Tetrahedron Letters No.39, pp. 3465-3472, 1965. Pergamon Press Ltd.
Printed in Great Britain.

# A NOVEL APPROACH TO THE C-TERMINAL DETERMINATION OF PEPTIDES: SELECTIVE $^{2}H$- AND $^{3}H$-LABELLING REACTION OF C-TERMINAL AMINO ACIDS THROUGH OXAZOLONE.

Hisayuki Matsuo, Yasuo Fujimoto and Takashi Tatsuno
The Institute of Physical and Chemical Research
Komagome, Bunkyo-ku, Tokyo, Japan

(Received 4 August 1965)

The carboxy-terminal amino acid determination is indispensable for the sequential analysis of peptides and proteins and a number of studies on this subject have been reported (1). However, only a few methods, such as the hydrazinolysis and the carboxypeptidase methods, have been successfully applied in practice (1). There still remains further investigation to be made on this problem.

Naturally, a chemical method for the determination of C-terminal amino acid should employ a chemical reaction that could discriminate the C-terminal from the other amino acids in a peptide chain.

In the field of peptide synthesis, it is generally believed that the racemization during peptide synthesis through the oxazolone intermediate occurs on the C-terminal amino acid(2). It is also well known that the optically active oxazolone derived from the corresponding N-acylamino acid by treatment

with acetic anhydride (3) or dicyclohexylcarbodiimide(DCCD)(4), undergoes racemization faster than the solvolytic ring-opening to afford a racemic product (3,5).

In the course of our study on the racemization of amino acids and peptides in deuterium-containing media, it was revealed that hydrogen-deuterium exchange occurs on the asymmetric carbon of amino acids under racemization conditions involving carbanion formation, and this deuteration reaction can be followed readily and precisely by the n.m.r. method (6).

On the basis of the foregoing considerations, the present authors carried out successfully selective deuteration of the asymmetric carbon of C-terminal amino acids in several N-acetylpeptides through the racemization reaction, involving the corresponding oxazolone formation, followed by the base-catalyzed hydrolytic ring-opening in deuterium oxide, so as to discriminate chemically the C-terminal from the others in the peptide chain. Deuterated products were characterized unambiguously by n.m.r. spectra.

This reaction was further extended to the radio-active tritium exchange, using tritium oxide in place of deuterium oxide in the reaction described above, and the authors found this method applicable to the C-terminal determination of peptides by a simple manipulation and in a micro-scale.

A) Deuteration Reaction :

As preliminary experiments, deuteration of several N-acetyl-amino acids, as a model of simple peptides, was carried out by the following procedure. Compounds used in this experiment

were as follows; acetyl-glycine, acetyl-DL-alanine, acetyl-L-valine, acetyl-L-leucine, acetyl-L-methionine, acetyl-L-glutamic acid and acetyl-L-phenylalanine.

Deuteration Procedure :

Method A : A solution of an N-acetylamino acid (0.5 mmole) and $Ac_2O$ (more than 0.5 mmole) in 5 ml. of anhydrous dioxane was brought to gentle boiling for several minutes. The mixture was allowed to cool to room temperature and evaporated in vacuo at 40° to afford a residue, whose IR spectrum showed characteristic strong absorption bands of oxazolone at 1832 (C=O) and 1660 (C=N) $cm^{-1}$ in $CHCl_3$. The residue thus obtained was dissolved in 2 ml. of dioxane and, after addition of one drop of pyridine, the solution was treated with more than 0.5 ml. of 99.5% $D_2O$ at room temperature for several minutes.
The solution was evaporated in vacuo to afford the deuterated N-acetylamino acid in a good yield. The product was characterized by comparison of its n.m.r. spectrum with that of the starting material.

Method B : The oxazolone formation reaction was carried out with more than 0.5 mmole of DCCD in place of $Ac_2O$ in Method A. The resulting mixture containing dicyclohexylurea formed was treated directly with pyridine and $D_2O$, successively, in a similar manner to Method A, and then worked up as usual to give the objective product in a good yield.

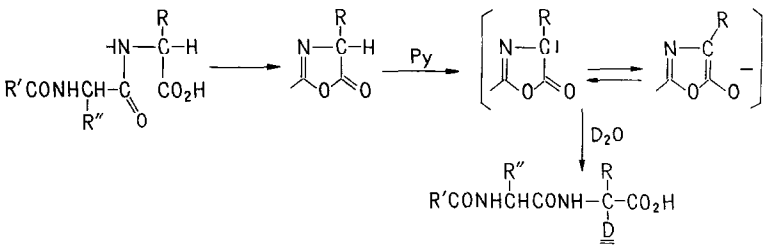
Typical n.m.r. spectra of the deuterated products thus obtained were listed in Fig. 1 (1 and 2). As shown in the figure, it is clear that α-proton signals (peak (a)) of the starting ma-

terials disappeared and the coupling mode of β-proton signals changed on completion of the deuterium exchange on their asymmetric α-carbon, while areal intensities of all other proton signals than α-proton did not decrease.

These results indicated that, by the present procedure, all the N-acetylamino acids, so far as the present work is concerned, underwent complete deuteration exclusively on their asymmetric α-carbon without any other deuterium exchange.

On the basis of preliminary data described above, the authors applied the same procedure to N-acetyl-glycyl-DL-alanine, N-acetyl-glycyl-DL-valine, N-acetyl-glycyl-L-leucine and N-acetyl-glycyl-L-leucyl-glycine and succeeded in the selective deuteration of C-terminal amino acids as expected. Typical n.m.r. data were shown in Fig. 1 (3 and 4). These results show that the present procedure is capable of discriminating the C-terminal amino acid from the others in a peptide chain.

The selective deuteration reaction seems to proceed as in the following scheme.



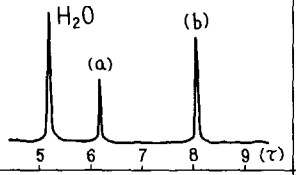
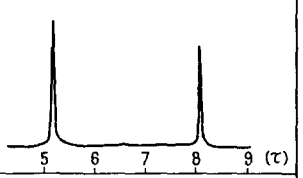
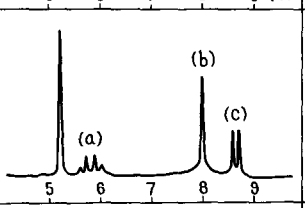
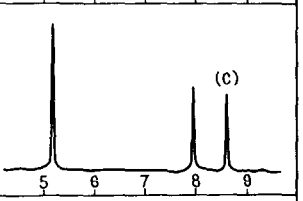
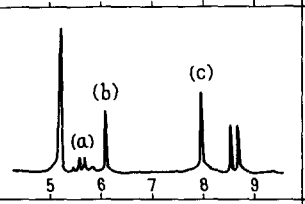
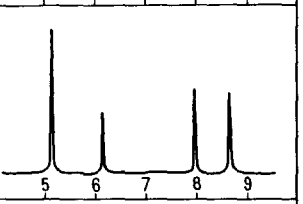
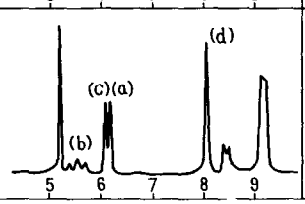
| Compound | Starting Material | After Deuteration |
|---|---|---|
| 1) Ac-Gly<br>$\underset{(a)}{CH_2}CO_2H$<br>$NH\underset{(b)}{COCH_3}$ |  |  |
| 2) Ac-Ala<br>$\underset{(c)}{CH_3}-\underset{(a)}{CH}CO_2H$<br>$NH\underset{(b)}{COCH_3}$ |  |  |
| 3) Ac-Gly-Ala(OH)<br>$\underset{(c)}{CH_3}CONH$<br>$\underset{(b)}{CH_2}CONH$<br>$CH_3-\underset{(a)}{CH}CO_2H$ |  |  |
| 4) Ac-Gly-Leu-Gly(OH)<br>$\underset{(d)}{CH_3}CONH$<br>$\underset{(c)}{CH_2}CONH$<br>$i\text{-}Bu-\underset{(b)}{CH}CONH$<br>$\underset{(a)}{CH_2}CO_2H$ |  |  |

FIG. 1

N.M.R. Spectra of Acetylamino Acids and Acetylpeptides at 60 Mc. in $D_2O$-NaOD* Internal Reference: DSS

*) Equimolar amounts of sample and base were used.

B) Tritiation Reaction as a Novel Method for C-Terminal Determination of Peptides :

For the purpose of improving the C-terminal determination method, the authors investigated an application of the C-terminal labelling procedure by the use of tritium oxide in place of deuterium oxide. In a model experiment with N-acetyl-glycyl-DL-valine, selective tritiation of the C-terminal valine was found to proceed in the same manner as in the deuteration. In this case, the C-terminal tritiation could be confirmed by detection of the radio-activity of an acid-hydrolysate of the tritiated acetyl-dipeptide on a paper-chromatogram.

Tritiation Procedure : A solution of acetyl-glycyl-DL-valine (5 mg.) and DCCD (10 mg.) in 0.5 ml. of anhydrous dioxane was heated at 80-90° for 40 mins. and allowed to cool to room temperature. After addition of one drop of pyridine, 0.2 ml. of dioxane containing 0.07 ml. of $T_2O$ (radio-activity: ca. 70 mc) was added and the whole was allowed to stand for 30 mins. at room temperature. Then, addition of ordinary $H_2O$ followed by evaporation, was repeated several times to remove completely the washable radio-isotope. The residue was hydrolyzed in 18% HCl. After completion of the hydrolysis and subsequent evaporation, the residue was basified with one drop of conc.$NH_4OH$. The sample thus obtained was subjected to paper partition chromatography (BuOH:EtOH:$H_2O$ = 4:2:1). The detection of radio-active spot on the paper chromatogram was performed by the aid of Radiochromatogram-scanner (Packard Model 7200).

The radio-chromatogram gave only a single radio-active spot

corresponding to the C-terminal valine (Rf 0.37), which was identified by direct comparison with a chromatogram coloured by the Ninhydrin reagent. Glycine (Rf 0.12) did not give any radio-activity.

These experimental results indicate a promising possibility for this procedure to develop into a novel method for the C-terminal determination, and the method will have following advantages.

By this method, both the C-terminal determination and identification of constituent amino acids of a peptide can be accomplished simultaneously in a simple manipulation and in the paper-chromatographic micro-scale.

Furthermore, the terminal amino acid can be identified as a radio-active amino acid and there is no need to identify through various derivatives.

Work is continuing in this laboratory to study further application, scope and limitation of this method.

The authors are greatly indebted to Professor Emeritus E. Ochiai and Professor S. Yamada of the Tokyo University and to Dr. A. Tahara of this Institute for their kind advice and encouragement. Thanks are also due to Mr. J. Uzawa of this Institute for the n.m.r. measurements.

References

1) J.P.Greenstein and M.Winitz, Chemistry of the Amino Acids Vol.2, p.1576. John Wiley & Sons,Inc., New York (1961).

2) A.Neuberger, Advances in Protein Chemistry Vol.IV, p.339. Academic Press, New York (1948).
G.T.Young, Collection Czech. Chem. Commun. 24, Special Issue, 39 (1959).

3) H.T.Clarke, The Chemistry of Penicillin p.742, Princeton Press, New York (1949).

4) I.Z.Siemion and K.Nowak, Roczniki Chem. 34, 1479 (1960).

5) A.L.Heard and G.T.Young, J. Chem. Soc. 1963, 5807.
M.W.Williams and G.T.Young, ibid. 1964, 3701.
M.Goodman and L.Levine, J. Amer. Chem. Soc. 86, 2918 (1964).

6) H.Matsuo, Y.Kawazoe, M.Ohnishi, M.Sato and T.Tatsuno, Abstracts of the 3rd Symposium on the Peptide Chemistry 1964, 30. ( Held at Osaka Univ., Osaka, Japan on Nov. 7, 1964.)