CHROM. 4558

The detection of peptide hydrazides on thin-layer chromatograms

One of the best coupling techniques in peptide synthesis is *via* the azide method[1], based on the oxidation of peptide hydrazides to their respective azides[2,3]. The latter are readily attacked by free amino terminals of peptides or amino acids resulting in peptide bond formation. The usefulness of this technique is clearly illustrated in the recent synthesis of ribonuclease[4].

Prior to the coupling step, some assurance of the purity of the peptide hydrazide should be obtained. For this purpose thin-layer chromatography (TLC) is particularly convenient but the reagents used at the present time for staining (I_2 vapour[5] and chlorine–*o*-toluidine[6]) will stain all peptide derivatives and therefore will not distinguish the peptide hydrazide from the others. Moreover, since spot tests for acid hydrazides, as described by FEIGL[7], are not applicable for TLC, it is therefore desirable to have a specific spray reagent for peptide hydrazides suited to TLC.

Since hydrazides are oxidizable, detection of these compounds by oxidants was studied. However, several amino acids also have this property, *e.g.* cysteine, cystine, methionine, tryptophan, tyrosine and histidine. The oxidizing spray reagent, therefore, must be able to distinguish between the oxidizable amino acid and the peptide hydrazide. Two such spray reagents as described by PATAKI[8] were not able to make this discrimination (see Tables I–III). However a 1 % (w/v) aqueous sodium dichromate solution was found to be ideal as a specific peptide hydrazide spray.

The amino acids, amino acid derivatives and peptides were spotted on Kieselgel (DC-Karten SI F) and Cellulose (DC-Karten CE F) both from Riedel-De Haen A.G. The plates were run in either chloroform–methanol (9:1) or *n*-butanol–acetic acid–water (BAW, 4:1:1). The plates run in the former solvent system were dried at room temperature for a few minutes while the plates run in BAW were dried at 110° for 15 min prior to staining. One set of plates was used for the detection of all the compounds by I_2 vapour[5]. The following solutions were used for spraying the plates: 1 % (w/v) sodium dichromate in water, 1 % (w/v) potassium permanganate in water, silver nitrate solution[9], 1 % (w/v) ceric ammonium nitrate, $FeCl_3$–$K_3[Fe(CN)_6]$[8] and methyl red–NaOBr[10].

From Table I, it can be seen that all the peptide hydrazides were stained a colour distinguishable from the background. This colour was observed readily with 0.02 μmoles of material and in the case of the silver nitrate spray 0.01 μmoles were easily detected.

Non-hydrazide peptides containing oxidizable side chains are with few exceptions stained the same colour as hydrazide peptides (Table I) with the permanganate, ceric ammonium nitrate, $FeCl_3$–$K_3[Fe(CN)_6]$, methyl red–NaOBr sprays (Table II). The silver nitrate spray also has this property but its occurrence is less frequent than with the above sprays (compounds 15, 17, 18 and 20, Table II). Only the dichromate spray does not stain the oxidizable peptide derivatives the blue-grey colour of the peptide hydrazides (Table I). Those peptide derivatives which are stained by dichromate produce an orange colour (compounds 12, 13, 15, 18, 24, Table II) or sometimes a faint white spot (compounds 16 and 21, Table II).

Table III shows the effect of the oxidizing sprays on non-oxidizable peptides.

TABLE I

THE DETECTION OF PEPTIDE HYDRAZIDES BY OXIDIZING SPRAYS ON THIN-LAYER CHROMATOGRAMS

No.	*Compound*[a]	*Chromate*[b]		*Permanganate*[b]	
		Silica[c]	*Cellulose*[c]	*Silica*[c]	*Cellulose*[c]
1	Z-Gly-hydrazide	blue-grey	brown	white	white
2	Z-Gly-Gly-hydrazide	blue-grey		white	
3	Z-Ala-Ala-hydrazide	blue-grey	brown	white	white
4	Z-Ser-hydrazide	blue-grey	brown	white	white
5	Z-Ser-Ser-hydrazide	blue-grey		white	
6	Z-(OBz-Ser)$_3$-Pro-hydrazide	blue-grey		yellow	
7	*t*-Boc-carbazide	blue-grey	brown	white	white
8	Z-Im-Bz-His-hydrazide	blue-grey		white	
9	Z-Asp-β-hydrazide	blue-grey		white	
10	Z-Phe-Phe-hydrazide	blue-grey		white	
11	Z-Phe-Gly-hydrazide	blue-grey		white	

[a] All amino acids except glycine were of the L-form. The abbreviations are: Z = carbobenzoxy; OBz = O-benzyl; *t*-Boc = *tert.*-butyloxycarbonyl; Im-Bz = N-benzylimidazole; Gly = glycine; Ala = alanine; Ser = serine; Pro = proline; His = histidine; Asp = aspartic acid; and Phe = phenylalanine. The hydrazides were dissolved in dimethylformamide.

[b] The spray reagents were aqueous solutions of 1% (w/v) sodium dichromate, 1% (w/v) potassium permanganate, basic silver nitrate solution[9], 1% (w/v) ceric ammonium nitrate, and 1 to 1 mixture of 1% ferric chloride in 2 *N* acetic acid and 1% (w/v) potassium ferricyanide in water[8]. The BRENNER AND HOFER reagent[10] involved spraying the thin-layer chromatograms first with 0.05% methyl red in 2 *N* sulfuric acid followed by spraying with 0.1 *N* sodium hypobromite. The colours of the background on either silica or cellulose plates were: yellow, violet (which lightens to a cream colour with time), very pale brown, cream, light green and faint pink, respectively.

[c] Silica refers to Kieselgel (DC-Karten SI F) while cellulose is DC-Karten CE F. The silica plates were run in either $CHCl_3$–CH_3OH (9:1) or BAW (4:1:1). The cellulose plates were run only in the former solvent system.

[d] These colours fade 15 to 30 min after the spraying operation.

TABLE II

THE EFFECT OF OXIDIZING SPRAYS ON OXIDIZABLE AMINO ACID AND PEPTIDE DERIVATIVES ON THIN-LAYER CHROMATOGRAMS

No.	*Compound*[a]	*Chromate*[b]		*Permanganate*[b]	
		Silica[c]	*Cellulose*[c]	*Silica*[c]	*Cellulose*[c]
12	Cys-SH	orange		white	
13	Glutathione (reduced)	orange	orange	white	brown
14	Z-Cys-S-Z	no stain	no stain	white	white[d]
15	Cys-S-S-Cys	light orange[d]	no stain	white	white
16	Z-Cys-S-S-Cys-Z	faint white		white	
17	Cloxacillin	no stain		white	
18	6-Amino-penicillanic acid	orange		white	
19	Z-Met-Ala	no stain	no stain	white[d]	faint white[d]
20	Z-Tyr-Tyr-OCH_3	no stain	no stain	white[d]	white[d]
21	Z-Trp	faint white		white	
22	Z-Phe-Phe-Trp-OCH_3	no stain	no stain	faint white[d]	faint white[d]
23	α-Im-di-Z-His-Trp-OCH_3	no stain		brown	
24	His	light orange[d]	no stain	white	faint white
25	N-Acetyl-His				

[a] See Table I footnote [a]. The abbreviations are: Cys = cysteine; Met = methionine; Tyr = tyrosine; and Trp = tryptophan. The blocked amino acids, peptides and cloxacillin were dissolved in dimethylformamide while 6-aminopenicillanic acid was dissolved in water.

For [b],[c] and [d] see the respective footnotes to Table I.

[e] These compounds are not initially stained but appear a few minutes after staining.

lver nitrate[b]		*Ceric ammonium nitrate*[b]*/silica*[c]	*$FeCl_3$–$K_3[Fe(CN)_6]$*[b]*/silica*[c]	*Methyl red–NaOBr*[b]*/silica*[c]
lica[c]	*Cellulose*[c]			
own	brown	white	blue	violet
own		white	blue	violet
own	brown	white	blue	violet
own	brown	white	blue	violet
own		white	blue	violet
own		white	blue	violet[d]
own	brown	white	blue	violet
own		white	blue	violet
own		white	blue	violet
own		white	blue	violet
own		white	blue	violet

ilver nitrate[b]		*Ceric ammonium nitrate*[b]*/silica*[c]	*$FeCl_3$–$K_3[Fe(CN)_6]$*[b]*/silica*[c]	*Methyl red–NaOBr*[b]*/silica*[c]
ilica[c]	*Cellulose*[c]			
rey		white	blue	violet
hite	white	white	blue	violet
ght brown[e]	grey[e]	faint white	faint blue[e]	pink
int brown	white	white	white[e]	no stain
hite	faint blue[e]	white	faint blue[e]	violet
rown		white	faint blue[e]	violet
int brown		white	faint orange	white[e]
hite	white	faint white	faint blue	violet
ght brown	no stain	white	faint blue	faint pink
int grey		brown	blue	violet
o stain	no stain	faint brown	blue	violet
			blue	violet
int grey[e]	white	faint white		
			white	no stain

TABLE III

THE EFFECT OF OXIDIZING SPRAYS ON NON-OXIDIZABLE AMINO ACID AND PEPTIDE DERIVATIVES ON THIN-LAYER CHROMATOGRAMS

No.	*Compound*[a]	*Chromate*[b]		*Permanganate*[b]	
		Silica[c]	*Cellulose*[c]	*Silica*[c]	*Cellulose*[c]
26	Z-Ser	no stain		no stain	
27	Z-Arg	no stain	no stain	no stain	no stain
28	Arg				
29	Z-Pro-Pro	no stain		no stain	
30	Z-Phe	faint white[d]	no stain	no stain	faint white[d]
31	Z-Gly-Gly-OCH_2CH_3	no stain	no stain	no stain	no stain
32	Leu	no stain	no stain	white[e]	no stain
33	*t*-Boc-Asn	no stain		brown[d]	
34	*t*-Boc-Gly-Gly	no stain		brown[d]	
35	Gly			white[e]	
36	Gly-Gly-Phe			white[e]	

[a] See footnote [a], Table I and II. The abbreviations are: Arg = arginine; Leu = leucine; and Asp = asparagine. The blocked amino acids and peptides were dissolved in dimethylformamide while the free amino acids were dissolved in water.
For [b], [c] and [d] see respective footnotes to Table I.
[e] See Table II, footnote [e].

The dichromate, silver nitrate, ceric ammonium nitrate and methyl red–NaOBr sprays, with few exceptions, do not stain these peptides at all. With the exception of the ceric ammonium nitrate spray, those peptides which are coloured by the above sprays are stained a different colour from hydrazide peptides. Unblocked α-amino peptides (compounds 32, 35 and 36) are stained white, as are hydrazides, while *t*-Boc-amino acids (compounds 33 and 34) are stained brown by the permanganate spray. The $FeCl_3$–$K_3[Fe(CN)_6]$ spray with few exceptions colours the peptides of Table III blue, the same colour produced by this spray with peptide hydrazides.

We would like to suggest, therefore, that simple oxidizing agents might be useful in staining peptide hydrazides. The 1% (w/v) aqueous sodium dichromate spray seems to be ideal in this respect since, apart from its ease of preparation and availability, it stains peptide hydrazides in trace amounts (0.02 μmoles). Furthermore in our experiments, the colour produced was clearly distinguishable from that caused by the oxidation of other functional groups. This spray reagent could therefore be useful to peptide chemists in determination of the purity of peptide hydrazides.

H. J. GOREN is grateful for the Post-doctoral Fellowship awarded him by the National Research Council of Canada, which enabled him to carry out this research.

Department of Biophysics, The Weizmann Institute of Science, Rehovot (Israel)

H. J. GOREN
M. FRIDKIN

1 E. SCHRODER AND K. LUBKE, *The Peptides*, Vol. I, Academic Press, New York, 1965.
2 T. CURTIUS, *Chem. Ber.*, 35 (1902) 3226.
3 Y. WOLMAN, P. M. GALLOP, A. PATCHORNIK AND A. BERGER, *J. Am. Chem. Soc.*, 84 (1962) 1889.
4 R. G. DENKEWALTER, D. F. VEBER, F. W. HOLLY AND R. HIRSCHMANN, *J. Am. Chem. Soc.*, 91 (1969) 502.
5 Y. S. KLAUSNER AND Y. WOLMAN, *J. Chromatog.*, 38 (1968) 152.

lver nitrate[b]		*Ceric ammonium nitrate*[b]*/silica*[c]	*$FeCl_3$–$K_3[Fe(CN)_6]$*[b]*/silica*[c]	*Methyl red–NaOBr*[b]*/silica*[c]
lica[c]	*Cellulose*[b]			
stain		no stain	faint blue[e]	faint orange
ıite	grey[e]	no stain		
			no stain	no stain
stain		no stain	faint blue[e]	no stain
stain	faint grey	faint white	faint blue[e]	orange
stain	no stain	faint white	faint white[e]	no stain
ıite	white	no stain		
			faint blue[e]	no stain
			no stain	no stain
			white	no stain

6 D. E. NITECKI AND J. W. GOODMAN, *Biochemistry*, 5 (1966) 665, and references cited there.
7 F. FEIGL, *Spot Tests in Organic Analysis*, Elsevier, Amsterdam, 1966, p. 274.
8 G. PATAKI, *Dünnschichtchromatographie in der Aminosäure und Peptid-Chemie*, Walter De Gruyter, Berlin, 1966, p. 126.
9 L. HOUGH, *Nature*, 165 (1950) 400.
10 M. BRENNER AND W. HOFER, *Helv. Chim. Acta*, 44 (1961) 1794.

Received November 26th, 1969

J. Chromatog., 47 (1970) 519–523

CHROM. 4553

The thin-layer chromatography of the hydroxyindole-3-carboxylic acids*

The importance of hydroxyindoles in metabolic processes in plants and animals is now generally recognized[1–3]. Recent progress in the development of new and improved methods, including chromatographic procedures, for the detection and determination of hydroxyindoles in biological materials have been reviewed by SANDLER[4,5].

Two of the main problems that are often encountered in the use of paper and thin-layer chromatographic procedures for the detection and identification of hydroxyindoles are the lack of suitable standard compounds and reliable data on their R_F values. The chromatographic behaviour of all four isomeric hydroxyindoles (hydroxylated in the benzene ring) has only been studied in a relatively few cases including indole[6], skatole[7,8], tryptamine[9,10], tryptophan[6], acetyltryptophan[10] and oxindole[11]. The 4-, 5-, 6- and 7-hydroxyindole-3-carboxylic acids have recently all been synthe-

* Issued as NRCC No. 11180.