Determination of Amino Acid Hydantoins by HPLC with Diode Array Detection†

Zhixian Huang and Cornelius S. Ough*

Department of Viticulture and Enology, University of California, Davis, California 95616

Eighteen amino acid hydantoins (AAHs) were synthesized from corresponding amino acids and potassium cyanate. The purity and structure of each hydantoin were confirmed by melting point measurement and by proton nuclear magnetic resonance spectroscopy (NMR). Some of the AAHs were also checked by ^{13}C NMR and by mass spectroscopy. AAHs were separated by reversed-phase HPLC and detected by a diode array detector at 225 nm. All AAHs tested were separated within 15 min with detection limits of 50 pmol. The stability of 12 AAHs was tested, and results indicated that most AAHs were stable in solutions during storage over a period of 6 months. This method was applied for peptide N-terminal determination. Examples of N-terminal amino acid determination of four peptides are shown.

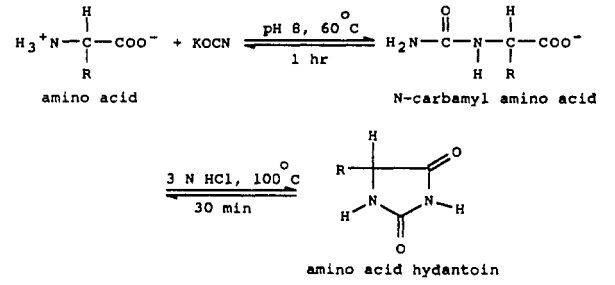
INTRODUCTION

Potassium cyanate (KOCN) reacts with the amino groups of amino acids, peptides, and proteins in a slightly basic solution, and the corresponding amino acid hydantoin is formed by subsequent acidification (Scheme I).

Amino acid hydantoins have been studied for their dissociation constants (Pickett and McLean, 1939), optical rotatory dispersion, circular dichroism (Suziki et al., 1973b), and mass spectra (Suzuki and Tuzimura, 1976). Hydantoins were also used for identification of carbonyl compounds through their conversion into hydantoins (Henze and Speer, 1942). At about the same time Edman first published the phenylthiohydantoin (PTH) amino acid method and reported a protein sequenator for the determination of the amino acid sequence in peptides and proteins (Edman, 1950; Edman and Berg, 1967), Stark and Smith (1963) reported the use of amino acid hydantoins in the determination of N-terminal amino acid groups of peptides or proteins. Stark identified the liberated AAHs by hydrolyzing them into the corresponding amino acids, which were detected with ninhydrin. His method had a total of six steps, which took more than 48 h for one analysis. Ultraviolet (UV) absorption has been used for the direct identification of AAHs in anion-exchange chromatography (Hagel and Gerding, 1969). The direct method involved fewer steps and avoided loss of AAHs during their hydrolysis to amino acids. However, classical anion-exchange chromatography took up to 30 h for each analysis. A thin-layer chromatography (TLC) method was developed by Suzuki et al. (1973a) for the detection of AAHs with *tert*-butyl hypochlorite as color reagent. This method was simple and sensitive but not quantitative.

In this work attempts were made to develop a method for direct determination of AAHs with sensitivity and short analysis time. Since amino acid hydantoins have strong UV absorbance at 225 nm and fairly low polarity, a reversed-phase HPLC method was developed. This method was applied to the determination of N-terminal amino acids in several peptides. Another use of this HPLC method was for identification of *N*-carbamyl amino acids (NCAAs) in wines, after NCAAs were converted into their corresponding AAHs. NCAAs are biologically important

Scheme I. Reactions for the Synthesis of Amino Acid Hydantoins



(Stryer, 1988) during yeast fermentation. The results for NCAAs are to be discussed in another paper. Experiments were also conducted to test the stability of 12 AAHs during storage.

EXPERIMENTAL PROCEDURES

Synthesis of Amino Acid Hydantoins. AAHs were prepared according to the modified methods of Hagel and Gerding (1969) and Suzuki et al. (1973b). A representative procedure is described here for the synthesis of hydantoins from alanine, phenylalanine, aspartic acid, glutamic acid, proline, serine, threonine, lysine, methionine, histidine, and cysteine. L-Alanine (4.45 g, 0.05 mol) and potassium cyanate (4.9 g, 0.06 mol) were dissolved in 30 mL of water. The solution was heated for 1 h at 60 °C to form *N*-carbamyl amino acid. The reaction mixture was refluxed for 30 min with 30 mL of hydrochloric acid (6 N). Then it was evaporated to about 40 mL over a hot plate. The solution was left overnight in a refrigerator, and the precipitated alanine hydantoin (Ala-H) was collected. Ala-H was recrystallized twice from hot water and then dried at 100 °C in an oven over the weekend. The white crystalline product (4.68 g) was collected and stored in a desiccator. The overall yield was 82.0% for Ala-H.

A somewhat different procedure was used for the synthesis of AAHs from neutral amino acids such as leucine, valine, isoleucine, tyrosine, tryptophan, and norleucine. Pyridine (about 25 mL) was added to help dissolve amino acid during carbamylation. Pyridine was then removed by extractions with ethyl acetate (300 mL in three portions). During acidification, glacial acetic acid (30 mL) was added along with hydrochloric acid (30 mL, 6 N). Acetic acid helped to dissolve *N*-carbamyl amino acids. For the reaction of L-leucine (6.56 g, 0.05 mol) and potassium cyanate (4.9 g, 0.06 mol), Leu-H was obtained as a crystalline product after recrystallization from hot ethanol–water and then hot ethanol. The dried Leu-H was 5.5 g with a yield of 70.3%.

Norleucine hydantoin (Nle-H) was synthesized as a potential internal standard for HPLC analysis. Glycine hydantoin was purchased from Sigma Chemical Co. (St. Louis, MO). Arginine

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hydantoin was synthesized according to the method of Boon and Robson (1935), but no pure form was obtained. Asparagine and glutamine hydantoins were not prepared because they would be hydrolyzed into Asp-H and Glu-H, respectively, during acidification. The purity and structure of all of the synthesized AAHs were checked by their melting points and by ^{1}H NMR. Some of them were also checked by ^{13}C NMR and mass spectroscopy (MS).

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR was carried out on a Nicolet NT-360 360-MHz spectrometer. Samples (about 20 mg) were dissolved in dimethyl-d_6 sulfoxide (DMSO-d_6, 0.5 mL, 99.9% atom D, Sigma). A small amount of dimethyl-d_5 sulfoxide (DMSO-d_5) in the solvent was used as the internal standard for chemical shift calibration at 2.49 ppm. ^{13}C NMR was run on a GE 300-MHz spectrometer (Model Omega 300). Samples (about 80 mg) were dissolved in 2.0 mL of deuterium oxide (D_2O) or DMSO-d_6. Solvent peak on ^{13}C NMR for samples in DMSO-d_6 appeared at 39.5 ppm.

Mass Spectroscopy. Electron impact (EI) mass spectra were obtained on a VG Masslab automated mass spectrometer in the Facility for Advanced Instrumentation at the University of California, Davis. Samples were introduced as solid by a probe. The probe temperature was set at 30 °C for 1 min, raised to 400 °C at a rate of 300 °C/min, and then held for 5 min.

High-Performance Liquid Chromatography. A Hewlett-Packard (Santa Clara, CA) 1090M HPLC apparatus with a diode array detector was used for analysis of the amino acid hydantoins. It was connected to a Hewlett-Packard 9000 Series 300 Chem-Station for data processing. An ODS Hypersil megabore column (200 × 2.1 mm, 5-μm particle size, Hewlett-Packard) was used for the separations. Column oven temperature was kept at 42 °C for all separations. Detection was carried out at 225 nm, and scanning range was from 200 to 400 nm. The detection range was set at 0.10 absorbance unit (AU). The mobile phase was filtered through 0.45-μm membrane filters (Millipore, Bedford, MA) and degassed with helium. Solvent A was 50 mM ammonium phosphate, pH adjusted to 3.0 with 85% phosphoric acid. Solvent B was 20% A in acetonitrile. Ammonium phosphate, phosphoric acid, and acetonitrile were of HPLC grade from Fischer Scientific (Fair Lawn, NJ). The water was of HPLC grade (18-Mohm resistivity) which was purified by a Milli-Q water system (Millipore). The gradient was 100–96% A from 0 to 4 min, 96–60% A from 4 to 12 min, and 60–0% A from 12 to 15 min. AAHs were eluted off within 15 min. The column was then washed with 100% solvent B and equilibrated with solvent A before the next sample was injected. The flow rate was 0.40 mL/min and injection volume 5 μL. Calibration curves were obtained by injecting 5 μL each of the standard AAH mixtures containing different concentrations of AAHs ranging from 0.02 to 0.60 mM. The calibration curves were repeated periodically to ensure satisfactory quantitation.

Stability Test of AAHs during Storage. Twelve AAHs were dissolved in 10% acetonitrile/water solutions for the stability tests during storage. Two starting concentrations of 0.50 and 0.20 mM for each AAH were tested. The solutions of the AAHs were kept in tightly sealed flasks and stored in a refrigerator (about 0–4 °C) and at room temperature (about 25 °C). Six months later, HPLC analyses were run in duplicate for each sample to determine the amounts of the AAHs remaining after storage.

Determination of Peptide N-Terminal Amino Acid. Peptides were treated by potassium cyanate under conditions similar to those for the synthesis of AAHs. A typical procedure was as follows (the scale was about 5 μmol, with Phe-Gly-Gly-Phe as an example): Peptide (3.7 mg) and KOCN (4.4 mg) were dissolved in 1 mL of water in a thick-wall reaction vial with Teflon-lined screw cap. Carbamylation was allowed to take place for 2 h at 50 °C. Hydrochloric acid (1 mL; 6 N) was added to the same vial, and acidification was allowed to proceed for 1 h at 105 °C in an oven. The reaction mixture was dried under a nitrogen stream at 50 °C and then redissolved in 2.0 mL of 10% acetonitrile/water solution. The solution was further diluted with water to have AAH concentrations around 0.4 mM and filtered through a 0.45-μm nylon membrane filter disk (Millipore) for HPLC analysis.

Table I. Melting Points of Amino Acid Hydantoins

name	mp, °C exptl	mp, °C lit.
Asp-H	210–212	210–213
Glu-H	175–177	175–176
Ser-H	186–187	187–188
Gly-H	221–223	223–225
Thr-H	193–195	193–196
Ala-H	167–168	174–177
Lys-H	184–185	193
Pro-H	155–157	155–157
Val-H	143	143–145
Met-H	112–114	104
Tyr-H	259–261	262
Ile-H	135–136	150–151; 125–126
Leu-H	213	212–214
Nle-H	137–139	
Phe-H	185–186	181–183
Trp-H	238–240	244
His-H	255 (decomp)	235
Cys-H	139–140	

RESULTS AND DISCUSSION

The synthesized amino acid hydantoins were in the form of white crystalline powder. Their melting points (Table I) agreed well with most of the reported values (Suzuki et al., 1973a; Hagel and Gerding, 1969). Melting points differed between experimental results and literature values for Ala-H, Lys-H, Met-H, Ile-H, and His-H. No literature report of melting points was located for Nle-H and Cys-H. Therefore, those seven hydantoins were further checked by ^{13}C NMR and MS in addition to ^{1}H NMR.

The ^{1}H NMR spectral data of the synthesized amino acid hydantoins are listed in Table II. Most of the data were quite interpretable. A typical spectrum would have two singlets for protons of imino groups at N_1 and N_3, which appeared around 7.9 and 10.5 ppm, respectively. Occasionally, the moisture of water also appeared as a small peak at about 3.50 ppm. The NMR spectra indicated the correct structures for AAHs and basically no impurities in the samples. However, there were a couple of exceptions. There was a small singlet at 5.85 ppm on the spectrum of Lys-H which could not be assigned. It was labeled an unknown in the table. This was possibly due to the reaction of the additional amino group on lysine with potassium cyanate. In the case of Thr-H, a certain extent of dehydration proceeded simultaneously, as reported by Suzuki et al. (1973b). The dehydrated Thr-H contributed a doublet (J = 7 Hz) at 1.78 ppm and a very small quartet at 5.55 ppm on the spectrum. This small amount of dehydrated Thr-H also showed up in the HPLC chromatograms, which will be discussed later. It is interesting to note that no dehydration was observed in the case of serine hydantoin, which also possessed a hydroxyl group. One of the possible explanations is that the primary hydroxyl group on serine is less liable to be dehydrated than the secondary hydroxyl group on threonine.

The ^{13}C NMR spectra data (Table III) showed two peaks for each of those seven AAHs around 160 and 180 ppm, representing C_2 and C_4 on the hydantoin ring, respectively. Those two peaks have also been shown by a reference ^{13}C NMR spectrum for Val-H (Sadtler Carbon-13 NMR 2303C, 1977). The remaining peaks were readily assigned for each individual carbon. The ^{13}C NMR spectral data indicated correct structures and good purity for those AAHs checked, although a bit higher baseline noise was observed on the spectrum of Lys-H.

Electron impact mass spectral data are given for those seven AAHs in Table IV. A fragmentation process of hydantoin (Scheme II) has been proposed (Suzuki and Tu-

Table II. ^{1}H NMR Spectral Data of the Synthesized Amino Acid Hydantoins

name	^{1}H NMR spectral data
Asp-H	2.15 ppm (d, 2 H, J = 5.7 Hz, HOOCC**H**$_2$CH), 3.70 (t, 1 H, J = 4.5 Hz, CH$_2$C**H**CO), 7.33 (s, 1 H, CHN**H**CO), 10.1 (s, 1 H, CON**H**CO), 11.9 (s, 1 H, **COOH**)
Glu-H	1.70 and 1.90 (dt, 2 H, CH$_2$C**H**$_2$CH), 2.30 (t, 2 H, J = 5.0 Hz, HOOCC**H**$_2$CH$_2$), 4.00 (t, 1 H, CH$_2$C**H**CO), 7.95 (s, 1 H, CHN**H**CO), 10.6 (s, 1 H, CON**H**CO), 12.2 (s, 1 H, **COOH**)
Ser-H	3.58 (dd, 2 H, J = 6.0 Hz, HOC**H**$_2$), 3.98 (t, 1 H, CH$_2$C**H**CO), 4.85 (s, 1 H, OH), 7.78 (s, 1 H, CHN**H**CO), 10.5 (s, 1 H, CON**H**CO)
Thr-H	1.14 (d, 3 H, J = 7.0, **CH$_3$**CHOH), 1.78 (d, J = 7.0 Hz, **CH$_3$**CH=C), 3.82 (d, 1 H, CHC**H**CO), 3.90 (m, 1 H, CH$_3$C**H**OH), 4.90 (s, 1 H, **OH**), 5.55 (q, J = 7.0 Hz, CH$_3$C**H**=C), 7.84 (s, 1 H, CHN**H**CO), 10.5 (s, 1 H, CON**H**CO)
Ala-H	1.20 (d, 3 H, J = 7.0 Hz, **CH$_3$**CH), 4.00 (q, 1 H, J = 7.0 Hz, CH$_3$C**H**), 7.90 (s, 1 H, CHN**H**CO), 10.6 (s, 1 H, CON**H**CO)
Lys-H	1.25 (m, 4 H, H$_2$NCH$_2$C**H**$_2$C**H**$_2$), 1.50–1.68 (m, 2 H, C**H**$_2$CHCO), 2.95 (m, 2 H, H$_2$NC**H**$_2$CH$_2$), 4.00 (t, 1 H, J = 3.0 Hz, CH$_2$C**H**CO), 5.36 (s, 2 H, NH$_2$), 5.85 (s, unknown), 7.90 (s, 1 H, CHN**H**CO), 10.6 (s, 1 H, CON**H**CO)
Pro-H	1.60–2.03 (m, 4 H, C**H**$_2$C**H**$_2$CH), 3.00 and 3.42 (q, 2 H, CH$_2$C**H**$_2$N), 4.10 (t, 1 H, J = 9.0 Hz, CH$_2$C**H**CO), 10.7 (s, 1 H, CON**H**CO)
Val-H	0.78 and 0.92 (d, 6 H, J = 7.0 Hz, **CH$_3$** × 2), 1.98 (m, 1 H, J = 7.0 Hz, CH$_3$C**H**CH$_2$), 3.90 (d, 1 H, J = 3.0 Hz, CHC**H**CO), 7.92 (s, 1 H, CHN**H**CO), 10.6 (s, 1 H, CON**H**CO)
Met-H	1.75 (m, 2 H, J = 7.0 Hz, SCH$_2$C**H**$_2$), 1.92 (t, 2 H, CH$_3$SC**H**$_2$), 2.04 (s, 3 H, **CH$_3$**), 4.08 (t, 1 H, J = 7.0 Hz, CH$_2$C**H**CO), 8.0 (s, 1 H, CHN**H**CO), 10.7 (s, 1 H, CON**H**CO)
Tyr-H	2.84 (d, 2 H, C**H**$_2$CHCO), 4.24 (d, 1 H, CH$_2$C**H**CO), 6.65 and 6.98 (d, 4 H, J = 8.0 Hz, C$_6$**H**$_4$), 7.84 (s, 1 H, CHN**H**CO), 9.22 (s, 1 H, **H**OC$_6$H$_4$), 10.4 (s, 1 H, CON**H**CO)
Ile-H	0.84 (t, 3 H, J = 6.3 Hz, **CH$_3$**CH$_2$), 0.90 (d, 3 H, J = 6.5 Hz, **CH$_3$**CH), 1.16–1.32 (m, 2 H, CH$_3$C**H**$_2$CH), 1.72 (m, 1 H, CH$_3$C**H**), 3.94 (d, 1 H, J = 2 Hz, CHC**H**CO), 7.88 (s, 1 H, CHN**H**CO), 10.6 (s, 1 H, CON**H**CO)
Leu-H	0.86 (dd, 6 H, J = 6.5 Hz, **CH$_3$** × 2), 1.38–1.46 (m, 2 H, C**H**$_2$CHCO), 1.75 (m, 1 H, CH$_3$C**H**CH$_3$), 4.0 (dd, 1 H, CH$_2$C**H**CO), 8.0 (s, 1 H, CHN**H**CO), 10.6 (s, 1 H, CON**H**CO)
Nle-H	0.88 (t, 3 H, J = 6.0 Hz, **CH$_3$**), 1.30 (m, 4 H, CH$_3$C**H**$_2$C**H**$_2$), 1.50 and 1.65 (m, 2 H, CH$_2$C**H**$_2$CHCO), 3.98 (t, 1 H, J = 3.0 Hz, CH$_2$C**H**CO), 7.86 (s, 1 H, CHN**H**CO), 10.5 (s, 1 H, CON**H**CO)
Phe-H	2.85 (t, 2 H, J = 3.0 Hz, C$_6$H$_5$C**H**$_2$CH), 4.34 (t, 1 H, J = 3.0 Hz, CH$_2$C**H**CO), 7.18–7.32 (m, 5 H, C$_6$**H**$_5$), 7.95 (s, 1 H, CHN**H**CO), 10.4 (s, 1 H, CON**H**CO)
Trp-H	3.06 (d, 2 H, J = 2.0 Hz, C**H**$_2$CHCO), 4.30 (t, 1 H, J = 2.0 Hz, CH$_2$C**H**CO), 6.95 and 7.05 (t, 2 H, J = 9.0 Hz, C$_6$**H**$_4$), 7.12 (s, 1 H, HNC**H**=C), 7.32 and 7.56 (d, 2 H, J = 9.0 Hz, C$_6$**H**$_4$), 7.85 (s, 1 H, CHN**H**CO), 10.4 (s, 1 H, CON**H**CO), 10.9 (s, 1 H, C=CN**H**C=C)
His-H	3.05 (dd, 2 H, J = 3.0 Hz, C**H**$_2$CHCO), 4.40 (t, 1 H, J = 3.0 Hz, CH$_2$C**H**CO), 7.40 (s, 1 H, =C**H**N=), 8.02 (s, 1 H, CHN**H**CO), 9.05 (s, 1 H, NHC**H**=N), 10.7 (CON**H**CO), 14.6 (s, 1 H, =CHN**H**C=)
Cys-H	2.76 (t, 2 H, **H**SCH$_2$CH), 3.26 (d, 1 H, HSC**H**$_2$), 4.27 (t, 1 H, CH$_2$C**H**CO), 7.85 (s, 1 H, CHN**H**CO), 10.7 (s, 1 H, CON**H**CO)

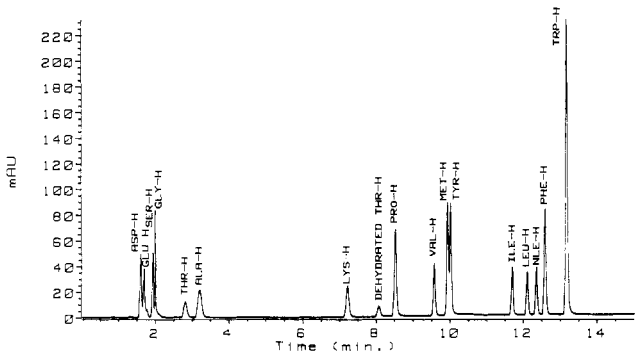


Figure 1. HPLC chromatogram of 16 amino acid hydantoins separated by a megabore C_{18} column using mobile phase A, 50 mM ammonium phosphate, pH 3.0, and mobile phase B, 80% acetonitrile in mobile phase A. Injection volume was 5.0 μL. Detection was at 225 nm. The gradient used was described under Experimental Procedures.

zimura, 1976). Ions of (M − 28) and (M − 43) were expected during ring breakage. A predominant peak at m/z 100 and molecular ion were observed for some of the AAHs.

Originally, 16 amino acid hydantoins were separated by the megabore reversed-phase column on HPLC. All 16 AAHs resolved within 15 min, with 10 of them having baseline separations (Figure 1). Peak 8 at retention time 8.15 min was the small amount of dehydrated Thr-H. The dehydrated Thr-H was not separated from Thr-H during recrystallizations. Therefore, it showed up on the HPLC chromatogram. However, this peak would not interfere with quantitation of Thr-H, presuming similar rate of dehydration occurred for samples under the same acidification conditions.

It was found during the chromatographic optimization that AAHs resolved best on the megabore C_{18} column with an injection volume of 5 μL when concentrations of AAHs ranged from 0.1 to 0.6 mM each for those 14 AAHs and from 0.02 to 0.12 mM each for Tyr-H and Trp-H. This is shown by a three-dimensional plot of HPLC chromatograms (Figure 2). It can be seen that resolutions were quite satisfactory at all four concentration levels. Peak names, retention times, calibration formulas, and correlation coefficients are given for those 16 AAHs (Table V). Correlation coefficients indicated very good linearity for all calibration curves. The detectable peak area for this HPLC method was Y = 5 for each AAH. This number corresponded to an average of about 10 pmol/μL (0.01 mM) for 11 AAHs, including Asp-H, Glu-H, Ser-H, Gly-H, Thr-H, Ala-H, Lys-H, Val-H, Ile-H, Leu-H, and Nle-H. Since the injection volume was 5 μL, the detectable amounts for these AAHs were about 50 pmol. Detection limits were around 25 pmol for Pro-H, Met-H, and Phe-H. Detection limits were 5 and 1 pmol for Tyr-H and Trp-H, respectively. The results also showed that this HPLC method had high precision and good accuracy, which were indicated by low relative standard deviations (an average of 1.55%, from a total of 44 data points) and small relative errors (an average of 2.0%, from 46 data points).

In the later stage of this work, His-H and Cys-H were synthesized and characterized by spectroscopy and then separated by HPLC. Both His-H and Cys-H eluted off early (Figure 3). The separation of His-H was satisfactory but not that of Cys-H. Cys-H basically coeluted with Ala-H under the same chromatographic conditions. The separation of His-H and Cys-H from the other AAHs could be improved possibly by fine-tuning the gradient and/or modifying the buffers.

The concentrations of AAHs found after storage and corresponding recoveries in percent for those 12 AAHs tested are listed in Table VI. Asp-H lost about 40% at both cold and ambient temperatures over 6 months. Glu-H decreased about 10% during the storage. It is interesting to note that Thr-H was recovered 94.3% in the cold and 84.8% at ambient temperature. The differences in recoveries indicated that the stability of Thr-H in solutions was probably more temperature dependent. There was about 5% loss for Ala-H and Pro-H when they were stored

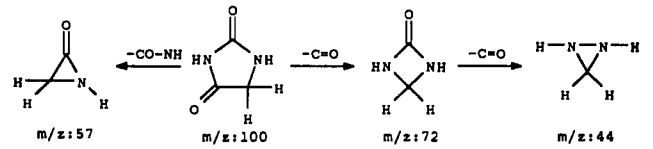
Table III. ^{13}C NMR Spectral Data for Some of the Synthesized Amino Acid Hydantoins

name	chemical shift, ppm (assignment)
Ala-H	17 (CH_3), 55 (CH_3CHCO), 160 (NHCONH), 180 (CHCONH)
Nle-H	14 (CH_3), 22 (CH_2CH_2), 26.5 ($CH_3CH_2CH_2$), 31.5 (CH_2CHCO), 58 (CH_2CHCO), 158 (NHCONH), 176 (CHCONH)
Ile-H	12 (CH_2CH_2), 14.8 (CH_3CH), 23.6 (CH_3CH_2), 36.8 (CH_3CH), 62.6 (CHCHCO), 158 (NHCONH), 176 (CHCONH)
Lys-H	22 ($NH_2CH_2CH_2CH_2$), 29 (CH_2CHCO), 31 ($NH_2CH_2CH_2$), 40 (NH_2CH_2), 59 (CH_2CHCO), 161 (NHCONH), 179 (CHCONH)
His-H	27 (CH_2), 59 (CH_2CHCO), 118 ($CH_2C{=}C$), 128 ($CH_2C{=}C$), 135 ($HNC{=}N$), 160 (NHCONH), 177 (CHCONH)
Met-H	15 (CH_3SCH_2), 29 (CH_2CHCO), 32 (CH_3SCH_2), 57 (CH_2CHCO), 158 (NHCONH), 176 (CHCONH)
Cys-H	25 ($HSCH_2$), 62 (CH_2CHCO), 161 (NHCONH), 177 (CHCONH)

Table IV. Characteristic MS Fragment Ions of Some of the Synthesized Amino Acid Hydantoins

name	MW	fragment ions, m/z (rel abundance, %)
Ala-H	114.09	43.37 (100), 114.02 (82.5), 42.36 (79.0), 86.12 (62.16), 44.36 (27.2), 71.36 (18.8)
Nle-H	156.17	1.00.40 (100), 43.47 (25.7), 41.47 (10.4), 113.34 (10.1), 156.5 (1.7)
Ile-H	156.17	100.19 (100), 57.49 (16.8), 70.42 (3.7), 82.28 (2.31)
Lys-H	171.19	43.38 (100), 55.42 (52.5), 83.48 (33.5), 113.35 (31.9), 125.39 (13.1), 154.49 (14.7), 171.42 (3.0)
His-H	180.16	81.11 (100), 82.46 (37.1), 180.23 (18.8), 54.39 (19.73)
Met-H	174.21	100.21 (100), 75.42 (33.8), 174.44 (20.6), 62.46 (22.25), 113.18 (18.3), 61.43 (18.3)
Cys-H	145.15	100.02 (100), 112.21 (3.93), 146.06 (2.7)

Scheme II. Fragmentation Process of Hydantoin



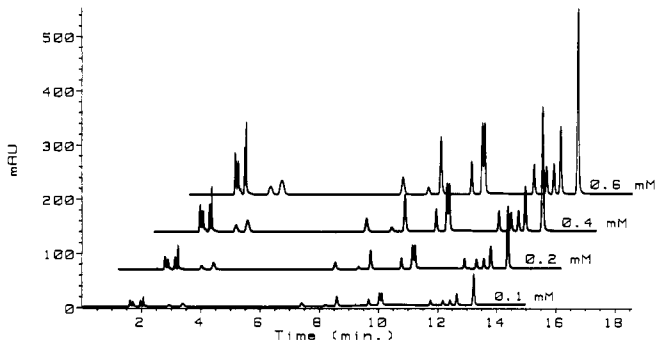


Figure 2. Three-dimensional plot of HPLC chromatograms of AAHs at different levels. Concentrations of each AAH are shown on the graph, except that of Tyr-H and Trp-H. Tyr-H and Trp-H were one-fifth as high in concentration as the other AAHs. They were 0.02, 0.04, 0.08, and 0.12 mM from low to high, accordingly. The HPLC conditions were given in Figure 1.

Table V. Calibration Data of Amino Acid Hydantoins

peak	Rt, min	peak name	calibration formula[a]	R^2 [b]
1	1.602	Asp-H	$Y = 383X + 0.063$	1.000
2	1.699	Glu-H	$Y = 307X + 4.13$	0.988
3	1.945	Ser-H	$Y = 313X + 2.45$	0.999
4	2.039	Gly-H	$Y = 402X - 0.626$	0.999
5	2.836	Thr-H	$Y = 210X + 0.941$	0.998
6	3.242	Ala-H	$Y = 405X + 0.669$	0.999
7	7.348	Lys-H	$Y = 351X - 0.858$	1.000
8	8.547	Pro-H	$Y = 766X - 0.923$	1.000
9	9.591	Val-H	$Y = 382X - 0.530$	1.000
10	9.966	Met-H	$Y = 776X - 2.27$	1.000
11	10.05	Tyr-H	$Y = 3729X - 1.82$	1.000
12	11.71	Ile-H	$Y = 353X + 0.282$	1.000
13	12.13	Leu-H	$Y = 324X + 0.320$	1.000
14	12.38	Nle-H	$Y = 357X - 0.035$	1.000
15	12.61	Phe-H	$Y = 818X - 1.06$	1.000
16	13.19	Trp-H	$Y = 11010X + 2.26$	1.000

[a] In the calibration formula, Y is the peak area and X is the concentration of AAH (millimolar). [b] R^2 is the correlation coefficient.

in solutions at room temperature. For the remaining AAHs tested, there was basically no loss during the storage at 0–4 °C. Overall results indicated that the majority of the AAHs tested were quite stable during storage at cold temperature. These included Ser-H, Gly-H, Thr-H, Ala-H, Pro-H, Val-H, Ile-H, Leu-H, Nle-H, and Phe-H. This is advantageous over phenylthiohydantoin (PTH) amino acids, which are not so stable even when stored at −20 °C, especially when in contact with oxygen (Zimmerman et al., 1977; Simpson et al., 1989). Asp-H was the single hydantoin tested that decreased drastically during storage in solutions.

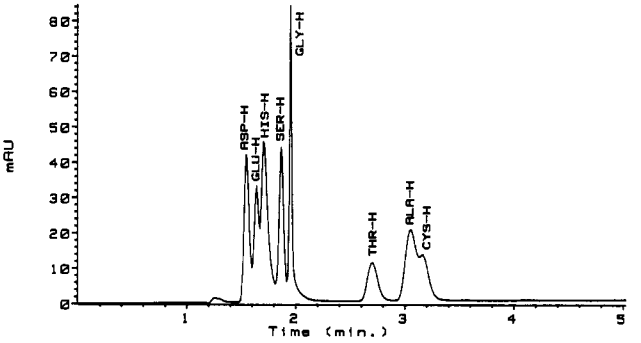


Figure 3. HPLC chromatogram showing the elution of His-H and Cys-H along with their neighboring AAHs. The HPLC conditions were the same as those given in Figure 1.

Table VI. Stability Test of AAHs during Storage

		0–4 °C, 6 months		ambient, 6 months	
name	start concn, mM	found concn, mM	recovery, %	found concn, mM	recovery, %
Asp-H	0.50	0.293	58.6	0.337	67.4
	0.20	0.119	59.5	0.109	54.5
Glu-H	0.50	0.449	89.8	0.459	91.8
	0.20	0.188	94.0	0.174	87.0
Ser-H	0.50	0.516	103	0.514	102
	0.20	0.205	102	0.188	94.0
Gly-H	0.50	0.544	108	0.531	106
	0.20	0.223	110		
Thr-H	0.50	0.468	93.6	0.431	86.2
	0.20	0.190	95.0	0.167	83.5
Ala-H	0.50	0.511	102	0.483	96.6
	0.20	0.206	101	0.189	94.5
Pro-H	0.50	0.491	98.2	0.477	95.4
	0.20	0.201	100	0.192	96.0
Val-H	0.50	0.530	106	0.512	102
	0.20	0.208	104	0.206	103
Ile-H	0.50	0.522	104	0.509	102
	0.20	0.215	107	0.207	103
Leu-H	0.50	0.493	98.6	0.480	96.0
	0.20	0.206	101	0.202	101
Nle-H	0.50	0.523	104	0.509	102
	0.20	0.209	105	0.206	103
Phe-H	0.50	0.511	102	0.498	99.6
	0.20	0.205	102	0.200	100

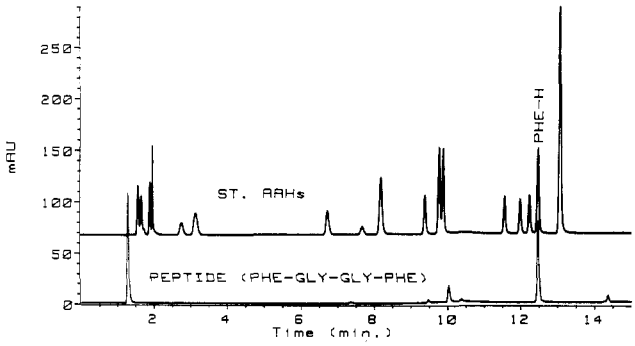


Figure 4. N-Terminal determination of a peptide by the AAH HPLC method. Phenylalanine was determined to be the N-terminal amino acid of the peptide (Phe-Gly-Gly-Phe).

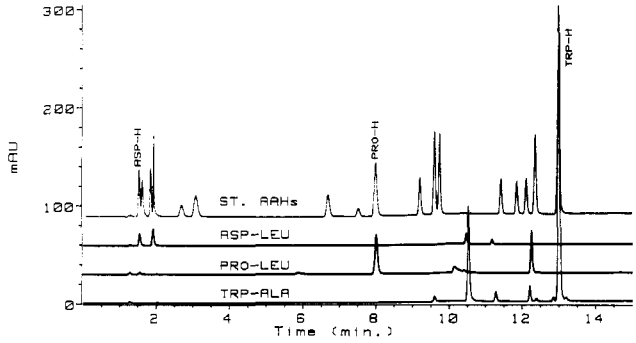


Figure 5. N-Terminal determination of three peptides by the AAH HPLC method. N-Terminal amino acids were shown to be Asp, Pro, and Trp for Asp-Leu, Pro-Leu, and Trp-Ala, respectively.

Figure 4 shows chromatograms of standard AAHs and a peptide (Phe-Gly-Gly-Phe) treated for N-terminal amino acid determination. The chromatograms indicated that phenylalanine hydantoin (Phe-H) was derived from the N terminus of the peptide. The UV spectrum of the sample peak at the retention time of Phe-H also matched that of the standard Phe-H. The yield of Phe-H formed was an average of 89.5% from two treatments of the same peptide. The other three peptides treated for N-terminal determination were Asp-Leu, Pro-Leu, and Trp-Ala. Their HPLC chromatograms were plotted together with a standard chromatogram of the AAHs (Figure 5). Asp-H, Pro-H, and Trp-H were identified as the N-terminal amino acids for those three peptides, respectively. There were some unknown peaks on the chromatograms, which were excluded as AAHs by matching both retention times and UV spectra. Diode array detection was a big help here because it aided in peak identifications by offering peak purity check as well as UV spectrum comparison between the unknown peaks and the standard AAH peaks.

This method did not succeed in the determination of N-terminal amino acids in proteins, because the relatively rough conditions used during acidification caused significant breakage of internal peptide bonds of proteins. This was inferior to the PTH amino acid method, which required milder conditions to release the thiohydantoin formed (Simpson et al., 1989). However, the amino acid hydantoin method was applicable for peptides. For those chemists who do not have an automated sequenater and have to carry out manual derivatization for the determination of N-terminal amino acids, the amino acid hydantoin method would be advantageous to use because AAHs are much more stable than PTH amino acids.

CONCLUSIONS

Amino acid hydantoins were readily synthesized from amino acids and potassium cyanate. The melting point and ^1H NMR data indicated that most hydantoins were obtained in pure form. AAHs were determined according to the megabore reversed-phase HPLC method with short analysis time and good sensitivity. Standard curves showed good linearity for AAHs in the range tested. AAHs were stable in solution during storage. This technique can be utilized for peptide N-terminal determination. This HPLC method can also be used for determination of *N*-carbamyl amino acids after they are converted into corresponding amino acid hydantoins (see Scheme I). Since NCAAs are of interest to us, research has been carried out to identify NCAAs as metabolic intermediates during wine yeast fermentation, and the results will be published.

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