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SYNTHESIS AND PMR PROPERTIES OF SOME DEHYDROALANINE DERIVATIVES

R. S. ASQUITH and P. CARTHEW

Department of Industrial Chemistry. The Queen's University of Belfast.
21 Chlorine Gardens. Belfast BT9 5EQ. N. Ireland

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Abstract- A number of new β-*N*-alkylamino acids have been synthesised from *N*-acetyldehydroalanine, and their chromatographic and PMR properties examined. A selected peptide *S*-DNP-glutathione has also been subjected to alkaline degradation and the formation of dehydroglutathione has been shown by PMR.

INTRODUCTION

DEHYDROALANINE has long been postulated as an intermediate in the degradation of cystine residues in proteins, after treatment with alkaline solutions. The formation of the final products of degradation, lanthionine,[1] lysinoalanine[2] and ornithinoalanine[3] and β-aminoalanine[4] can only be easily explained by a preliminary decomposition of the cystine residues to dehydroalanine residues, followed by addition of thiol and amines *e.g.* cystine, ε-amino groups of lysine or ammonia.

Whilst direct evidence has not been obtained to substantiate the presence of this amino acid residue during the decomposition, much indirect evidence that it occurs is available. In particular, the presence of free organic bases or thiols in the alkaline medium has been shown[5-7] to lead to a variety of products, dependent on the structure of these materials.

The absolute identification of these final products requires their synthesis as model compounds *N*-Acetyldehydroalanine or its ethyl ester has been used to synthesise lysinoalanine[2] and lanthionine[8] and some β-sulphoamino-acids[9,10] and *N*-acetyl-β-aminoalanine have also been prepared.[11-13]

The purpose of this paper has been to extend the range of β-*N*-alkylamino-acids prepared in this way and to determine their electrophoretic properties and characteristic PMR spectra, and to further examine a selected peptide of cysteine in order to determine by a PMR technique the presence of the dehydroalanine residue after alkaline decomposition.

RESULTS AND DISCUSSION

The synthesis of acetyldehydroalanine as given in Greenstein and Winitz[16] was found to be more difficult and gave no better yield than the method of Kil'disheva[14] which is a one step procedure. The formation of amine adducts yielding the α-acetyl derivatives of the amino acids proceeded smoothly, but isolation of the product in a crystalline form was impossible without prior removal of all traces of excess amine. Volatile amines were removed by repeated rotary evaporation, non volatile *i.e.* ethanolamine, by extraction of the basified reaction mixture with organic solvent.

Clear electrophoretic separation of the substituted β-amino-acids from one another

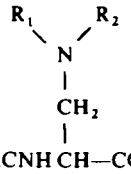

TABLE 1. NEW β-*N*-ALKYL-α-*N*-ACYLAMINO-ACIDS

R_1	R_2	Yield %	m.p. °C	CH[b]	CH_2N[b]	Required %			Found %		
						C	H	N	C	H	N
Et[a]	H	66	164–5	4·51	3·35	43·8	8·33	14·59	43·88	8·3	14·47
Pr	H	55	167–8	4·49	3·35	51·0	8·5	14·9	50·6	8·43	14·3
But	H	49	170–1	4·48	3·33	53·4	8·9	13·7	53·57	8·62	13·66
Amyl	H	46	174–5	4·50	3·33	55·4	9·25	12·95	55·42	8·99	12·71
Et	Et	50	163–4	4·57	3·42	51·86	9·0	14·25	52·5	8·9	13·85
EtOH	H	52	166–7	4·43	3·23	43·9	7·28	14·46	44·2	7·43	14·76

[a] Crystallized as monohydrate.

[b] Chemical shifts in ppm from DSS.

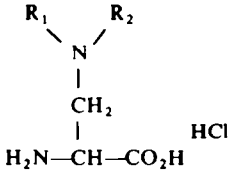

TABLE 2. NEW β-*N*-ALKYLAMINO-ACIDS

R_1	R_2	m.p. °C	CH[c]	CH_2N[c]	Required %				Found %			
					C	H	N	Cl	C	H	N	Cl
Et[a]	H	154–5	4·04	3·4	33·8	7·9	15·8	20·0	34·27	7·7	15·97	19·86
Pr	H	165–6	3·81	3·24	38·8	8·1	15·1	19·45	39·2	8·15	15·5	19·54
But	H	177–8	3·6	3·23	42·8	8·65	14·25	18·05	42·69	8·56	14·15	18·18
Aryl	H	183–4	3·74	3·24	45·6	9·0	13·3	16·85	45·62	9·1	13·38	16·80
EtOH[b]	H	157–8	3·38	3·73	29·6	7·4	13·85	17·5	30·0	7·49	14·0	17·5

[a] Crystallized containing 0·5 mole H_2O.

[b] Crystallized containing 1 mole H_2O.

[c] Chemical shifts in ppm from DSS.

can be effected over a wide range of pH. When present in protein hydrolysates, however, some overlap occurs with other amino acids (Table 3). It can be seen that at pH 1·85 the Rm values for a homologous series of basic amino acids are separated by a characteristic distance, while the diethyl and β-hydroxy-ethyl derivatives lie at intermediate values.

TABLE 3. RM VALUES OF NEW BASIC AMINO ACIDS RELATIVE TO GLYCINE AND ALANINE AT PH 1·85

Amino acid	Rm (to Glycine)	Rm (to Alanine)
β-Ethyl-amino-dl-alanine	1·13	1·3
β-Propyl-amino-dl-alanine	1·07	1·21
β-Butyl-amino-dl-alanine	1·00	1·12
β-Amyl-amino-dl-alanine	0·95	1·065
β-Diethyl-amino-dl-alanine	1·04	1·16
β-(Hydroxyethyl)-amino-alanine	1·1	1·23

Effective separation of all the amino acids in a protein hydrolysate can be effected by careful choice of electrophoretic pH (*e.g.* pH 1·1 for the butyl and amyl products).

Synthesis of the β-diethylaminoalanine product from the acteyl derivative, was obtained pure by the method outlined, failed. All attempts to hydrolyse it resulted in partial breakdown to give two products on electrophoresis, alanine and a product with the expected electrophoretic properties of the diethyl derivative (Table 3).

The PMR spectral data of the α-*N*-acylamino-acids is given in Table 1. Alkyl methylene groups are not given due to poor resolution of the overlapping groups (except the propyl derivative). The δ-methylene protons all occurred at approximately 3·12 ppm relative to DSS, while in the free amino acids at about 3·15 ppm to DSS. The α-CH proton signals are all quartets, deshielded to approximately 4·50 ppm as would be expected due to the carbonyl group.[17] The coupled splitting of the β-CH_2 protons overlaps in all cases with the γ-methylene protons to give a 12 line multiplet. Expansion yielded separation of all 12 lines and treatment of the —CH—CH_2 group as an ABX case gave agreement with expected values on computer analysis.[19] The PMR of the amino acids themselves are also very characteristic[19,20] and could be treated as ABC cases. The exception to the general finding are the protons of the hydroxyethyl compound which are at slightly lower field. This could be expected if the hydroxyl group is ionically linked to the acylamino group and removal of the acyl group does indeed shift the CH—CH_2 protons further downfield (0·3 and 0·5 ppm).

PMR data of dehydroalanyl protons is not readily available. However, the transformation of *S*-DNP-glutathione to dehydroglutathione is readily followed by PMR. The spectra of *S*-DNP-glutathione showed the characteristic positions of the dinitrophenyl protons 7·76, 8·36, 8·83 ppm and the CH—CH_2 for the cysteine residue at 4·75 and 3·61 respectively. After elimination however, all these absorptions

disappeared and were replaced by a doublet at 5·48 ppm which integrated as 2 protons with respect to glycine.

This compares favourably with the absorption of α-acyldehydroalanine (doublet at 5·78 ppm) and acyl-dehydroalanylethylester (6·21 ppm).

It may be possible in view of the separation of the signals of the 2 dehydroprotons from all other common PMR signals of amino acid in D_2O, to use this approach to identify the same characteristic splitting in alkali treated proteins of high sulphur content and so obtain independent physical evidence of the formation of dehydroalanine from cystine under these conditions. Further work on this is being carried out.

EXPERIMENTAL

M.ps were taken using a hot stage microscope and are uncorrected.

PMR spectra of the amino acids were recorded in D_2O on a Varian 60 spectrometer with DSS as internal standard. The ethyl derivative was examined at pD 10·1 all the others were examined at pD's between 8·0 and 8·5.

Chemical shifts are quoted in p.p.m. relative to DSS.

The PMR of acetyl derivatives were recorded at 100MHz using a Perkin Elmer R14 with DSS as internal standard at neutral pD.

Acetyldehydroalanine was prepared by the method of Kil'disheva *et al.* m.p. 198–200° (lit.[14] 198–200°).

S-Dinitrophenyl-glutathione was prepared by the method of Sadeh *et al.*[15] modified as follows:

Glutathione (1 g) was dissolved in 0·1 M $KHCO_3$ (10 ml) and fluorodinitrobenzene (0·65 g) in MeOH (5 ml) added. After stirring (10 min) at room temp. MeOH was removed at room temp. and the *S*-DNP-glutathione precipitated by addition of dilute HCl to pH 2. Recrystallisation from water yielded 0·9 g (90%) m.p. 211° (lit.[15] m.p. 211°).

Preparation of β-N-*alkyl and* N-*acylamino-acids.* Acetyldehydroalanine (5 g) was dissolved in a 30% soln (50 ml) of the amine in water and kept at 40–50° for 72 hr.

The excess amine was removed by rotary evaporation and repeated washing with water followed by further rotary evaporation. The yellow oils were treated with acetone and chilled to precipitate the amino-acid derivative which was recrystallized from MeOH/Et_2O. Yields and m.ps are in Table 1.

Preparation of β-N-*Alkylamino-acids.* The *N*-acyl derivatives were refluxed with 15 fold excess of 2 M HCl for 3 hr to ensure complete removal of the acyl group. The soln was evaporated at 40° to dryness and water washed repeatedly and evaporated again. The residue (after taking up in a little water) was applied to a column of Amberlite 1R4B to remove excess HCl and the eluate evaporated to small bulk. The amino acids were precipitated as monohydrochlorides by addition of acetone and chilling then. Recrystallized from water and acetone. M.ps are in Table 2.

Synthesis of 2-(2′-*hydroxy-ethyl*)-*amino*-1-*acetyl*-DL-*alanine.* Acetyldehydroalanine (5 g) was dissolved in a 20% ethanolamine soln (50 ml) and kept at 45° for 72 hr. The soln was basified with NaOH, extracted with EtOAc several times, passed down a column of Amberlite 1RC50 to remove sodium ions and evaporated at 40° to small bulk. The product was precipitated by addition of acetone (Table 1) then hydrolysed to the amino acid as above.

Preparation of dehydroglutatione from S-*DNP-glutathione.* S-DNP-glutathione (60 mg) was dissolved in a 1:1 mixture of D_2O and DMSO–d_6 (0·5 ml) and made 0·5 M in NaOD by addition of 40% NaOD in D_2O. The soln rapidly became dark brown and was kept at 40° for 3 hr prior to filtration and PMR spectroscopic examination (Varian 60, DSS).

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