(14) D. E. Schmidt, Jr., and F. H. Westheimer, *Biochemistry*, **10**, 1249 (1971).
(15) R. Kluger and K. Nakaoka, *Biochemistry*, **13**, 910 (1974).
(16) P. Lee, Ph.D. Thesis, Harvard University, 1965, quoted in ref 6.
(17) M. Dixon and E. C. Webb, "Enzymes", 2nd ed, Academic Press, New York, N.Y. 1964, pp 63–70.
(18) W. Tagaki and F. H. Westheimer, *Biochemistry*, **7**, 895 (1968).
(19) I. A. Rose and S. V. Rieder, *J. Biol. Chem.*, **231**, 315 (1958).
(20) S. Benson, "The Foundations of Chemical Kinetics", McGraw-Hill, New York, N.Y., 1960, p 39ff.
(21) J. Hine, J. C. Kaufman, and M. S. Cholod, *J. Am. Chem. Soc.*, **94**, 4590 (1972).
(22) J. Hine, M. S. Cholod, and J. H. Jensen, *J. Am. Chem. Soc.*, **93**, 2321 (1971).
(23) J. Hine, M. S. Cholod, and R. A. King, *J. Am. Chem. Soc.*, **96**, 835 (1974).
(24) S. J. Coutts, Ph.D. Thesis, Harvard University, 1967.
(25) P. A. Frey, F. C. Kokesh, and F. H. Westheimer, *J. Am. Chem. Soc.*, **93**, 7266 (1971); F. C. Kokesh and F. H. Westheimer, ibid., **93**, 7270 (1971).
(26) M. H. O'Leary and R. L. Baughn, *J. Am. Chem. Soc.*, **94**, 626 (1972).

Model Ligands for Copper Proteins. Proton Magnetic Resonance Study of Acetylhistamine and Acetylhistidine Complexes with Copper(I)

P. A. Temussi* and A. Vitagliano

Contribution from Istituto Chimico, University of Naples, 80134 Naples, Italy. *Received May 6, 1974*

Abstract: The complexes (acetylhistamine)$_2$copper$^+$ and (acetyl-L-histidine)$_2$copper$^+$ have been studied in aqueous solutions by means of proton magnetic resonance spectroscopy. The amide group proved to be unable to complex Cu^+ both alone and in conjunction with the nonspecific imidazole ligand. The Cu^{2+} specific carboxylate group, on the other hand, appears to be a possible site of coordination also for Cu^+ when coupled with the imidazole ligand. These results are interpreted with respect to the problem of the active site of copper proteins with redox activity.

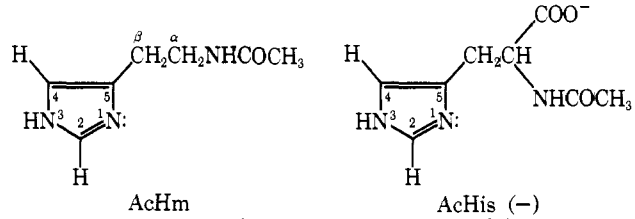
Copper-containing proteins have been the subject of intensive studies[1] during the last few years, both because of their great biological importance (mainly in respiratory chains) and also because of the intrinsic interest posed by the difficult problem of identifying the location of copper in the protein.[2] In fact, this problem is much more difficult than for other metal proteins, *e.g.*, iron heme proteins. In the case of these last proteins, at least four of the six ligands of iron are easily identified upon isolation and characterization of the prosthetic group. On the other hand, in copper proteins, each copper atom may have three to six ligands, all supplied by the residues of the peptide chains,[2] and, accordingly, only direct studies of the proteins can lead to the identification of the ligands. In the case of relatively small proteins, an unequivocal answer to this problem may come from single-crystal X-ray diffraction studies, but it is rather unlikely that any such study will even be attempted for the gigantic molecules of hemocyanins in the near future.

All direct spectroscopic studies[3] have failed to give clear evidence on the structure and composition of the active site of copper proteins (or even for that matter, on the oxidation states of the different copper atoms of a given protein). On account of all these difficulties, indirect studies on model compounds may be of great significance for identifying the probable copper ligands in copper proteins.

In the absence of prosthetic groups, the choice of ligands can be restricted to the side chains of naturally occurring amino acid residues and/or to the peptide chain groups themselves. These ligands can be further subdivided, on the basis of many physicochemical studies, into Cu^{2+} specific, Cu^+ specific, and nonspecific.[4] A very stringent requirement on the composition of the active site of copper proteins involved in oxidation-reduction reactions is that electron transfers occur without ligand displacement. This amounts to saying that the ligands we are looking for must be good for both Cu^{2+} and Cu^+.

In line with these ideas, several researchers in the past have proposed either the sulfhydryl group of cysteine[5] or the imidazole ring of histidine[6] as the linkage of copper to protein in hemocyanins. Other studies, *e.g.*, the effect of photooxidation and histidine reagents on *Murex trunculus* hemocyanin,[7] point to the imidazole ring as a probable ligand rather than the sulfhydryl group. Accordingly we focused our attention on models in which the nitrogen atoms of the imidazole ring could be complexed to Cu^+ or Cu^{2+} together with other ligands very common in protein molecules such as the amide group and/or the carboxylate group which, although specific for Cu^{2+}, might take part in Cu^+ complexation if coupled with a possible Cu^+ ligand.

Acetylhistamine (henceforth referred to as AcHm) was chosen as a model for the case of imidazole ring plus amide group and acetyl-L-histidine (henceforth referred to as AcHis) as a model for the case of imidazole ring plus carboxylate group (and amide group).



Nmr spectroscopy techniques were employed in our study since they are probably unique in giving direct structural information on complexes containing an ion such as Cu^+ which have been generally studied with physicochemical methods that give only very indirect structural information (*e.g.*, potentiometry).

Experimental Section

Materials. Acetonitrile was purchased from C. Erba (Milano, Italy) and purified by distillation over molecular sieves. *N,N*-Dimethylacetamide was obtained from Fluka AG (Buchs, Switzerland) and distilled twice before use. Powdered Cu and $CuSO_4 \cdot 5H_2O$ were from C. Erba. $CuSO_4 \cdot 5D_2O$ was prepared from $CuSO_4 \cdot 5H_2O$ by exhaustive dehydration and subsequent recrystallization from D_2O (99.7%, from C. Erba).

Table I[a]

	$\delta H(2)$	$\delta H(4)$	$\delta \alpha\text{-}CH_2$	$\delta \beta\text{-}CH_2$	δCH_3
$AcHmH^+$	8.66_5	7.33	3.53	2.975	1.98
AcHm	7.66_5	6.92_5	3.45	2.80	1.98
$AcHmCu^+$ (pD 6.88)	8.11_5	7.15	3.54	2.95	1.98
$AcHmCu^+$ (pD 7.77)	8.05	7.11_5	3.53	2.91_5	1.98
$AcHmCu^+$ (pD 8.43)	7.99	7.10	3.53	2.90	1.98
$(AcHm)_2Cu^+$	8.06	7.13	3.54	2.92	1.98

[a] Proton chemical shifts of acetylhistamine and its Cu(I) complex in parts per million. Figures of third, fourth, and fifth rows refer to solutions with AcHm/Cu(I) ratios of 2.5:1. The figures of the last row are extrapolated values for the pure 2:1 complex.

Acetylhistamine was prepared from histamine hydrochloride (Fluka AG) according to ref 8. Acetyl-L-histidine was prepared from L-histidine (Fluka AG) according to the method of ref 9.

Sample Preparation. Cu(I) complexes of AcHm and of AcHis were prepared by reduction of Cu^{2+} with metallic Cu in solutions containing appropriate amounts of the ligands, in a carefully controlled inert atmosphere. In a typical preparation, 100 mg of powdered Cu was added to 3.0 ml of degassed D_2O containing 78.0 mg (0.300 mol) of $CuSO_4 \cdot 5D_2O$, 230.5 mg (1.504 mmol) of AcHm, and 20 μl of 0.821 M D_2SO_4. The suspension was stirred for 60 min under argon atmosphere at 60° (decoloration, however, usually was complete after a few minutes). Part of the filtered solution was then transferred to an nmr tube, whereas the remaining portion was used for pH measurement. Both for AcHm and AcHis solutions, the measured pD values were consistent with the presence in solution of 2:1 complexes (ligand:Cu ratio) as the predominant species. For instance, in the quoted preparation of $(AcHm)_2Cu^+$, a pD value of 8.43 was determined. From the pK_a value of AcHm in D_2O solution (7.53, *vide infra*) and the pD of the solution, one obtains the ratio between the amounts of free and protonated AcHm, $n_B/n_A = 8.0$. Assuming n_A to be given, as a first approximation, by the amount of D_2SO_4 added, it is possible to calculate the number of moles of AcHm bound to Cu^+, on the average (n_C). In our example, $n_C = n_{tot} - n_A - n_B = 1.21$ mmol, *i.e.*, twice the number of Cu moles (0.600 mmol). In any case, as it is clear from the nmr data (*vide infra*), the presence in solution of complexes with different ligand:Cu(I) ratios would not affect the interpretation of the results in terms of the ligand–Cu(I) interactions since fast exchange phenomena are always present in our systems.

AcHis was actually used as its potassium salt.

Measurements. All pD measurements were performed with a Radiometer pH 4 pH meter. In all cases, the readings of the pH meter plus 0.4 were used to calculate pD according to the relationship: pD = pH(read) + 0.4.[10] For internal consistency, pK values of AcHm and AcHis were redetermined in D_2O solutions and found to be 7.53 and 7.58, respectively. Most nmr spectra were recorded at probe temperature with a Varian A-60-A spectrometer. Some spectra, in particular those necessary for the ABX analysis, were also recorded with a Varian HA-100-15 spectrometer. DSS was used as internal standard and/or as lock signal.

Results and Discussion

Figure 1 shows a comparison of the spectra of the ligand AcHm, both neutral and protonated, and of the $(AcHm)_2Cu^+$ complex. The fine structure of the imidazole C(2) H peak and the sharpness of all peaks in the spectrum of $(AcHm)_2Cu^+$ indicate that the concentration of Cu^{2+} in solution is negligible (*vide infra* for a semiquantitative estimate of the Cu^{2+} content). It can be noted that the only peaks appreciably affected by complexation are those of the imidazole ring C(2) H and C(4) H, and the values of their chemical shifts are intermediate between those of the neutral and protonated ligand. All the relevant chemical shift data are reported in Table I. The figures under the heading $(AcHm)_2Cu^+$ are extrapolated values for the pure complex (δ_C) extracted from the equation

$$\delta_{obsd} = x_A\delta_A + x_B\delta_B + x_C\delta_C$$

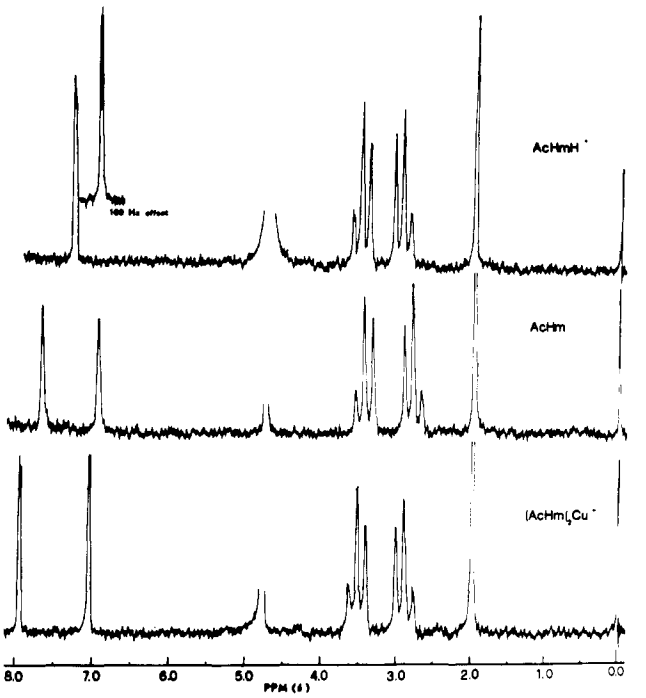


Figure 1. Comparison of the 60-MHz spectra of AcHm (free base), $AcHmH^+$ (sulfate salt) and $(AcHm)_2Cu^+$ (pD 8.43). The spectrum of $(AcHm)_2Cu^+$ shows that the sample is free from Cu^{2+}.

where the indexes A, B, C refer to protonated ligand, free base ligand, and complexed ligand, respectively. The values of x_A and x_B were determined from pD measurements and the stoichiometry of the solutions. It is apparent that the major cause of chemical shift differences among the ring protons in the three chemical environments A, B, and C is the influence of the positive charge either from H^+ or Cu^+. In fact, the approximate halving of this effect in the complex chemical shifts with respect to those of $AcHmH^+$ is consistent with the sharing of one positive charge by two imidazole groups in the $(AcHm)_2Cu^+$ molecule. The influence of Cu^+ on the two methylene groups is much smaller so that the slightly larger effect on the α-CH_2 with respect to the β-CH_2 group cannot be used for diagnostic purposes since the differences are almost comparable to experimental errors. Rather it is safe to state that the amide group is not involved in complexation, because in such a case the chemical shifts of either the methyl or the α-methylene group would be affected.[11-13]

We wish to emphasize the results of recording proton spectra of Cu^+ complexes in aqueous solution. Usually it is extremely difficult to obtain good spectra of Cu^+ complexes since the presence of even traces of Cu^{2+} ions can broaden the ligand peaks to a considerable extent. In order to estimate the effect of Cu^{2+} on the spectrum of our ligand, we recorded pmr spectra of AcHm doped with Cu^{2+} concentrations ranging from 10^{-4} to 10^{-5} M. Even a concentration of 2×10^{-5} M is sufficient to appreciably broaden the peaks of the imidazole protons.

Figure 2 shows a comparison of the spectra of $AcHis^{\pm}$ (the second model ligand of our study) and of its complex with Cu^+. As for $(AcHm)_2Cu^+$, in this case the only chemical shifts appreciably affected by the complexation are those of the imidazole ring. This result substantiates the finding (*vide supra*) that the amide group is not involved in the coordination. All the relevant chemical shift data are reported in Table II. The values for the pure $(AcHis)_2Cu^+$ complex were calculated in a manner analogous to that used for the corresponding data of Table I. Besides the chemical shift data, Table II reports some coupling con-

Table II[a]

	H(2)	H(4)	H_X	H_A	H_B	CH_3	J_{AB}	$J_{AX}+J_{BX}$	J_{BX}	J_{AX}
$AcHis^+$	8.74	7.44	4.81	3.43	3.30	2.08	15.4	13.8	9.7	4.1
$AcHis^{\pm}$	8.70	7.38	4.61	3.37	3.20	2.07_5	15.2	13.3	8.8	4.5
$AcHis^-$	7.78	7.02	4.52_5	3.19	3.04	2.04	15.0	13.4	9.8	3.6
$AcHis^-Cu^+$ (pD 7.88)	7.98	7.08	4.59	3.22_5	3.14	1.98	15.5	12.4	8.3	4.1
$AcHis^-Cu^+$ (pD 9.10)	7.92_5	7.05	4.58	3.17		1.96_5		12.4		
$(AcHis^-)_2Cu^+$	7.98	7.06	4.59	3.22	3.15	1.95_5	15.6	12.1		

[a] Proton chemical shifts in parts per million and coupling constants in hertz of acetylhistidine and its Cu(I) complex. Figures of fourth and fifth rows refer to solutions with AcHis/Cu(I) ratios of 2.5:1. The figures of the last row are extrapolated values for the pure 2:1 complex.

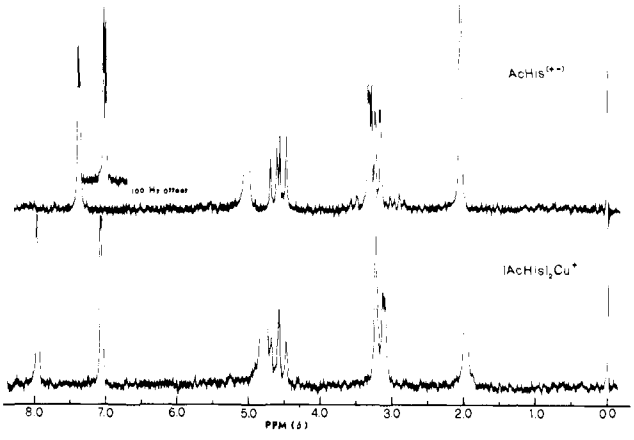
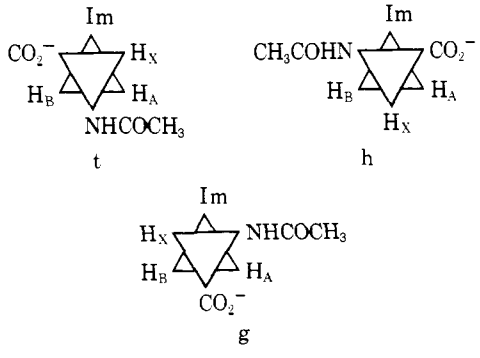


Figure 2. Comparison of the 60-MHz spectra of AcHis (zwitterionic form) and $(AcHis)_2Cu^+$ (pD 7.88).

stant data obtained from the analysis of the ABX system of the CH_2 and CH groups of the side chain. These coupling constants, as is well known, might be of diagnostic value with respect to the conformation of the side chain. Unfortunately, the individual coupling constants (J_{AX} and J_{BX}) of the complex cannot be determined with reasonable accuracy owing to the shape of the AB part of the spectrum (see Figure 2). However, it is possible to use the sum $J_{AX} + J_{BX}$ to estimate the relative abundance of one of the rotamers of the ethane-like grouping. In fact, the values of $J_{AX} + J_{BX}$ in both the spectra of the complex and of the free ligand can be determined with the same good accuracy (0.2 Hz) since they are directly measured from the separation of the outer lines of the X part of the spectrum.

Let us call t, g, and h the three possible staggered conformations and *t*, *g*, and *h* their relative abundances.



It is then possible to write down the well-known approximate equations:

$$J_{AX} = tJ_g + gJ_t + hJ_g$$
$$J_{BX} = tJ_t + gJ_g + hJ_g \qquad (1)$$

where J_t and J_g are, as usual, the coupling constants of vicinal hydrogens in trans and gauche positions, respectively. Rearranging eq 1 together with the relation $t + g + h = 1$, it is easy to show that

$$h = \frac{(J_t + J_g) - (J_{AX} + J_{BX})}{J_t - J_g} \qquad (2)$$

i.e., the relative abundance of rotamer h depends only on the value of the sum $J_{AX} + J_{BX}$ and on the values of J_t and J_g, while the populations of the other two rotamers depend on the individual values of J_{AX} and J_{BX}. The absolute value of *h* depends in a critical way on the choice of J_g and J_t. Many different values have been proposed in the literature;[15a] a reasonable choice, because of the similarity of the compounds, may be to use the values proposed by Martin and Mathur in a study of the rotamer populations of cysteine and histidine derivatives.[15b] Using 2.0 and 12.0 Hz for J_g and J_t, respectively, as suggested in ref 15b, the following set of *h* populations would be obtained for our compounds: $AcHisH^+ = 0.02$, $AcHis^- = 0.06$, $AcHis^{\pm} = 0.07$, $(AcHis)_2Cu^1 = 0.19$. A more sophisticated approach to the evaluation of rotamer populations in histidine derivatives has been recently published by Weinkam and Jorgensen.[16] The effect of the electronegativity of the substituents on the coupling constants is accounted for in great detail by means of calculations analogous to those described by Pachler[17] for fluoroethane. By using eq 14 and 15 of ref 16, we calculated 3.2 and 11.5 Hz for J_g and J_t, respectively and, accordingly, the following *h* values: $AcHisH^+ = 0.11$, $AcHis^- = 0.16$, $AcHis^{\pm} = 0.17$, $(AcHis)_2Cu^1 = 0.31$. Although the absolute values of the *h*'s are quite different in the two cases shown above, it is easy to see that in going from the free ligand to the complex there is a large increase in the value of *h* in both cases. The increased relative stability of rotamer h in the complex can be interpreted to reflect an interaction between Cu^+ and the carboxylate group.

The binding by carboxylate, suggested by the rotamer analysis, can be demonstrated by an independent piece of information. It is well known that the chemical shifts of the imidazole protons of histidine are very sensitive to changes of the net charge present on the ring. In the case of the complex $(AcHm)_2Cu^+$, it was shown that the chemical shift difference between the complexed ligand and the free ligand ($\delta_C - \delta_B$) is approximately one half the difference between the protonated ligand and the free ligand ($\delta_A - \delta_B$) both for C(2) H and C(4) H ring protons. It can be seen from Table III that the corresponding ratios for $(AcHis)_2Cu^1$ are much smaller. This effect can be taken as a strong evidence of a smaller charge fraction on the imidazole rings of $(AcHis)_2Cu^1$ and, indirectly, as an evidence of complexation of the (negative) carboxylate group to Cu^+.

This result is particularly relevant since, to the best of our knowledge, coordination of Cu(I) to oxygen in aqueous solution is unknown.[18]

Conclusions

The main conclusions of the present investigation are the following. (1) A ligand such as carboxylate, although

Table III[a]

	$\left(\frac{\delta_C - \delta_B}{\delta_A - \delta_B}\right)_{H(2)}$	$\left(\frac{\delta_C - \delta_B}{\delta_A - \delta_B}\right)_{H(4)}$
AcHm	0.40 ±0.03	0.51 ±0.08
AcHis	0.22 ±0.03	0.11 ±0.08

[a] Comparison of chemical shift changes of imidazole ring protons caused by Cu^+ and H^+ for AcHm and AcHis. The ratios of such changes, as defined in the headings of the table, are proportional to the charge fraction carried by the imidazole ring. Errors are maximum errors calculated on the basis of an average error of 0.02 ppm in the individual chemical shifts.

Cu(II) specific, can take part in Cu(I) complexation if coupled with a nonspecific ligand such as the imidazole ring of histidine. This finding should be taken into account for the identification of active sites of Cu proteins with redox activity; spatial proximity of an histidyl residue (present in nearly all such proteins) and of the ubiquitous carboxylate group may be considered as a sufficient requirement for a redox active site. (2) The negligible tendency of the amide group toward Cu(I) complexation[4] is confirmed also for cases in which it is coupled with the imidazole ring. (3) The simple application of nmr spectroscopy used in this study turned out to be very effective in revealing a complexation to Cu(I) that would probably be undetectable by techniques such as potentiometry, commonly employed in the study of complexes with poor optical spectral properties. In fact, in the specific case of $(AcHis)_2Cu^1$, a hypothetical potentiometric study of the Cu^+-carboxylate interaction should be performed in a pH range in which the whole complex is not stable owing to protonation of the imidazole nitrogen atoms.

References and Notes

(1) (a) J. Peisach, P. Alsen, and W. E. Blumberg, Ed., "The Biochemistry of Copper," Academic Press, New York, N.Y., 1966; (b) G. L. Eichorn, Ed., "Inorganic Biochemistry," Elsevier, Amsterdam, 1973.
(2) D. C. Gould and H. S. Mason in ref 1a, p 35.
(3) (a) T. Nakamura and H. S. Mason, *Biochem. Biophys. Res. Commun.*, **3**, 297 (1960); (b) W. E. Blumberg in ref 1a, pp 49–65; (c) R. Malkin in ref 1b, p 689.
(4) P. Hemmerich in ref 1a, p 15.
(5) I. M. Klotz and T. A. Klotz, *Science*, **121**, 477 (1955).
(6) (a) R. Lontie, *Clin. Chim. Acta*, **3**, 68 (1958); (b) R. Lontie and R. Witters in ref 1b, pp 353–354.
(7) E. J. Wood and W. H. Bannister, *Biochim. Biophys. Acta*, **154**, 10 (1968).
(8) D. P. Van der Merwe, *Hoppe-Seyler's Z. Physiol. Chem.*, **177**, 308 (1933).
(9) M. Bergmann and L. Zervas, *Biochem. Z.*, **203**, 284 (1929).
(10) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).
(11) A. Fratiello, D. P. Miller, and R. Schuster, *Mol. Phys.*, **12**, 111 (1967) and references quoted therein.
(12) P. A. Temussi, T. Tancredi, and F. Quadrifoglio, *J. Phys. Chem.*, **73**, 4227 (1969).
(13) As an independent test of the ligand ability of the amide group towards Cu^+, we tried to observe the complex of Cu^+ with *N,N*-dimethylacetamide, a simple amide. The Cu^+ ion was introduced in aqueous DMA solutions as acetonitrile complex[14] with molar ratios Cu^+/DMA up to 10:1. Both the special features of all DMA peaks and the coalescence temperature of the methyl peaks were totally unaffected. This result, probably, reflects the greater stability of the acetonitrile complex with respect to the hypothetical amide complex.
(14) P. Hemmerich and C. Sigwart, *Experientia*, **19**, 488 (1963).
(15) (a) See, for instance, A. A. Bothner-By, *Advan. Magn. Resonance*, **1**, 201 (1965); (b) R. B. Martin and R. Mathur, *J. Amer. Chem. Soc.*, **87**, 1065 (1965).
(16) R. J. Weinkam and E. C. Jorgensen, *J. Amer. Chem. Soc.*, **95**, 6084 (1973).
(17) K. G. R. Pachler, *Tetrahedron Lett.*, 1955 (1970).
(18) H. C. Freeman in ref 1b, p 158.

Preparation of a New *o*-Nitrobenzyl Resin for Solid-Phase Synthesis of *tert*-Butyloxycarbonyl-Protected Peptide Acids

Daniel H. Rich* and S. K. Gurwara

Contribution from the School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706.

Received October 23, 1974

Abstract: A new resin—3-nitro-4-bromomethylbenzoylamide polystyrene resin—was prepared. This resin is suitable for the synthesis of Boc-protected peptides possessing a free C-terminal carboxyl group. The protected peptide acid is removed from the resin by photolysis at 3500 Å. These conditions do not cleave acid-labile protecting groups nor decompose aromatic amino acids. The application of this resin to the synthesis of Boc-Leu-Arg(Tos)-Pro-Gly, Boc-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly, pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly, and Boc-Ser(Bzl)-Tyr(Bzl)-Gly is described.

The solid-phase method of peptide synthesis, introduced by Merrifield in 1963,[1] has proven to be an effective method for the rapid synthesis of peptides. However, the products prepared by this method are often difficult to purify. Impurities, such as failure sequences[2] which are caused by changes in the physical-chemical properties of the polymer,[3] accumulate during a stepwise synthess and can be difficult to remove. Several authors[4-7] have suggested that a more homogeneous final product might be isolated by coupling pure protected peptide fragments on the solid support. Failure sequences formed during a synthesis using fragment coupling would differ substantially from the desired product and would be more readily removed during purification. The basic trypsin inhibitor from bovine pancreas, synthesized by fragment coupling on a polymer, was reported to be purer[8] than the product obtained by stepwise synthesis.[9] However, if the fragment coupling approach is to become generally useful, a convenient method for preparing Boc peptide acids is needed. These derivatives have been prepared by solid-phase synthesis but either transesterification[10,11] or hydrazinolysis[12] reactions were required to remove the Boc-protected peptides from the resin and these conditions are not always applicable. In this paper we describe the synthesis and use of a new resin **7** from which protected peptide acids can be removed by photolysis under conditions which do not destroy aromatic residues nor cleave acid- or base-labile protecting groups.

Recently we reported that protected amino acids and