks I, II and III and the results are summarized in Table 3. I am unable to explain the low yields obtained with peaks II and III. The yields were not improved by changing the hydrolysis conditions nor by utilizing the Zn^{2+}/trifluoroacetic acid method of Edmonson *et al.* [6]. Nevertheless, it is concluded that peak I is due to Ac-His(1HONBzl), peak II to Ac-His(3HONBzl) and peak III to Ac-His[1,3(HONBzl)$_2$]. This conclusion is supported by the mass spectrophotometric and infrared studies reported in the Appendix. The chemical structures of the HONBzl-substituted histidines are given in Fig. 2.

Characterization of His-32(HONBzl)

His-32(HONBzl), isolated from both bovine and human α-lactalbumins, is indistinguishable from His-(1HONBzl) by gel filtration, thin-layer chromatography, electrophoresis and light absorption properties (Table 1). These results, together with those reported in the Appendix, show that His-32(HONBzl) and His(1HONBzl) are identical compounds. His(3HONBzl) or His[1,3(HONBzl)$_2$] could not be detected in either α-lactalbumin modified with HONBzl-Br at pH 7.0.

The Reactivity of His-32 of Bovine α-Lactalbumin towards HONBzl-Br under Various Experimental Conditions

When bovine α-lactalbumin was treated with HONBzl-Br in the presence of 8 M urea, His-32 was not modified (Table 4, expt 2). Further, His-32 was unreactive in reduced and carboxymethylated α-lactalbumin and in α-lactalbumin already labelled with the HONBzl group at Trp-26, Trp-104 and Trp-118 (expt 3 and 4). His-32 is not carboxymethylated under the conditions used to carboxymethylate the eight sulphydryl groups of reduced α-lactalbumin [1].

DISCUSSION

Unlike the reaction of HONBzl-Br with tryptophan, which is very complex [3], that with histidine yields only three products: His(1HONBzl), His-(3HONBzl) and His[1,3(HONBzl)$_2$]. This reaction is that expected of an alkyl halide but because of the high reactivity of HONBzl-Br with water [11], it can take place only in a suitable organic solvent. Thus, HONBzl-Br does not react at all with histidine in aqueous solutions buffered at pH 3, 7 or 11.3 [11] or at pH 6 [1]. The reaction of His-32 of α-lactalbumin with HONBzl-Br is, therefore, very interesting. This reaction depends on the native conformation of the α-lactalbumin molecule and it is restricted to position 1 of the imidazole nucleus. His-32 of human α-lactalbumin also reacts with diethyl pyrocarbonate [12] and it may be at or near the area on the α-lactalbumin surface which is involved in the formation of the lactose synthetase complex [12]. This residue is conserved in at least four α-lactalbumins (human, bovine, guinea-pig and kangaroo [2]). Browne *et al.* [13] propose that His-32 is in the α-lactalbumin cleft and that it may replace the catalytically important Glu-35 of lysozyme. Although this conclusion differs from that of Warme *et al.* [14], who propose that the side chain of His-32 points away from the cleft region, both models place His-32 in a hydrophobic area. Such an area is indicated by the recent proton magnetic resonance studies of Bradbury and Norton [15] and it would at once explain the reaction of His-32 with HONBzl-Br (a reagent known to have an affinity for hydrophobic region [3]) and the ability of 1-anilino-8-naphthaline sulphonic acid to abolish this reaction [1]. It is noteworthy that His-57 of chymotrypsin, a residue which is also associated with a hydrophobic area, does not react with HONBzl-Br [16]. Indeed, in so far as we are aware, His-32 of native α-lactalbumin is uniquely reactive towards HONBzl-Br.

Table 4. *The distribution of the HONBzl group in various bovine α-lactalbumin preparations treated with HONBzl-Br*
The α-lactalbumins were treated with HONBzl-Br at pH 7 [5] and analyzed for incorporated HONBzl as described previously [1]

Expt	α-Lactalbumin	Reagent/protein in reaction mixture	HONBzl incorporated into α-lactalbumin		
			total[c]	tryptophan	His-32
		mol/mol			
1.	Native	40	0.91	0.81	0.10
2.	Native in 8 M urea	40	0.63	0.63	≯0.01
3.	Reduced and carboxymethylated	80	3.27	3.27	≯0.01
4.	HONBzl (1.9)[a]	40	2.47[b]	2.47	≯0.01

[a] Native α-lactalbumin modified at pH 2.7 (remaining native protein = 7% [10]).
[b] This value includes HONBzl incorporated at pH 2.7.
[c] Of the reduced and carboxymethylated protein.

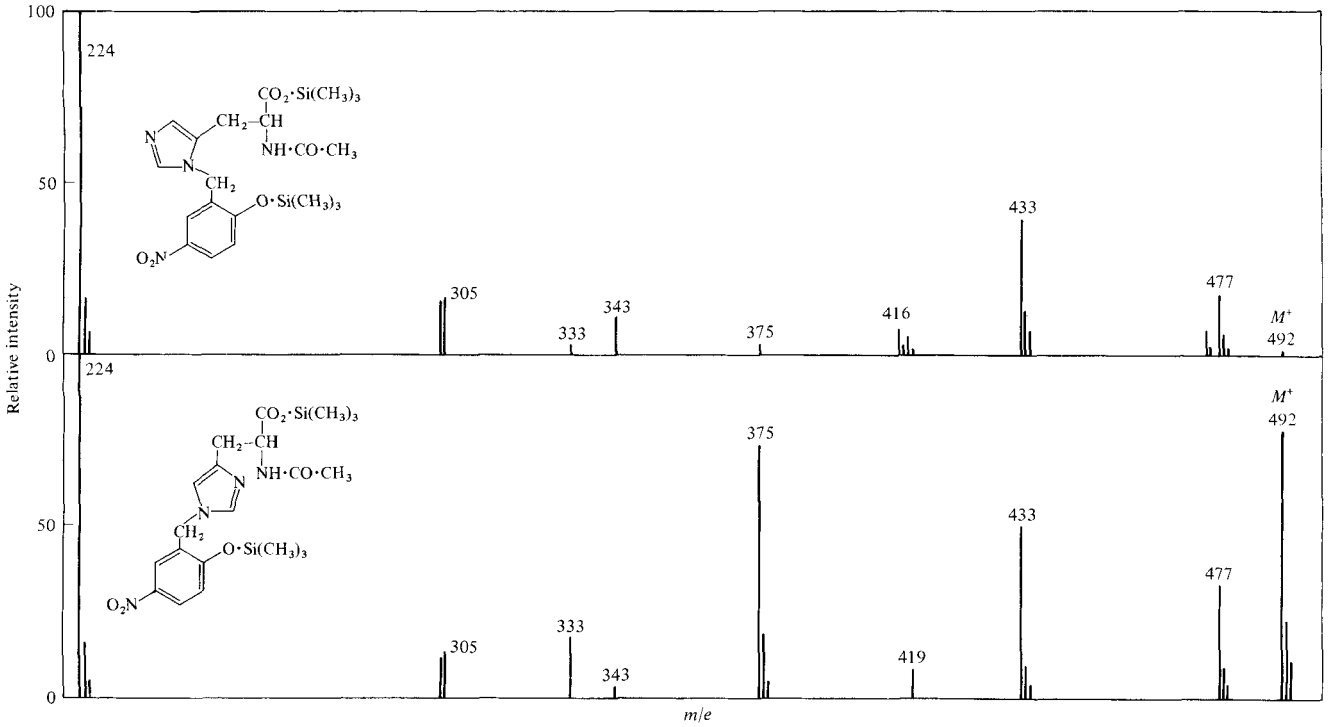

Fig. 3. *Mass spectra of silylated Ac-His(1HONBzl) and Ac-His(3HONBzl)*

APPENDIX

Donald J. MANNING and John C. PRICE

Spectral Characterization of His-32(HONBzl)

In order to characterise His-32(HONBzl) it was first necessary to establish the structures of the isomeric N-1 and N-3 derivatives of histidine. These compounds were prepared from acetylated histidine as described above and their mass spectra were recorded prior to deacetylation. To increase their volatility the samples were silylated with bis(trimethylsilyl)-trifluoroacetamide in pyridine. Low-resolution and high-resolution mass spectra were recorded on an AEI MS 902 mass spectrometer operated at 8 kV and an ionisation beam energy of 70 eV. Samples were introduced into the ion source using a direct insertion probe.

Normalised mass spectra of the HONBzl derivatives of N^{α}-acetyl-histidine above *m/e* 220 are shown in Fig. 3, the abundance of each peak shown in relation to the intensity of the peak at *m/e* 224. The base peak at *m/e* 73 could not be used as a reference since it was also present in the background from the silylating reagent. A fragmentation scheme for the breakdown of the acetylated HONBzl-substituted histidines is shown in Fig. 4.

Although the fragmentation of the two isomers was similar, the relative abundances of certain ions were

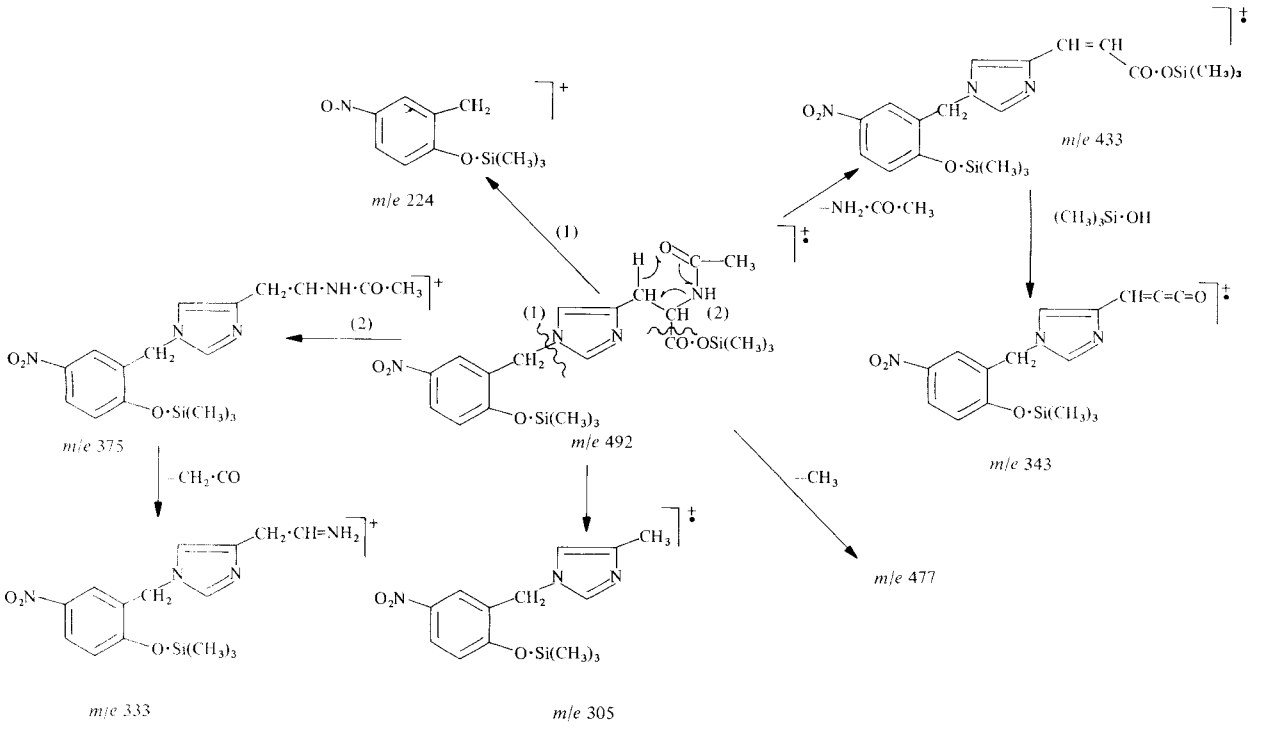

Fig. 4. *Fragmentation scheme for silylated Ac-His(3HONBzl)*

different in the two spectra. In particular, the lower stability of the molecular ion of Ac-His(1HONBzl) at *m/e* 492 relative to that of the N-3 isomer is consistent with steric hindrance occurring between the groups in the 1 and 5 positions. The fragmentation pattern, together with accurate mass measurements of the molecular ions, confirmed that the two compounds were Ac-His(1HONBzl) and Ac-His(3HONBzl). A mass spectrum could not be obtained from Ac-His[1,3(HONBzl)$_2$] presumably due to lack of volatility of the sample even after silylation.

Infrared spectra of the His(1HONBzl), His(3-HONBzl), His[1,3(HONBzl)$_2$] and His-32(HONBzl) (isolated from bovine α-lactalbumin treated with HONBzl-Br, see above) were recorded on KBr discs of a Perkin-Elmer 197 spectrophotometer. The fingerprint regions of the spectra of the four components from 800 – 1200 cm^{-1} are shown in Fig. 5. It was found that the method previously used for differentiating between the histidines substituted at N-1 and N-3 [17] based upon the presence of a peak at 830 cm^{-1} in the N-1 isomer could not be used since the benzene ring of the HONBzl-substituted histidines also produced a peak at about 830 cm^{-1} due to the two adjacent hydrogens on the ring [18]. However, the infrared spectra of His(1HONBzl) and His-32(HONBzl) were sufficiently similar to conclude that these compounds are identical. Minor differences between the spectra are attributed to the use of scale expansion to obtain the spectrum of His-32(HONBzl). The infrared spectrum

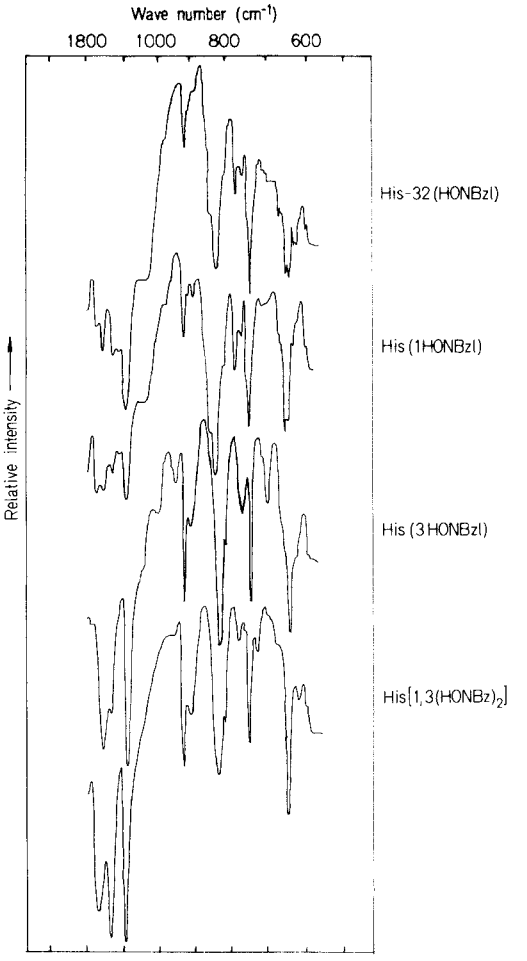

Fig. 5. *Infrared spectra of His(1HONBzl), His(3HONBzl), His-[1,3(HONBzl)$_2$] and His-32(HONBzl)*

of His[1,3(HONBzl)$_2$] is sufficiently close to that of His(3HONBzl) to suggest that the compounds are similar, but a positive identification of the bis-substituted compound could not be obtained.

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REFERENCES

1. Barman, T. E. (1972) *Biochim. Biophys. Acta, 257*, 297–313.
2. Hill, R. L. & Brew, K. (1975) *Adv. Enzymol. 43*, 411–490.
3. Loudon, G. M. & Koshland, D. E. (1970) *J. Biol. Chem. 245*, 2247–2254.
4. Barman, T. E. (1973) *Eur. J. Biochem. 37*, 86–89.
5. Bell, J. E., Castellino, F. J. Trayer, I. P. & Hill, R. L. (1975) *J. Biol. Chem. 250*, 7579–7585.
6. Edmondson, D. E., Kenney, W. C. & Singer, T. P. (1976) *Biochemistry, 15*, 2937–2945.
7. Barman, T. E. & Perry, R. A. (1977) *Biochim. Biophys. Acta, 494*, 314–318.
8. Findley, J. B. C. & Brew, K. (1972) *Eur. J. Biochem. 27*, 65–86.
9. Stark, G. R. (1967) *Methods Enzymol. 11*, 125–138.
10. Barman, T. E. & Bagshaw, W. (1972) *Biochim. Biophys. Acta, 278*, 491–500.
11. Horton, H. R. & Koshland, D. E., Jr. (1965) *J. Am. Chem. Soc. 87*, 1126–1132.
12. Schindler, M., Sharon, N. & Prieels, J. P. (1976) *Biochem. Biophys. Res. Commun. 69*, 167–173.
13. Browne, W. J., North, A. C. T., Phillips, D. C., Brew, K., Vanaman, T. C. & Hill, R. L. (1969) *J. Mol. Biol. 42*, 65–86.
14. Warme, P. K. Momany, F. A., Rumball, S. V., Tuttle, R. W. & Scheraga, H. A. (1974) *Biochemistry, 13*, 768–782.
15. Bradbury, J. H. & Norton, R. S. (1975) *Eur. J. Biochem. 53*, 387–396.
16. Keil-Dlouha, V. & Keil, B. (1973) *J. Mol. Biol. 87*, 381–394.
17. Crestfield, A. M., Stein, W. H., Moore, S. & Jaffe, H. (1963) *J. Biol. Chem. 238*, 2413–2420.
18. Bellamy, L. J. (1958) *The Infrared Spectra of Complex Molecules*, 2nd edn, p. 65, John Wiley and Sons, New York.

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