CHROM. 3810

THIN LAYER CHROMATOGRAPHY OF N-THIOBENZOYLAMINO ACID ANILIDES

G. C. BARRETT AND A. R. KHOKHAR
Department of Chemistry, West Ham College of Technology, Romford Road, London E.15 (Great Britain)

(First received May 2nd, 1968; revised manuscript received September 25th, 1968)

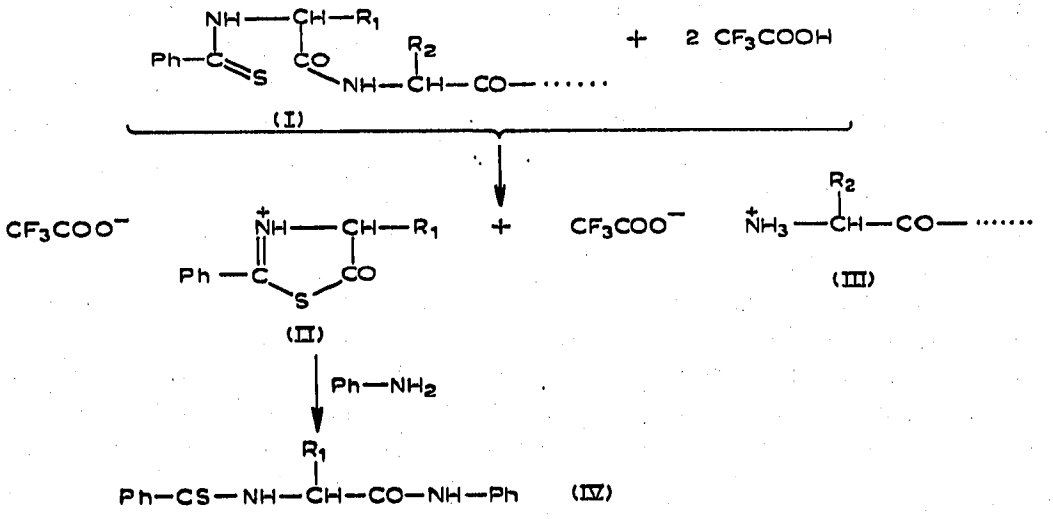
SUMMARY

The thin-layer chromatographic behaviour of a series of N-thiobenzoylamino acid anilides (TBA-amino acids) has been studied. Techniques for the separation of these derivatives by thin-layer chromatography have been established, permitting the identification of a TBA-amino acid obtained in a cycle of a new stepwise degradation of polypeptides.

Model experiments with bovine insulin have demonstrated the efficiency of an N-terminal analysis method in which the N-terminal amino acid residue of a polypeptide is isolated as its TBA-derivative, and identified as such by thin-layer chromatographic techniques.

INTRODUCTION

The title compounds, first prepared in 1955[1], have recently become of renewed interest in connection with a new stepwise degradation of polypeptides[2]. The terminal N-thiobenzoyl derivative (I) of a polypeptide, on treatment with trifluoroacetic acid, yields a 2-phenylthiazol-5(4H)-one trifluoroacetate (II), identification of which defines the N-terminal amino acid residue of the polypeptide.



Thiobenzoylation of the shortened peptide (III), and repetition of the trifluoroacetic acid cleavage step, constitutes the second cycle of the stepwise degradation of the polypeptide. Recently, mass-spectrometric identification of 2-phenylthiazolones cleaved from terminal N-thiobenzoyl polypeptides in this way has been demonstrated[3], but chromatographic methods appear promising also, judging by the success of such methods[4] for the identification of 3-phenyl-2-thiohydantoins (PTH-amino acids) released during the formally similar Edman stepwise degradation of polypeptides[5].

A sequence analysis of a polypeptide based on N-thiobenzoyl derivatives (I) may offer specific advantages[2]; therefore, we have followed up our earlier suggestion[2] that N-thiobenzoylamino acid anilides (TBA-amino acids; IV), obtained from corresponding 2-phenylthiazolones (conjugate base of II) by brief treatment with aniline in boiling toluene[1], should lend themselves well to chromatographic identification. We now report a study of the thin-layer chromatographic (TLC) behaviour of a number of TBA-amino acids.

EXPERIMENTAL

2-Phenylthiazol-5(4H)-ones from N-thiobenzoylamino acids

N-Thiobenzoylamino acids may be converted into the corresponding 2-phenylthiazolones through the use of one of a number of cyclising agents[1]. Of those which have been proposed[1], we have found phosphorus tribromide to be the most convenient in practice; during the course of this work, it was found that anhydrous trifluoroacetic acid was also capable of effecting this cyclisation reaction, and this reagent was used in a number of cases.

With phosphorus tribromide. A solution of the N-thiobenzoylamino acid[6] in anhydrous ether was treated with excess phosphorus tribromide during 10–15 min, until no further 2-phenylthiazolone hydrobromide separated out. The solid hydrobromide was washed by decantation with several portions of anhydrous ether to remove excess reagent, and was then treated with excess aqueous sodium hydrogen carbonate and extracted into ether.

With anhydrous trifluoroacetic acid. A solution of the N-thiobenzoylamino acid in excess trifluoroacetic acid was kept at room temperature overnight, and was then evaporated *in vacuo*. The residue was treated with excess aqueous sodium hydrogen carbonate and extracted into ether; the derivatives of tyrosine, asparagine, aspartic acid, glutamine, glutamic acid, isoleucine, proline, hydroxyproline, histidine, and arginine were converted into 2-phenylthiazolones using this reagent, the sodium hydrogen carbonate treatment being omitted in the case of the arginine derivative.

Preparation of TBA-amino acids from 2-phenylthiazolones

Ethereal solutions of 2-phenylthiazolones prepared as described in the foregoing paragraph were dried ($MgSO_4$) and evaporated. Excess aniline in toluene was added to the residual thiazolone, and the resulting solution was heated to boiling during 10 min, and was then evaporated to dryness *in vacuo*. The TBA-amino acid was crystallised from ether or from ethanol; derivatives obtained in this way are listed in Table I (satisfactory analytical and physical data verifying the structures of the TBA-amino acids and the intermediate 2-phenylthiazolones will be reported elsewhere).

The TBA-derivatives of glycine, leucine, valine, phenylalanine, and alanine are known through the work of JEPSON, LAWSON, AND LAWTON[1], who reported that they had prepared 2-phenylthiazolones from N-thiobenzoyl-DL-amino acids by acetic anhydride cyclisation; in our hands, acetyl derivatives of the desired thiazolones were obtained by this method[3], and treatment of these products with aniline gave poor yields of TBA-amino acids*.

TABLE I

THIN-LAYER CHROMATOGRAPHIC DATA FOR TBA-AMINO ACIDS

Solvents: (1) chloroform–carbon tetrachloride (1:1), (2) chloroform–ether (1:1), (3) ether, (4) ether–petrol (b.r. 40–60°) (1:1), (5) benzene–methanol (20:1), (6) ethyl acetate, (7) chloroform–dichloromethane (1:1), (8) ethyl acetate–methanol (3:1).

N-Thiobenzoyl anilide of	*R_F values in solvents 1–8*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Tyrosine	0.00	0.43	0.48	0.12	0.28	0.58	0.08	0.62
Asparagine	0.01	0.10	0.17	0.02	0.17	0.40	0.03	0.56
Aspartic acid	0.03	0.18	0.22	0.03	0.38	0.51	0.10	0.30
Glutamine	0.03	0.33	0.39	0.05	0.43	0.57	0.10	0.61
Glutamic acid	0.00	0.06	0.08	0.01	0.12	0.30	0.02	0.26
Glycine	0.09	0.44	0.47	0.15	0.49	0.57	0.20	0.52
Alanine	0.14	0.48	0.51	0.22	0.52	0.58	0.28	0.61
Methionine	0.24	0.50	0.53	0.23	0.58	0.59	0.36	0.62
Valine	0.26	0.52	0.54	0.27	0.57	0.59	0.38	0.62
Leucine	0.28	0.53	0.56	0.32	0.58	0.59	0.41	0.61
Isoleucine	0.30	0.54	0.56	0.34	0.59	0.59	0.43	0.60
Phenylalanine	0.31	0.55	0.56	0.32	0.60	0.59	0.46	0.62
Proline[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.58
Hydroxyproline[a]	0.00	0.00	0.00	0.00	0.06	0.01	0.00	0.29
Histidine	0.00	0.03	0.03	0.00	0.10	0.09	0.00	0.52
Arginine[b]	0.00	0.00	0.00	0.00	0.01	0.04	0.00	0.05

[a] As meso-ionic thiazolones (V).
[b] As trifluoroacetate salt.

Behaviour of TBA-amino acids during thin-layer chromatography

Pre-coated silica gel TLC plates incorporating a fluorescent agent were used (Merck Kieselgel F_{254}). TBA-amino acids were located on the chromatograms as either (a) yellow-brown spots after exposure to iodine vapour during 10–30 sec, or (b) dark spots on a green background in ultraviolet light (365 mμ). The limits of sensitivity of detection involved sub-microgram quantities for each TBA-amino acid studied; exposure to iodine vapour followed by illumination with ultraviolet light enhanced the contrast between the spot and background adsorbent, and appeared to increase the sensitivity of detection[7].

R_F values relate to separations on TLC plates de-activated by exposure to atmospheric moisture during at least three days.

TBA-glycine and TBA-phenylalanine from bovine insulin

Insulin (B.D.H. Ltd., Poole, Dorset, England; 0.124 g) was suspended in water

* Racemisation occurs at the α-carbon atom in N-thiobenzoyl-L-α-amino acids during their conversion into TBA-amino acids following the procedures described here.

(5 ml), then brought into solution (pH 8–9) by the addition of 0.1 *N* sodium hydroxide. To this solution was added a solution of thiobenzoylthioglycollic acid[8] (0.020 g) in one equivalent of aqueous sodium hydroxide. Next day, the reaction mixture was exactly neutralised (0.1 *N* hydrochloric acid), and was evaporated to dryness *in vacuo* at room temperature. The residue was washed with ether, and was dissolved in anhydrous trifluoroacetic acid (5 ml). The resulting solution was evaporated *in vacuo* without delay; the residue was treated with excess aqueous sodium hydrogen carbonate, and was extracted with ether. The dried ($MgSO_4$) ethereal solution was evaporated, and the residue was treated with excess aniline in boiling toluene during 10 min. The resulting solution containing TBA-derivatives of the N-terminal amino acid residues of insulin, was evaporated *in vacuo*; the residue yielded two-spot chromatograms with solvents 1–8 (Table I), R_F values corresponding with those established in this study for TBA-glycine and TBA-phenylalanine.

DISCUSSION

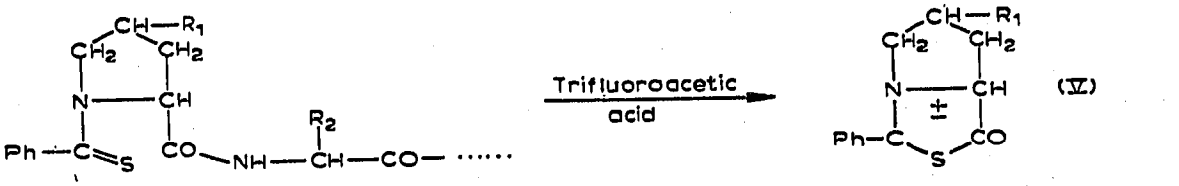
The TLC behaviour of a variety of amino acid derivatives has been studied in many laboratories, and several solvent systems have been found which give generally satisfactory separations of these derivatives[4]. In particular, the establishment of adequate chromatographic methods for the separation of PTH-amino acids has been intensively studied over a number of years, since the small-scale identification of these derivatives forms the basis of the widely-used Edman method[5] for the sequential analysis of polypeptides. Recently[9,10], improved methods involving multiple development techniques have been established for the TLC analysis of PTH-amino acids, allowing unambiguous identification of the PTH-derivatives of all the "natural" amino acids.

It was anticipated that accumulated chromatographic data for PTH-amino acids would be helpful in the present study of the TLC behaviour of TBA-amino acids, and indeed, a benzene–methanol (20:1) mixture[4] was fairly satisfactory for the purpose. However, solvent systems used for the separation of PTH-amino acids[4] were not generally suitable for TBA-analogues, rather less-polar solvents being more appropriate, though general trends in R_F values are similar for the two series. In the cases of proline and hydroxyproline, meso-ionic thiazolones (V) are formed during the trifluoroacetic acid cleavage reaction with the N-thiobenzoyl derivatives of peptides in which one or other of these amino acids occupies the N-terminal position[3]; these thiazolones are not susceptible to aminolysis by aniline under the conditions used with the other 2-phenylthiazolones (Experimental section), and, although they show distinctive TLC behaviour, they are not structurally comparable with TBA-amino acids (IV), or with PTH-amino acids.

Solvents 1–8 (Table I) were found to be the most satisfactory of a large number tried in addition to those recommended[4] for separations of PTH-amino acids. Partition TLC techniques (*e.g.* with the solvents *n*-butanol–acetic acid–water, 6 : 2 : 2, and *n*-butanol–pyridine–water, 2 : 1 : 2) were unsatisfactory, except for the separation of TBA-derivatives of amino acids with the more hydrophilic side chains (histidine, arginine), this particular group of TBA-amino acids being separated best by methanol-containing solvents (*e.g.* solvent 8, Table I). Solvent 7 established a satisfactory separation of the group of amino acid derivatives carrying simple aliphatic or aromatic

side chains (glycine, alanine, methionine, valine, leucine, isoleucine, and phenylalanine), from each other and from the more polar amino acids represented in the present series. Solvents 5 and 8, though incapable of resolving the alanine–valine–methionine–leucine–isoleucine group (TLC separation of some members of this group has been satisfactorily achieved only recently[9,10]), were suitable for the separation of all the other TBA-amino acids in the present series.

A procedure of the identification of "unknown" TBA-amino acids obtained through a sequence determination of a peptide involving N-thiobenzoyl derivatives[2] would therefore first involve development of the TLC plate with a relatively non-polar solvent (solvent 7; Table I). Where examination in ultraviolet light indicates no movement in the non-polar solvent, the same chromatogram may be developed again, in the same direction, with a methanol-containing solvent (solvents 5 or 8; Table I).



V; from proline, $R_1 = H$
V; from hydroxyproline, $R_1 = OH$

In particular cases where doubt remains about the identities of TBA-amino acids on a TLC chromatogram, the technique[11] involving the extraction of unresolved PTH-amino acid mixtures from the chromatogram, followed by hydrolysis to the constituent amino acids and subsequent chromatographic identification, appears to be applicable also to TBA-amino acids. Although this problem does not appear likely to arise with the TBA-amino acids used in the present study, since differences in R_F values are adequate for identification purposes, and problems of this type can be overcome with multiple development techniques, there remains the general problem of the uncertainties in TLC interpretations due to the presence of impurities and artifacts introduced through the chemical procedures involved in the sequence degradation of a peptide. This problem is referred to in relation to the Edman method in a recent review[12]. An N-terminal analysis of bovine insulin was performed to demonstrate that, as expected[2], no artifacts are introduced during the trifluoroacetic acid cleavage of N-terminal amino acid residues from N-thiobenzoyl peptides, and the conversion of the resulting 2-phenylthiazolones into TBA-amino acids. Application of this procedure to insulin was expected to give a mixture of TBA-glycine and TBA-phenylalanine, since these amino acids are known[13] to be the N-terminal amino acid residues of this polypeptide. In the event, TLC of the TBA-amino acid mixture from insulin in solvents 1–8 (Table I) gave clean two-spot chromatograms, with satisfactory concordance of R_F values with those established for TBA-glycine and TBA-phenylalanine.

The 2-phenylthiazolones (conjugate base of II) cleaved from terminal N-thiobenzoyl peptides do not appear to be suitable for chromatographic identification purposes since bearded spots[14] were obtained in preliminary studies of the TLC behaviour of 2-phenyl-4-isobutylthiazol-5(4H)-one[3] in various solvent systems, possibly due to the potential keto-enol tautomerism of thiazol-5-ones. 2-Phenylthiazol-5(4H)-

ones can be converted[1] into a range of derivatives Ph–CS–NH–CH(R)–CO–X by reaction with ammonia (X = NH_2), or with a primary or a secondary amine (X = NH–R′ or NR′R″), and although aniline has been used in the present work with satisfactory results, the use of another amine might be found to give derivatives with improved chromatographic properties. Such a flexibility of approach is not available in the treatment of amino acid residues cleaved from peptides as 2-anilinothiazol-5-ones (II; Ph–NH in place of Ph) through the Edman procedure[15], because these intermediates rapidly undergo isomerisation into the corresponding PTH-amino acids.

REFERENCES

1 J. B. JEPSON, A. LAWSON AND V. D. LAWTON, *J. Chem. Soc.*, (1955) 1791.
2 G. C. BARRETT, *Chem. Commun.*, (1967) 487.
3 G. C. BARRETT AND J. R. CHAPMAN, *Chem. Commun.*, (1968) 335.
4 G. PATAKI, *Dünnschichtchromatographie in der Aminosäure- und Peptid-chemie*, W. de Gruyter, Berlin, 1966, p. 181–185.
5 J. I. HARRIS AND V. M. INGRAM, in P. ALEXANDER AND R. J. BLOCK (Editors), *A Laboratory Manual of Analytical Methods of Protein Chemistry*, Vol. II, Pergamon, Oxford, 1960, p. 424.
6 G. C. BARRETT, *J. Chem. Soc.*, (1965) 2825; *ibid.*, *J. Chem. Soc. (C)*, (1966) 1771.
7 B. V. MILBORROW, *J. Chromatog.*, 19 (1965) 194.
8 F. KURZER AND A. LAWSON, *Org. Syn.*, 42 (1962) 100.
9 J.-O. JEPPSSON AND J. SJÖQUIST, *Anal. Biochem.*, 18 (1967) 264.
10 G. PATAKI, *Helv. Chim. Acta*, 50 (1967) 1069.
11 D. G. SMYTH AND D. F. ELLIOTT, *Analyst*, 89 (1964) 81.
12 F. SANGER AND H. TUPPY, *Biochem. J.*, 49 (1951) 463, 481.
F. SANGER AND E. O. P. THOMPSON, *Biochem. J.*, 53 (1953) 353, 366.
13 M. BRENNER, A. NIEDERWIESER AND G. PATAKI, in E. STAHL (Editor), *Thin-layer Chromatography, a Laboratory Handbook*, Springer-Verlag/Academic Press, New York, 1965, p. 432.
14 I. M. HAIS AND K. MACEK, *Handbuch der Papierchromatographie*, Gustav Fischer Verlag, Jena, 1958, p. 147.
15 D. ILSE AND P. EDMAN, *Australian J. Chem.*, 16 (1963) 411.
D. BETHELL, G. E. METCALFE AND R. C. SHEPPARD, *Chem. Commun.*, (1965) 189.