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ACETYLATION OF PHENYLTHIOHYDANTOINS OF AMINO ACIDS

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SUMMARY

The acetylation of all the common amino acid phenylthiohydantoins has been studied and the chromatographic behaviour of the derivatives compared with that of the phenylthiohydantoins. All of the phenylthiohydantoins (except that of proline) examined could be converted into acetylated products but a variety of reaction conditions was necessary. In some cases, these acetylated derivatives were better suited than the parent phenylthiohydantoins to gas chromatographic analysis.

INTRODUCTION

One of the major limiting factors in the extended sequencing of a protein in the protein sequenator[1] is the difficulty of identifying and quantitating some of the 3-phenyl-2-thiohydantoin amino acid derivatives (PTHs) following the Edman degradation. Paper and thin-layer chromatographic techniques[2] are conventionally applied for this purpose but the former are not convenient for quantitative determinations. We have found that amino acid analysis after hydrolysis of the PTHs with hydriodic acid[3] is perhaps the best procedure to use, although the method is time consuming and does not differentiate between the PTHs of serine, alanine and S-carboxymethylcysteine. Gas chromatographic techniques[4] have been shown to be well suited to providing both qualitative and quantitative results, but they are unable to handle certain involatile PTH-derivatives. This problem can be partially overcome by increasing the volatility of the PTHs. Trimethylsilyl derivatives[4,5] have proved to be very useful in this regard, particularly with the "on-column" derivatization procedure[6]; however, the silylated PTHs have limited stability, exhibit widely differing molar responses to flame ionization detectors and PTH-arginine does not yield a volatile product.

Recently, two papers have appeared[7,8] in which acylation is advocated as a means of decreasing the polarity of the PTHs and obtaining more desirable gas chromatographic properties. Both of these papers concentrated on the acylation of the most volatile PTHs but did not demonstrate whether this approach was generally applicable to all PTHs. We were interested to investigate this reaction further and to ascertain whether it offered any advantages for the identification of the more difficult PTH-derivatives by either thin-layer or gas chromatographic techniques.

EXPERIMENTAL

N-Acetylation of PTH-amino acids

PTHs were purchased from the Pierce Chemical Co., Rockford, Ill., U.S.A., and all other reagents were of analytical reagent grade. Typically, 3 mg of PTH-amino acid was treated with 100 μl of acetic anhydride–pyridine (4:1) reagent in a stoppered 3-ml test-tube. The reaction was terminated by the addition, with shaking, of 1 ml of water and 1 ml of ethyl acetate, the aqueous layer was separated and the organic phase was further extracted with four 1-ml portions of water in order to remove pyridinium salts completely.

Thin-layer chromatography

Thin-layer chromatography was carried out on aluminium-backed plates coated with silica gel containing a fluorescent dye (Kieselgel $60F_{254}$, E. Merck, Darmstadt, G.F.R.[9].)

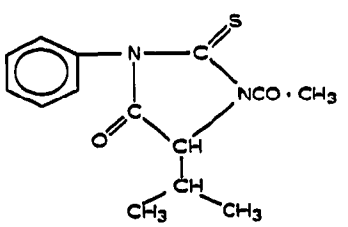
Gas chromatography

A Hewlett-Packard Model 7620A gas chromatograph with dual flame ionization detectors (Hewlett-Packard, Avondale, Pa., U.S.A.) was used. The injection temperature was 270° and the detector temperature 280°. A 1 m×6 mm (2 mm bore) glass column containing 5% Dexsil 300 GC on Chromosorb W (acid washed and silanized) was operated at 165° for 2 min initially, programmed at 8°/min to 210°, then programmed at 10°/min to 290° and maintained at this temperature for 4 min. The helium carrier gas flow-rate was 25 ml/min.

RESULTS

Acetylation of PTH-valine

PTH-valine was used as a model for initial studies of the acetylation reaction. Complete reaction to give acetyl-PTH-valine, as judged by both gas and thin-layer chromatography, was observed after 10 h at 20°, 1 h at 50° or 5 min at 100°. The acetyl-PTH-valine was isolated and recrystallized (m.p. 92–93°) from ethanol by dropwise addition of water. Thin-layer and gas chromatography confirmed that this compound was pure. The mass spectrum indicated a molecular weight of 276. The nuclear magnetic resonance spectrum revealed a sharp singlet at δ 2.88 ppm which signified that N-acetylation at the imino group of the thiohydantoin ring had taken place. These data confirm that acetyl-PTH-valine has the following structure:



The ultraviolet spectrum has a major absorption band at 280 nm (*cf.* 269 nm for PTH-valine) with an extinction coefficient similar to that of PTH-valine. A smaller band is present at 240 nm, but the large absorption around 220 nm characteristic of PTHs is absent in the acetylated derivative.

Acetylation of other PTHs

As expected, all acetylated derivatives exhibited a reduction in their polarity when examined by both chromatographic techniques, and a summary of their properties is presented in Table I.

Table I shows that different reaction conditions are desirable for optimum

TABLE I

ACYLATION CONDITIONS AND CHROMATOGRAPHIC PROPERTIES FOR ACETYLATED PHENYLTHIOHYDANTOINS

Properties of unacetylated PTHs are given in parentheses.

PTH	*Reaction time at 20°*	*Mass spectral analysis*	*GC retention times (min)*		*TLC R_F values*	
Ala	14 h	Monoacetylated	5.04	(5.28)	0.61	(0.40)
Ser	10–40 min	Mono- + di-acetylated	5.60		0.51 0.68 *	(0.11)
Gly	14 h	Monoacetylated	5.64	(5.96)	0.58	(0.35)
CmCys **	10 min–6 h	Mono- + di-acetylated	5.80	Weak response	0.31 0.50	(0.16)
Val	14 h	Monoacetylated	6.08	(6.44)	0.65	(0.46)
Pro	—	Not determined	—	(7.20)		(0.55)
Ile	14 h	Monoacetylated	7.20	(7.52)	0.65	(0.50)
Leu	14 h	Monoacetylated	7.20	(7.52)	0.67	(0.52)
Thr	6 h	Diacetylated	7.00 7.36 ***		0.49	(0.18)
Hyp	14 h	Not determined	9.76 10.28	(10.00)	0.54 *** 0.48	(0.28)
Met	14 h	Monoacetylated	9.88	(10.24)	0.63	(0.44)
Glu	6 h	Monoacetylated	10.24		0.36	(0.20)
Phe	14 h	Monoacetylated	10.40	(11.36)	0.66	(0.45)
Tyr	14 h	Diacetylated	13.12	(13.96)	0.62	(0.25)
Lys-PTC	14 h	Diacetylated	13.16		0.63	(0.36)
Trp	6 h	Monoacetylated	14.88 *** 17.28	(17.68)	0.52	(0.41)
Asp	6 h	Not determined			0.32	(0.14)
Asn	14 h	Monoacetylated			0.28	(0.08)
Gln	14 h	Monoacetylated			0.27	(0.09)
His	3 h–6 days	Not determined	13.43 ***,§ 13.94			(0) (0)
Arg	3 h–6 days	Not determined	12.50§			(0)

* Spot with R_F = 0.51 gradually decreases as second spot increases.

** CmCys= S-carboxymethylcysteine.

*** Major of two peaks.

§ Must be injected in a fresh aliquot of acetic anhydride for "on-column" conversion.

yields of the various acetylated derivatives. The formation of monoacetylated PTH-serine is very rapid (less than 10 min at 20°). The amount of diacetylated product gradually increases with time until 40 min, after which decomposition of the products occurs and multiple spots begin to appear on the thin-layer plate. PTH-S-carboxymethylcysteine reacts similarly but the mono- and di-acetylated products formed are considerably more stable and appear to be present in equal amounts. The PTHs of S-carboxymethylcysteine, threonine, tryptophan, aspartic acid and glutamic acid all showed clean thin-layer chromatograms of the acetylated derivatives after 6 h at 20° but thereafter decomposition of the derivatives began to occur, as manifested by extra spots on the plates. The acetylated derivatives of alanine, glycine, methionine, isoleucine, leucine, tyrosine, phenylalanine, asparagine, glutamine, lysine and hydroxyproline were stable after 14 h at room temperature. Histidine and arginine did not acetylate smoothly. Even after more stringent reaction conditions (4 h at 100° in a sealed tube), we did not detect a major product, although six definite spots were obtained on the thin-layer plate from PTH-arginine. However, both PTH-arginine and PTH-histidine showed evidence of an acetylation product when the mixture was injected into the gas chromatograph with additional acetic anhydride. As expected, PTH-proline did not acetylate.

The number of acetyl groups incorporated into the PTHs could be conveniently determined from the mass of the parent ion in the mass spectrometer. All of the acetylated derivatives studied gave strong parent ions together with another ion 43 mass units lower (*i.e.*, loss of a $-COCH_3$ group). Most derivatives were monoacetylated, as indicated in Table I, while the PTHs of lysine, tyrosine and threonine were the only derivatives to undergo complete diacetylation, presumably forming N,N^{ε}- or N,O-diacetyl products. Mass spectral analysis also indicated that the acetylated products from PTH-serine and PTH-S-carboxymethylcysteine were mixtures of mono- and di-acetyl derivatives, in accord with the thin-layer chromatographic data.

DISCUSSION

Although previous workers[8] have carried out acetylations in the absence of pyridine, this omission is detrimental, as acetylation of PTH-valine, as judged by thin-layer chromatography, was not observed. However, on injecting the acetic anhydride–PTH mixture into the gas chromatograph, a high percentage of the acetyl derivative is formed. This "on-column reaction" technique is a very convenient means of preparing some acetyl-PTH derivatives and we were able to form volatile derivatives from PTH-histidine and PTH-arginine only by this method. However, the PTHs of threonine, serine and S-carboxymethylcysteine all gave rise to a product that had the same chromatographic properties as acetyl-PTH-glycine. Also, we were unable to completely acetylate many PTH-derivatives by this technique; approximately 30% of PTH-valine, for example, did not undergo acetylation. As other workers[8] did not observe unreacted PTH-valine on the gas chromatogram, we wonder whether their use of stainless-steel instead of glass columns is the reason for this difference. It has been noted[8] that some PTHs are not eluted from metal columns, although in our experience PTH-valine (and PTH-proline, -isoleucine, -leucine, -glycine and -alanine) can be successfully subjected to gas chromatography from stainless-steel

columns. In an attempt to achieve better "on-column" acetylations, acetyl chloride was tried instead of acetic anhydride but this was unsuccessful and caused poisoning of the column.

In general, the acetylated PTHs exhibit approximately a 6% reduction in their retention times over their unacetylated parents and a 2- to 3-fold improvement in their response in the flame ionization detector. The detection of the acetylated PTHs from glutamic acid, serine, threonine and lysine, in particular, is vastly superior in the gas chromatograph. Moreover, the PTHs from arginine and histidine can be acetylated by the "on-column" procedure to give characteristic peaks, although these peaks give only about one tenth of the response observed from acetyl-PTH-valine. On the other hand, the acetylated PTHs of aspartic acid, asparagine and glutamine are not suitable for gas chromatography, although thin-layer chromatography and mass spectral analysis confirm that these derivatives have been smoothly acetylated. Nor were the acetyl derivatives from PTH-leucine and PTH-isoleucine easier to separate from one another than were their unacetylated parent compounds, although different column packings (*e.g.*, OV-210 as recommended by Pisano and Bronzert[4]) could overcome this problem.

The conditions used for the thin-layer chromatography were clearly not ideal for acetylated PTHs because many acetylated products had similar R_F values. For the main part, thin-layer chromatography was used only to follow the course of the reaction and to assist in the interpretation of the gas chromatographic results. Although modifications to the solvent system were not tried, it was useful for identifying the slow-moving derivatives (*i.e.*, the acetylated PTHs of aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine and S-carboxymethylcysteine). The acetylated derivatives gave very weak ninhydrin colours when treated with the spray reagent of Roseau and Pantel[10], indicating that the imino group in the thiohydantoin ring is largely responsible for the colours observed.

CONCLUSIONS

Although it is not a completely satisfactory approach, the acetylation of the PTHs offers advantages for the identification of some PTH-amino acids by thin-layer and gas chromatography. The PTHs of histidine and arginine are the most difficult to acetylate but they do yield volatile derivatives by "on-column" reaction in the gas chromatograph. However, it is desirable to increase the yield of this reaction and/or the limits of detection. Further studies with other acylating reagents could be fruitful in this direction. The acetylated derivatives from all other PTHs could be identified using the above combined chromatographic techniques.

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