A NEW METHOD FOR THE SYNTHESIS OF GLUTAMATE 1-SEMIALDEHYDE. CHARACTERIZATION OF ITS STRUCTURE IN SOLUTION BY NMR SPECTROSCOPY

by

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Glutamate 1-semialdehyde is formed in high yields by ozonolysis of 4-vinyl-4-aminobutyric acid and purification of the reaction products by cation exchange chromatography. The structure of the product and its pyrrole derivative have been confirmed by ^1H -NMR and ^{13}C -NMR spectroscopy, and by its conversion to δ -aminolevulinate by purified glutamate 1-semialdehyde aminotransferase, and to uroporphyrinogen with porphobilinogen and uroporphyrinogen synthetase. The NMR spectra provide evidence for the presence in solution of a hydrate of glutamate 1-semialdehyde. Concentration dependent formation of dimers of the hydrate was also shown. It is suggested that chemical conversion of glutamate 1-semialdehyde to δ -aminolevulinate proceeds through dimeric intermediate stages.

1. INTRODUCTION

Glutamate 1-semialdehyde (GSA) is the immediate precursor of δ -aminolevulinate in plants and many bacteria. In these cells glutamyl tRNA^{glu} can be reduced to GSA (11), which is converted into δ -aminolevulinate by GSA aminotransferase (17). Studies on the semialdehyde have been made difficult by the low yields and complicated synthetic processes required by methods previously reported (6, 7, 10). In this paper we describe a new rapid synthetic method utilising ozonolysis of 4-vinyl-4-aminobutyric

acid (Fig. 1), an inhibitor of γ -aminobutyric (GABA) aminotransferase. This inhibitor also called vigabatrin has been suggested for use in the treatment of epilepsy (e.g. 16). The present method gives much higher yields than those previously reported.

2. MATERIALS AND METHODS

2.1. Chemicals and thin layer chromatography materials

4-Vinyl-4-aminobutyric acid was kindly provided by Merril Dow, Paris, France. δ -

Abbreviations: ALA = δ -aminolevulinate; COSY = homo- and heteronuclear 2D correlated spectroscopy; GSA = glutamate 1-semialdehyde; TLC = thin layer chromatography.

Figure 1. Synthesis of glutamate 1-semialdehyde by ozonolysis.

Aminolevulinate was from Sigma, St. Louis, Miss., USA. Thin layer plates used were Kieselgel 60 F254, Merck, Darmstadt, W. Germany. Pyrroglutamate aldehyde (glutamate 1-semialdehyde lactam) was synthesized from pyrroglutamic acid by a method analogous to that described in (7).

2.2. ^1H and ^{13}C -NMR spectra

^1H and ^{13}C spectra were recorded using a 500 MHz Bruker AM 500 spectrometer operating at 300 K in a volume of 0.5 ml D_2O in a 5 mm dual probe. Sweep widths were for ^1H spectra, 5000 Hz and for ^{13}C , 31250 Hz. Sample amounts used: purified ozonolysis synthetic product 15 mg (pH 1.2), in the COSY and heteronuclear COSY spectra 19 mg, ammonium salt of the pyrrole derivative 4.8 mg, of the δ -aminolevulinate-pyrrole derivative, 12.8 mg.

2.3. Ozonolysis

Ozone was prepared using an Ozone Generator, Fischer, Bonn, W. Germany. The rate of ozone production was measured by bubbling the ozone stream through 100 ml of 10% KI for 1 min. Five μl of the iodine solution produced was diluted to 3 ml with 10% KI and the iodine determined spectrophotometrically using a Varian 2200 spectrophotometer. Iodine produced (mmoles), equal to the O_3 (mmoles) passed, equal to double bonds oxidised (mmoles) was determined from $E_{\text{mm}352\text{nm}} = 26.2$. Typically the ozone was passed through a solu-

tion of 1 mmole (129 mg) 4-vinyl-4-aminobutyric acid in 1 ml of 1 M HCl cooled in an ice bath.

2.4. Purification of synthetic glutamate 1-semialdehyde by cation exchange chromatography

A 1×10 cm column (Biorad Econocolumn) packed with Dowex 50W-X8, was washed with 1 M HCl and then with 50 mM HCl. The ozonolysis products were passed through the column and it was then washed with 10 ml of 50 mM HCl. The GSA together with any glutamate formed by oxidation was then eluted with twenty 2 ml fractions of 0.5 M HCl (6). Under these conditions 4-vinyl-4-aminobutyric acid remains on the column but can be eluted, when present, with 10 ml of 1 M HCl. The fractions containing semialdehyde were detected by enzyme assay and by thin layer chromatography using 1 μl portions of each fraction. Active fractions were combined and freeze-dried to constant weight.

2.5. Enzymatic assays

GSA was assayed using GSA aminotransferase. The enzyme was partially purified (17) by passing soluble proteins from greening barley chloroplasts through Sephacryl S-300, Blue-Sepharose, matrix gel red A and chlorophylline-Sepharose. The fraction which does not bind to the affinity matrices contains GSA aminotransferase. It also contains activities which convert formed δ -aminolevulinate to uroporphyrinogen. For quick assay of substrate fractions during purification they were incubated for 20 minutes at 29 $^\circ\text{C}$ in 1 ml 25 mM MgCl_2 , 1 mM dithiothreitol, 0.3 M glycerol, 0.1 M Tricine, pH 7.9, cooled on ice, 20 μl of perchloric acid added and the tube allowed to stand for 5 min on an ultraviolet light source used for detection of nucleic acid in ethidium bromide-stained gels. Uroporphyrinogen formation in the samples was detected by the red fluorescence of the uroporphyrin formed by the irradiation. Enzymatic conversion of GSA to δ -aminolevulinate was studied by the addition of 5 mM levulinate to the above assay. In some cases GSA aminotransferase prepared by

preparative isoelectric focusing using the Rotaphor apparatus of Biorad was used (5).

2.6. Preparation of pyrrole derivatives of glutamate 1-semialdehyde and δ -aminolevulinate

Pyrrole derivatives were prepared of the synthetic GSA, purified by Dowex chromatography or of δ -aminolevulinate (Sigma, St. Louis, Miss., USA) used without purification. GSA (15 mg) or δ -aminolevulinate (15 mg) were dissolved in 1 ml water and the pH adjusted, respectively, to 1.0 (2) with H_3PO_4 or 4.6 (14) with H_3PO_4 and acetic acid. Acetyl acetone (0.5 ml) was added and the mixture boiled for 20 min on a water bath to form the pyrrole derivatives. The pH of the solutions was brought to 7.0 and the acetyl acetone removed by three extractions with equal volumes of chloroform. The pH was then adjusted to 4.0 with H_3PO_4 and the pyrroles extracted by three extractions with equal volumes of ether. Two drops of concentrated ammonia were added and the combined ether extracts reduced

to dryness under a stream of nitrogen. On a weight basis the yield of the pyrrole derivative of GSA, 3-carboxymethyl-2-methylpyrrole-5-propionate, was 30%. The yield of 3-carboxymethyl-2-methylpyrrole-4-propionate (the δ -aminolevulinate derivative) was 87%. The purity of the pyrroles was checked by thin layer chromatography (TLC).

2.7. Thin layer chromatography

Separation of 4-vinyl-4-aminobutyric acid, glutamic acid and glutamate 1-semialdehyde was performed on silica gel (Kieselgel 60 F254, Merck) using the solvent system butanol, acetic acid, water 60:20:20 v/v/v (6, 7). The R_f of standards in this systems were: glutamate 1-semialdehyde, prepared as described (10), 0.54, 4-vinyl-4-aminobutyric acid 0.41, glutamic acid 0.22. The detection system used was ninhydrin. For detection of the semialdehyde two cycles of ninhydrin spraying and heating were necessary. The pyrrole derivatives of GSA and δ -aminolevulinate were separated on a stationary

Figure 2. The ^1H -NMR spectrum of synthetic glutamate 1-semialdehyde in D_2O , pH 1.2.

Figure 3. The ^{13}C -NMR spectrum of synthetic glutamate 1-semialdehyde in D_2O , pH 1.2. Inset, monomeric and dimeric glutamate semialdehyde structures.

phase of commercial silica gel with solvent system (8) methyl acetate, isopropanol, 25% ammonia, 43:33:20, v/v/v. Pyrroles were detected by their characteristic absorption of 254 nm ultraviolet light and by spraying with modified Ehrlich reagent (14). The R_f of 3-carboxymethyl-2-methylpyrrole-4-propionic acid, the δ -aminolevulinate pyrrole derivative prepared from standard δ -aminolevulinate, was 0.38 in this system.

3. RESULTS

3.1. Identification of the product formed by ozonolysis as GSA

3.1.1. Chromatographic and enzymatic identification of the ozonolysis product

The Dowex column fractions of the ozonolysis products were analysed by TLC. The fractions containing the main ninhydrin reactive substance had an R_f identical to GSA. These fractions were also active as substrate for gluta-

Table I. NMR resonances assigned to structures A and B of glutamate semialdehyde. Parameters for the structures shown in Figure 2 are assigned.

^{13}C	Structures A		Structures B		^1H	Structures A		Structures B	
	δ		δ			δ		δ	
	ppm		ppm			ppm		ppm	
						J		J	
						Hz		Hz	
C 1	167.1		177.6,	177.5					
C 2	30.5		30.4,	30.3	H 2	2.61	7.5	2.62	7.5
					H 3a	2.09	7.4	2.26, 2.21	
C 3	24.0		24.0,	25.7					
					H 3b	1.92	7.4	2.14	
C 4	56.0		55.3,	55.2	H 4	3.28	6.6	3.43	
C 5	88.9		90.7,	90.7	H 5	5.19	4.8	5.24, 5.26,	2.8, 3.8,
									4.3, 4.3

Figure 4. The heteronuclear $^1\text{H}/^{13}\text{C}$ COSY spectrum of glutamate 1-semialdehyde in D_2O , pH 1.2.

mate 1-semialdehyde aminotransferase prepared by isoelectric focusing (5).

3.1.2. The ^{13}C and ^1H -NMR spectra of the synthetic product

The ^1H - and ^{13}C -NMR spectra of the GSA are shown in Figures 2 and 3 and the spectral data are summarised in Table I. The covalently linked carbon and protons were identified by heteronuclear $^1\text{H}/^{13}\text{C}$ COSY (Fig. 4), and the proton connectivities through a $^1\text{H}/^1\text{H}$ COSY

experiment (Fig. 5). The low field resonance of the carbonyl carbon is seen at δ 167.1 and it shows the characteristic low intensity typical of slowly relaxing carbons with no coupled protons. No low field resonance which can be attributed to an aldehydic proton were seen in the ^1H -NMR spectrum but the low field doublet δ 5.19 in the proton spectrum (1, 6, 12) and the resonance at δ 88.9 in the ^{13}C spectrum are attributed to a hydrated aminoaldehyde proton and carbon atom, respectively. The signal at δ 56 is assigned to the A4 carbon and resonances at

higher fields are attributed to the methylene carbons, i.e. δ 24.0 to A3 and δ 30.4 to A2. The COSY spectrum shown in Figure 5 indicates that the proton on an adjacent carbon has δ 3.28 attributed to $-\text{CHNH}_2$. This proton gives crosspeaks with signals δ 1.92 and 2.09, m, thus identifying them as $-\text{CHH}-\text{CH}_2-\text{COOH}$. Finally, the COSY spectra identify the adjacent $-\text{CH}_2-\text{CHH}-\text{COOH}$ protons as δ 2.61, t. These spectral data serve to identify the synthetic product as glutamate 1-semialdehyde. The large splitting of the signal of the $-\text{CHH}-\text{CH}_2-\text{COOH}$ protons is not seen with the homologous β protons of glutamate (6, 18) but does resemble that seen

with the β protons of proline (18). The resonances labelled A2 to A5 are attributed to the monomeric linear hydrate of GSA. GSA could also exist as a cyclic lactone or lactam as indicated by the carbon chemical shift of the signal A1 at 167 ppm (Fig. 2). However, it would be expected that two anomeric lactones should be formed but signals attributable to only one are observed. The lactam, on the contrary, would only give rise to one compound. The resonances labelled B1 to B5 and in Figures 2 and 3 as given in Table I are attributed to cyclic dimeric forms of glutamate 1-semialdehyde. The intensity of B4 in Figure 2 is about 20% of A4. The B5 and

Figure 5. The homonuclear COSY spectrum of synthetic glutamate 1-semialdehyde in D_2O , pH 1.2.

B4 signals disappear after a 4-times dilution of the glutamate 1-semialdehyde as would be expected for a concentration dependent dimer to monomer conversion. The identities of the B5 and B4 proton resonances are established in an entirely analogous way to the above. For the B5 proton δ 5.24, 5.26, the multiplet signal indicates at least four forms. The $^1\text{H}/^1\text{H}$ COSY spectrum shows that the B4, like the A4 proton, gives cross-peaks with two signals in the region where the B3 protons are expected. The carboxylic acid residues of the dimeric structure could form lactone rings with the remaining aldehyde hydroxyls, however, the ^{13}C -chemical shifts are not in favour of such an interpretation. The resonances at δ 4.0 and 2.2 (labelled G in Figure 2), are due to a small amount of glutamate as was shown by the addition of glutamate to the sample and the increase of signal intensity of these resonances. The intensity of the δ 2.2 signal which is attributed to CHH-COOH of glutamate (6) is 8% of that of the two signals attributed to the homologous protons of glutamate 1-semialdehyde.

3.2. Chromatographic parameters for the acetyl acetone pyrrole of GSA

The reaction mechanism for the formation of the pyrrole derivatives of GSA and aminolevulinic acid is given in Figure 6. Thin layer chromatography of the pyrrole prepared from GSA showed one ultraviolet absorbing spot with R_f 0.46. When mixed with the δ -aminolevulinic acid-pyrrole two distinct spots could be seen, the δ -aminolevulinic acid-pyrrole having the lower R_f .

Figure 6. Formation of acetyl acetone pyrroles of glutamate 1-semialdehyde and δ -aminolevulinate.

On spraying with Ehrlich reagent the ultraviolet absorbing spot of the GSA pyrrole developed a weak pink colour, whereas the δ -aminolevulinate-pyrrole became strongly purple in colour. The GSA-pyrrole also contained a small amount of an Ehrlich positive substance having the same R_f as δ -aminolevulinate-pyrrole. The δ -aminolevulinate-pyrrole contained a small amount of substance giving an orange colour with Ehrlich reagent and with the R_f of the GSA-pyrrole.

3.2.1. The ^{13}C and ^1NMR spectra of the acetyl acetone pyrrole derivatives of GSA and ALA

In Table II is shown the NMR spectral data of the 3-carboxymethyl-2-methyl pyrrole propionates: The GSA- and the δ -aminolevulinate-pyrrole derivatives. The numbering system is indicated in Figure 6. The methyl protons at position 10 δ 2.41 and at position 11 δ 2.44 of GSA-pyrrole give the most intense signals. If they are attributed to three protons the following other assignments can be made. δ 2.48, t to two protons at position 7, δ 2.78, t to protons at position 6. In the ALA-pyrrole the signal δ 2.88 can be assigned to the methylene protons of position 6 because the ring current is expected to down field shift the signal and more so if the interaction occurs with position 4 than with position 5. The other assignments for the ALA-pyrrole can then be made as follows. δ 2.44, 2.43, s with unresolved shoulder are methyl protons at positions 11 and 10 and methylene protons at position 7. Characteristic low field resonances of the α -proton (position 5) of δ -aminolevulinate-pyrrole (δ 6.51, s) and of the β -proton (position 4) of GSA-pyrrole (δ 6.33, s) are seen (9). As expected (9) the α proton resonance in δ -aminolevulinate-pyrrole is shifted to a lower field relative to the β -proton of GSA-pyrrole. In the ^{13}C spectra of the GSA pyrrole a weak signal is seen for the slowly relaxing C5 carbon at δ 132.5 and an intense signal for the protonated C4 at δ 107.1. In δ -aminolevulinate-pyrrole, as expected, this situation is reversed. The resonance of the carbon at position 5 (δ 116.0) which has an attached proton, is intense while that at C4 (δ 125.7) is weak.

Table II. NMR resonances assigned to 3-carboxymethyl-2-methyl-5 and 4-propionate pyrrole derivatives of GSA and ALA, respectively.

¹³ C	ALA-pyrrole		GSA-pyrrole	¹ H	ALA-pyrrole		GSA-pyrrole	
	δ				δ	J	δ	J
	ppm		ppm		ppm	Hz	ppm	Hz
C 2	139.4		138.1 ^a					
C 3	120.0		120.6					
C 4	125.7		107.1	H 4			6.33	
C 5	116.0		132.5 ^a	H 5	6.51			
C 6	24.7		24.2	H 6	2.88	7.4	2.78	7.6
C 7	38.4		37.7	H 7	2.43		2.48	7.6
C 8	183.7		182.9					
C 9	200.8		200.5					
C 10	30.6		28.4	H 10	2.43 ^b		2.41 ^b	
C 11	15.2		14.0	H 11	2.44 ^b		2.44 ^b	

a and b) The assignments may be reverse

3.3. The effect of different ozonolysis conditions

In order to determine the optimum conditions of ozonolysis an empirical study of the effect of different times of ozonization on the yield and purity of glutamate 1-semialdehyde was performed (Fig. 7). In this experiment the rate of ozone production was measured as 0.33 mmole per minute. The amount of 4-vinyl-4-aminobutyrate used was 1 mmole. Figure 7 shows that the yield of semialdehyde increased with time up to 8 minutes. After eight minutes the yield was 66%. Examination of the fractions by TLC revealed that in the enzymatically active

chromatography fractions, amounts of ninhydrin positive products with R_f values lower and higher than GSA increased with time. No quantitative determination was made of the amounts of these substances. A sample ozonised with 4× excess of ozone showed ninhydrin positive spots with the R_f of glutamic acid indicating that oxidation of the aldehyde formed could occur with longer periods of ozonization. Total yield of purified GSA was only 51% in this case. A sample ozonolysed with 0.63 mmoles of ozone, at a rate of ozone production of 0.19 mmoles per minute, gave 0.63 mmoles of semialdehyde. Preparation of semialdehyde by this method is therefore optimal for rates of ozone formation not exceeding 0.19 mmoles per minute.

Figure 7. Yield of glutamate 1-semialdehyde versus time of ozonolysis.

4. DISCUSSION

The starting material 4-vinyl-4-aminobutyric acid, used for the synthesis of GSA has been synthesised from 4-acetylenic-4-aminobutyric acid (13) which in turn has been prepared by Michael reaction of a protected propargylamine cation with methyl acrylate (15). The method for GSA synthesis presented here represents an extremely simple and reproducible method. The product is potent as an enzyme substrate. Typically 2 mg of the GSA hydrochloride, dissolved in 5 ml can be used for 500 routine 1 ml

assays of GSA aminotransferase. This solution can be frozen for months without loss of activity. Potentially unlimited amounts of GSA are now available by using this method and so it has been possible to present natural abundance ^{13}C NMR spectra. Glutamate 1-semialdehyde could conceivably associate to give a cyclic lactone or lactam in solution. The lactam is not considered to be present since synthetically prepared lactam is not active as an enzyme substrate until it has been boiled for 2 h in 2 M HCl. At concentrations of 200 mM, 15% of the glutamate 1-semialdehyde is present as aminoacetal dimers. As shown in Figure 8, this could explain how the previously observed (6) chemical conversion of GSA to δ -aminolevulinate takes place. The latter was shown to be a non-linear function of GSA concentration which would be expected if the formation of δ -aminolevulinate takes place with a dimeric form of GSA as an intermediate. It is suggested that the aminohemiacetal I, (Fig. 8), loses water to give a double Schiff base II. This imine is tautomeric with the less stable

eneamine forms III and IV. The formation of the important tautomer III may be expected to be increased at high pH since protonation of the secondary ring nitrogen of III and of the propionate residues might inhibit the formation of III and IV. The chemical formation of GSA from δ -aminolevulinate has been observed to be stimulated at pH 8.2 relative to 6.8 (6). The hydrolysed form of V is the aminohemiacetal of δ -aminolevulinate. The proposed mechanism is similar to the Amadori rearrangements observed for sugars when treated with amines. It is conceivable that GSA aminotransferase catalysed conversion of GSA to δ -aminolevulinate takes place by a similar process of dimerisation and exchange of amino-groups between two molecules of glutamate 1-semialdehyde. In this case a cofactor such as pyridoxamine phosphate may not be necessary. In agreement with this suggestion spectral examination of active barley glutamate 1-semialdehyde aminotransferase has failed to show substrate catalysed absorption changes (5) typical of pyridoxamine phosphate

Figure 8. Suggested reaction scheme for chemical conversion of glutamate 1-semialdehyde to δ -aminolevulinate.

enzymes (4). The substrate dependent inhibition observed with gabaculin (6) can be ascribed to formation of a covalently linked substrate inhibitor molecule on the enzyme. In contrast to the dimeric barley enzyme, purified glutamate 1-semialdehyde aminotransferase from the cyanobacterium *Synechococcus* is a monomer and requires pyridoxamine phosphate for activity (3). This intimates different catalytic mechanisms for glutamate 1-semialdehyde aminotransferases of different organisms.

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