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IMPROVED CHROMATOGRAPHIC IDENTIFICATION OF COLOURED AMINO ACID THIOHYDANTOINS

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SUMMARY

A new N-terminal reagent for peptides and proteins, 4-N,N-dimethylamino-naphthylazobenzene-4'-isothiocyanate, is described which gives purple thiohydantoin derivatives; chromatographic separation of 24 amino acid thiohydantoin is reported. Such standard purple derivatives can be used as markers in the separation of the red 4-N,N-dimethylaminoazobenzene-4'-thiohydantoin. Conversely, standard red thiohydantoin can be used as markers in the separation of unknown purple amino acid thiohydantoin.

In two-dimensional thin-layer chromatography, the precision of identifying the unknowns can be greatly improved by running markers with a colour different from the unknowns on the same side of the sheet.

INTRODUCTION*

The introduction of DABITC as a coloured N-terminal reagent¹ and the combined use of DABITC and phenyl isothiocyanate in the extended manual sequence determination of peptides and proteins² has demonstrated that DABITC possesses several advantages over conventional methods³. The yield of the acid-labile N-terminals, such as asparagine, glutamine and tryptophan, and the colour change among isothiocyanate, thiocarbamoyl and thiohydantoin derivatives, which provides a rapid identification of N-terminal amino acids, were the major advantages over the dansyl method^{4,5}.

Due to the small size of the polyamide sheets used for the identification of the amino acid thiohydantoin (3 × 3 cm) and the difficulty in running markers on the back of the sheet due to differential solvent migration on the front and back, we wished to improve the ease and reliability of identification of the thiohydantoin. The generation of some blue markers in the DABITC method which could be used

* Abbreviations: DABITC = 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate; DABTC = 4-N,N-dimethylaminoazobenzene-4'-thiocarbamoyl; DABTH = 4-N,N-dimethylaminoazobenzene-4'-thiohydantoin; DANABITC = 4-N,N-dimethylaminonaphthylazobenzene-4'-isothiocyanate; DANABTC = 4-N,N-dimethylaminonaphthylazobenzene-4'-thiocarbamoyl; DANABTH = 4-N,N-dimethylaminonaphthylazobenzene-4'-thiohydantoin.

to distinguish immediately most of the DABTH-AAs (amino acids) suggested the idea of replacing the application of standard markers on the reverse side of the sheet by internal markers of contrasting colours.

Under the existing procedure, although identification between DABTH-Asn and DABTH-Ser and DABTH-Gln and DABTH-Thr could be achieved solely by judging their relative positions to the major blue by-product E, we wish to establish further criteria for the rapid and accurate confirmation of DABTH-Asn, DABTH-Ser, DABTH-Gln, and DABTH-Thr by locating some authentic purple markers around these four red DABTH-AAs.

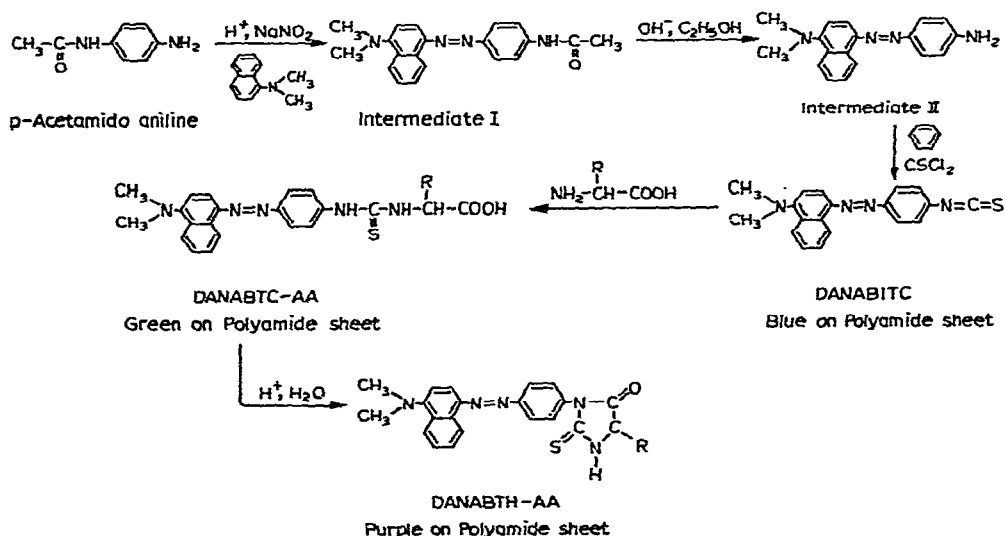
For this purpose, we synthesized another azo dye isothiocyanate, DANABITC, which gives rise to purple amino acid thiohydantoin derivatives. The purple-coloured DANABTH-Asp and DANABTH-Ser were selected as two markers to distinguish red DABTH-Asn and DABTH-Ser and DABTH-Gln and DABTH-Thr (see Fig. 1) in the DABITC method. Conversely, the red-coloured DABTH-Gly and DABTH-Hyp were used as the authentic markers to help the identifications of DANABTH-AAs (see Fig. 2).

MATERIALS AND METHODS

Peptides and proteins were the highest purity obtainable from normal commercial sources. Other chemicals used in the synthesis of DANABITC and for thin-layer chromatography (TLC) were A.R. grade. Chen Chin polyamide sheets were from Pierce (Rockford, Ill., U.S.A.).

Synthesis of DANABITC

The preparation of DANABITC (Scheme 1) was similar to the synthesis of



Scheme 1. Preparation of 4-N,N-dimethylaminonaphthylazobenzene-4'-isothiocyanate (DANABITC) and 4-N,N-dimethylaminonaphthylazobenzene-4'-thiohydantoin amino acids (DANABTH-AA). The colour appeared after exposure to HCl vapour.

DABITC¹, except for the following modifications. (a) The precipitation of intermediates I and II was found to be difficult. Two portions of ethyl acetate (500 ml) were used to extract both intermediate I and intermediate II from the aqueous layers. The extracts were evaporated to dryness in vacuum, giving the creamy intermediates I and II. The synthesis could be continued without purifying the crude intermediates. (b) The conversion of intermediate I to intermediate II was accomplished by refluxing with ethanol (100 ml) and 11 *N* NaOH (20 ml) for 1 h. (c) The final product, DANABITC, after having been eluted from a silica gel column by benzene, was dried (also a creamy product) and redissolved in the minimum volume of acetone. Deep red crystals precipitated when left in the refrigerator overnight (m.p. 125–126°). Its IR spectrum had a strong $\text{N}=\text{C}=\text{S}$ peak at 2110 cm^{-1} .

Analysis. Calc. for $\text{C}_{19}\text{H}_{16}\text{N}_4\text{S}$: C, 68.67; H, 4.82; N, 16.87; S, 9.64. Found: C, 68.76; H, 4.96; N, 16.51; S, 9.44%.

Microsynthesis of DANABTH amino acids

A solution of 150 nmoles amino acid in 40 μl buffer (50 ml acetone + 50 ml distilled water + 0.2 ml triethylamine + 5 ml 0.2 *M* acetic acid; pH 9.65) was treated with 40 nmoles of DANABITC in 20 μl of acetone (2 nmoles/ μl) and heated at 50° for 75 min. The mixture was dried in a vacuum over P_2O_5 and taken up in a mixture of 20 μl of distilled water and 40 μl of acetic acid saturated with HCl. The solution was allowed to stand at 50° for 45 min, and then dried again in the desiccator. The residue was redissolved in 40 μl of ethanol for TLC. 0.01–0.02 μl of each DANABTH-AA was applied on a 3.75 × 3.75 cm polyamide sheet.

Special buffer with higher pH value (0.4 ml triethylamine + 5 ml 0.2 *M* acetic acid + 50 ml acetone + 50 ml distilled water; pH 10.4) was used for the synthesis of DANABTH-Glu, DANABTH-Asp and DANABTH-Cys(O_3H), due to the higher pK_a values of glutamic acid, aspartic acid and cysteic acid.

N-Terminal determination of peptides and proteins by the DANABITC method—TLC separations

The separations^{1,2} were performed as described previously^{1,2}.

RESULTS AND DISCUSSION

Double-sided coated polyamide sheets are commercially available^{6,7}, and both sides can be used independently. They offer another advantage by applying known standards on the reverse side, followed by correlating the unknown spot to those standards. In our work¹ of separating DABTH-AAs on a small-size polyamide sheet (such as 2.5 × 2.5 cm), however, it was found that the speed of solvents was always different on the opposite sides when the sheet was not developed in a precisely vertical position. Hence, we prefer to apply standards on the same side of the polyamide sheet and then identify the unknowns by relating their positions to the applied markers. The appearance of the major blue by-product E fortuitously meets this requirement. By its help, the discrimination of threonine-serine; glutamine-asparagine and glutamic-aspartic pairs was clear. However, the distinction between asparagine and serine and between glutamine and threonine could be improved, even though they are well separated on two-dimensional TLC. This problem arises mainly

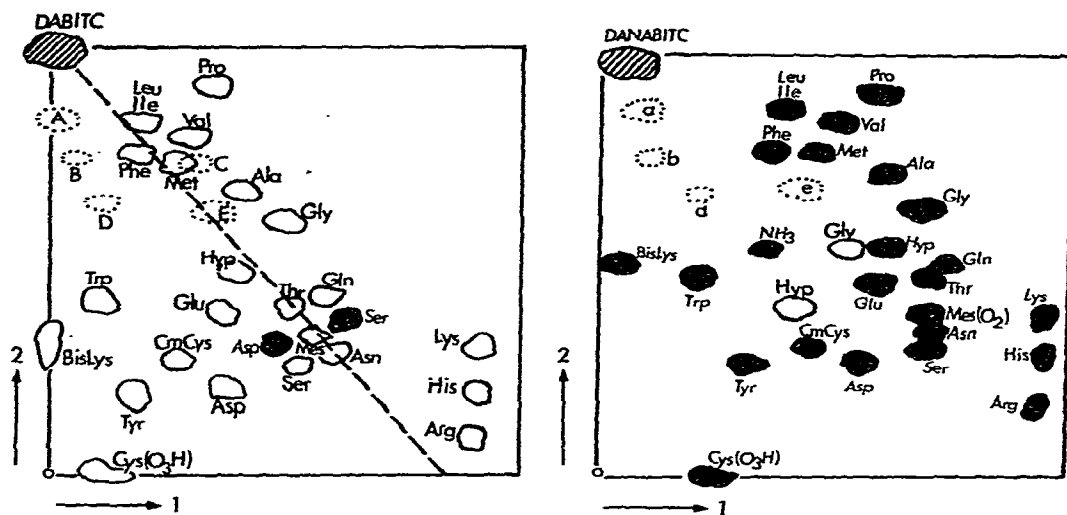


Fig. 1. Tactics of discriminating thiohydantoin derivatives of glutamine and threonine and asparagine and serine using the DABITC method. For details, see text and ref. 1. Solvents: (1) water-acetic acid (2:1); (2) toluene-*n*-hexane-acetic acid (2:1:1). The striped area was the unreacted DABITC. Abbreviations: BisLys = α -DANABTH-(ϵ -DANABTC)-lysine; CmCys = carboxymethylcysteine; Cys(O₃H) = cysteine acid; Mes = methionine sulphone; A, B, C, D and E = blue by-products in the DABITC method.

Fig. 2. Two-dimensional TLC separation of DANABTH amino acids and DANABTC-NH₂ (blackened areas) on a polyamide sheet using the DANABITC method. All the blackened areas were of purple colour except that BisLys was greenish purple and NH₃ (DANABTC-NH₂) was green. The greenish by-products (broken circles; a, b, d and e) and the red spots (unbroken circles; DABTH-Gly and DABTH-Hyp) were used as the reference markers. The striped area was the unreacted DANABITC. Solvents as in Fig. 1. Mes(O₂) = methionine sulphone; other abbreviations as in Fig. 1.

because the size of the polyamide sheet used was only 2.5 × 2.5 cm. Two criteria are proposed to help in resolving this problem:

(1) Extrapolation of the line joining the centre point of unreacted DABITC and spot E (Fig. 1) should go through the centre point of DABTH-Thr and DABTH-Asn and leave the DABTH-Gln and DABTH-Ser located outside this line. This is the easiest way, which has been successfully employed during the sequence determination of glucagon and insulin A chain.

(2) Application of the purple standards DANABTH-Ser and DANABTH-Asp, together with the unknowns at the origin when one of those four DABTH-AA is suspected. Fig. 1 shows the relative positions of the red-coloured DABTH-Gln, DABTH-Asn, DABTH-Thr and DABTH-Ser to the two purple markers, DANABTH-Asp and DANABTH-Ser (blackened areas). The colour difference between markers and unknowns offers an advantage in that, even though they partly overlap, one can still tell their relative positions by the heterogeneous colour intensity. When this criterion is to be applied, the diameter of the origin spot should be as small as possible. The amount of authentic markers applied should be less than 20 picomoles for a 2.5 × 2.5 cm sheet.

The colour changes of DANABITC (blue), DANABTC derivatives (green) and DANABTH derivatives (purple), as indicated in scheme 1, were the major

TABLE I
COLOURS IN THE DABITC AND DANABITC METHODS

<i>Azo dye derivative</i>	<i>Colour</i>	<i>Azo dye derivative</i>	<i>Colour</i>
DANABITC	Blue	DABITC	Purple
DANABTC-AA	Green	DABTC-AA	Blue
DANABTH-AA	Purple	DABTH-AA	Red

differences between the DABITC and the DANABITC method. As anticipated, the replacement for the benzene ring in DABITC by the naphthalene ring in DANABITC shifted the absorptions to longer wavelengths. The colours of DANABITC, DABITC and their derivatives are shown in Table I for comparison.

Fig. 2 shows the two-dimensional TLC separation of 25 different DANABTH-AAs. We found that they had very similar chromatographic behaviour to DABTH-AAs. The only amino acid pairs which could not be satisfactorily separated were: isoleucine and leucine, glutamine and threonine; asparagine, methionine sulphone and serine. The generation of the green by-products a, b, d, e was the same as those blue by-products produced in the DABITC method¹. The major green by-product e, in addition to the two selected red DABTH-Gly and DABTH-Hyp, fulfilled the requirement of markers to distinguish most of the DANABTH-AAs.

We have successfully applied this new reagent on the N-terminal amino acid determination of peptides, Gly-Ala, Gly-Leu, His-Gly, Trp-Gly, Ala-Gly, Leu-Tyr, hexapeptide (Leu-Trp-Met-Arg-Phe-Ala), bradikinin (arginine), insulin A chain (glycine), glucagon (histidine), insulin (phenylalanine and glycine), ribonuclease and lysozyme (both with α -DANABTH-(ϵ -DANABTC)-lysine as the major cleaved N-terminal and α -DANABTH-lysine as the minor products), and human serum albumin (aspartic acid) with only 2 nmoles as the starting materials. The purple colour of the DANABTH-AAs makes the spots readily detectable on the white background of a polyamide sheet.

We are also using DANABITC to complement the DABITC method for the assessment of peptide purity and for the N-terminal map of tryptic digests of proteins which gave preliminary information about the amino acids which follow lysine and arginine. It appears that DANABITC is a very promising N-terminal reagent; it offers an alternative spectrum of amino acid derivative colours to the DABITC procedure.

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