4-*NN*-Dimethylaminoazobenzene 4'-Isothiocyanate, A New Chromophoric Reagent for Protein Sequence Analysis

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4-*NN*-Dimethylaminoazobenzene 4'-isothiocyanate was synthesized for the purpose of improving the ease and sensitivity of peptide sequence analysis. The method of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate synthesis, the preparation of 24 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin derivatives of amino acids and their t.l.c. separation are described. All the thiohydantoin derivatives, except those of leucine and isoleucine, could be satisfactorily separated by chromatography on a two-dimensional polyamide sheet. The sensitive azo group permits the detection of 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin derivatives of amino acids as red spots down to pmol amounts directly on the sheet. A simple sensitive method for sequencing dipeptides and the first two or three *N*-terminal amino acids of proteins is also reported. The colour change of the spots from purple to blue to red after being exposed to HCl vapour, corresponding to the chemical change from 4-*NN*-dimethylaminoazobenzene-4' isothiocyanate to the 4-*NN*-dimethylaminoazobenzene-4'-thiocarbamoyl amino acid derivative to the 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivative, reveals a very interesting and valuable feature of this reagent.

Several modified Edman reagents have been reported for sequential degradation of peptides, and some of them present a variety of advantages, such as 4-sulphophenyl isothiocyanate (Birr *et al.*, 1970), methyl isothiocyanate (Vance & Feingold, 1970), pentafluorophenyl isothiocyanate (Lequin & Niall, 1972), α -naphthyl isothiocyanate (Deyl, 1970), *p*-phenylazophenyl isothiocyanate (Deyl, 1970) and 4-*NN*-dimethylamino-1-naphthyl isothiocyanate (Ichikawa *et al.*, 1970). The last compound is virtually a combination of the Edman reagent and dansyl chloride. However, owing to the replacement of the sulphonyl group by isothiocyanate, only the thiocarbamoyl amino acid derivatives show fluorescence, and hence the cleaved thiozolinone ring should be reopened in order to obtain maximum sensitivity (Ichikawa *et al.*, 1970). Several workers have improved the sensitivity of the detection of the phenylthiohydantoin derivatives of amino acids on t.l.c. to 0.2-0.05 nmol, by using fluorescent indicators (Summers *et al.*, 1973; Kuble, 1974). We describe here a new reagent, 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate, which is also capable of stepwise degradation of minute quantities of peptides, producing coloured 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin derivatives of amino acids, with the sensitivity of 1 pmol.

Experimental

Materials

L-Amino acids and glucagon were Sigma products, St. Louis, MO, U.S.A. The hexapeptide Leu-Trp-Met-Arg-Phe-Ala and insulin A chain were purchased

from Schwarz/Mann Co., Orangeburg, NY, U.S.A. Dipeptides were from Nutritional Biochemical Corp., Cleveland, OH, U.S.A., Sigma or Schwarz/Mann Co. All other chemicals and solvents (redistilled before use) were of commercial analytical grade. Cheng-Chin polyamide sheets were purchased from Pierce Co., Rockford, IL, U.S.A.

Synthesis of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate

The summarized preparation of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate is shown in Scheme 1. *p*-Acetamidoaniline (3 g) was dissolved in 25 ml of water. The solution was heated and stirred vigorously for 10 min, and then cooled to room temperature (20°C). Conc. HCl (5 ml) was added, and the beaker was immersed in an ice bath, and cooled until the temperature of the stirred solution fell below 5°C. Then 1.47 g of NaNO₂ in 3 ml of water was added very slowly to the cold acetamidoaniline hydrochloride, and kept stirring for 10 min (temperature was not allowed to rise above 10°C). To this diazonium chloride solution was added 3.2 ml of *NN*-dimethylaniline; stirring was continued for 20 min and a solution of 4 g of sodium acetate, dissolved in the minimum amount of water, was then added. The azo-dye intermediate I (Scheme 1) was precipitated, filtered and dried under vacuum over P₂O₅ overnight. Crude intermediate I (1 g) was dissolved in a solution consisting of 10 ml of methanol, 5 ml of water and 5 ml of conc. HCl. The solution was refluxed until it changed from deep purple to deep red (about 1 h). After cooling, 80 ml of 1 M-NaOH was added and the

Scheme 1. Synthesis of 4-NN-dimethylaminoazobenzene 4'-isothiocyanate and 4-NN-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives

The colours appeared after exposure to HCl vapour.

mixture was stirred for 20 min. The precipitated intermediate II (Scheme 1) was again collected in a Buchner funnel and dried overnight. The final step was accomplished by refluxing 1 g of intermediate II in 40 ml of benzene and 0.12 ml of thiophosgene (CSCl_2) for 1–1.5 h. The mixture was cooled, filtered, and the filtrate, which mainly contained 4-NN-dimethylaminoazobenzene 4'-isothiocyanate and intermediate II, was kept, concentrated and applied to a column (10 cm $\times$ 20 cm) packed with silica gel (100–200 mesh). Separation of 4-NN-dimethylaminoazobenzene 4'-isothiocyanate from intermediate II was achieved by using benzene as the elution solvent. 4'-NN-Dimethylaminoazobenzene 4'-isothiocyanate was eluted first, whereas intermediate II moved only slowly from the top of the column. The benzene was

evaporated under reduced pressure, and the 4-NN-dimethylaminoazobenzene 4'-isothiocyanate was recrystallized from pure ethanol. The deep-orange leaflet crystals (m.p. 169–170°C) gave an i.r. spectrum with a very strong $\text{N}=\text{C}=\text{S}$ characteristic peak at 2150 cm^{-1} . (Analysis: Calc. for $\text{C}_{15}\text{H}_{14}\text{N}_4\text{S}$: C, 63.8; N, 19.9; S, 11.3; H, 5.00. Found: C, 63.75; N, 19.59; S, 10.94; H, 5.20.)

Preparation of 4-NN-dimethylaminoazobenzene-4'-thiohydantoin derivatives of amino acids, and their separation on polyamide sheets

A solution of $0.5\text{ }\mu\text{mol}$ of amino acid in $100\text{ }\mu\text{l}$ of buffer (2 ml of 2 M-acetic acid + 1.2 ml of triethylamine + water to 25 ml + 25 ml of acetone; pH 10.1) (Sjoquist,

1960) was treated with 100 μ l of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate solution (2 μ mol/ml in acetone), and incubated for 1 h at 50°C. (A molar ratio of isothiocyanate/amino acid of 2:5 was used to avoid interference by excess of reagent with the subsequent t.l.c.) The solution was then dried under vacuum over P₂O₅. Water (200 μ l) and acetic acid saturated with HCl (400 μ l) were added and the solution was left for 50 min at 50°C and then dried again under vacuum over P₂O₅. Ethanol (400 μ l) was added to the residue to extract the 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives for identification by t.l.c. About 0.03–0.05 μ l (15–25 pmol) of each of a 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin of an amino acid solution was successively applied on the origin of a 5 cm $\times$ 5 cm polyamide sheet. A hair drier was used to confine the diameter of the spot to 1.0–1.4 mm. Two-dimensional development in a covered jar was performed by ascending solvent flow; solvent 1, water/acetic acid (2:1, v/v) for the first-dimensional development, and solvent 2, toluene/*n*-hexane/acetic acid (2:1:1, by vol.) for the second-dimensional development. For the preparation of 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin-(ϵ -4-*NN*-dimethylaminoazobenzene-4'-thiocarbamoyl)-L-lysine, the molar ratio of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate/amino acid was adjusted to 5:1. The developed sheet was dried, and exposed to HCl vapour, when the spots turned from yellow to red.

Sequence determination of dipeptides and the first two or three amino acids of peptides and proteins

A portion (5 nmol) of dipeptide, peptide or protein was placed in a Pyrex test tube (4 mm $\times$ 5 cm) and dried over P₂O₅. Then 20 μ l of triethylamineacetic acid buffer, pH 10.1 (as described above), and 20 μ l of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate solution (2 μ mol/ml in acetone) were added. The mixture was incubated at 50°C for 75 min, and then dried under vacuum. The residue was taken up in 20 μ l of water and 40 μ l of acetic acid saturated with HCl, and left for 50 min at 50°C; it was then dried again under vacuum. The dried residue was dissolved in 50 μ l of ethanol, and about 20 pmol (0.2 μ l) was removed to identify the *N*-terminal amino acids (as described above, but a 3 cm $\times$ 3 cm polyamide sheet could be used instead of a 5 cm $\times$ 5 cm size). The remainder was dried and subjected to the next cycle, except that only 10 nmol of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate was added in the following cycle instead of the 40 nmol in the first cycle.

Results and Discussion

Three azo-dye isothiocyanates were synthesized in our laboratory, *p*-phenylazophenyl isothiocyanate

(Deyl, 1970), 4-phenylazonaphthyl isothiocyanate and 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate. The last reagent, owing to the presence of the *NN*-dimethylamino group, allows the generation of resonance structures produced by the combination of the azo group with a proton; thus absorption is shifted to longer wavelengths in acidic medium, and this increases the sensitivity in the visible region. According to our observation, the detection limits of the above mentioned azo-dyes were 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate (1 pmol), 4-phenylazonaphthyl isothiocyanate (0.5 nmol) and phenylazophenyl isothiocyanate (1 nmol).

The colour change of various azo-dye derivatives exhibits an outstanding feature, which facilitates both synthesis of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate and identification of 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives. During the conversion from 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate into 4-*NN*-dimethylaminoazobenzene-4'-thiocarbamoyl amino acid derivatives and then into 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives (all of which appeared as yellow spots on the polyamide sheet before exposure to HCl vapour), the colour changes from purple to blue to red (Scheme 1), which simplified the identification of the 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives on the sheet by eliminating the ambiguous contamination caused by the unchanged 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate and 4-*NN*-dimethylaminoazobenzene-4'-thiocarbamoyl amino acid derivatives. There are four blue by-products, which always appeared during the *N*-terminal analysis when 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate is in excess in the reaction mixture, but those blue spots could be used as markers and do not confuse the identifications (Fig. 1).

The satisfactory separation of various 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives on a two-dimensional polyamide sheet is a significant advantage of this reagent. Fig. 1 shows the schematic representation of 24 different 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives on a two-dimensional sheet. The blue spots (dotted), unchanged purple 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate (hatched) and purple-red 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin-(ϵ -4-*NN*-dimethylaminoazobenzene-4'-thiocarbamoyl)-L-lysine could be readily discriminated from the other 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives. Except for leucine and isoleucine, all other 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives could be readily separated. It is possible to achieve acceptable separation of leucine and isoleucine on a one-dimensional separation with

Fig. 1. Two-dimensional separation of 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives on Cheng-Chlin polyamide sheet

Solvent: first dimension, water/acetic acid (2:1, v/v); second dimension, toluene/*n*-hexane/acetic acid (2:1:1, by vol.). Abbreviations: Cys(O₃H), cysteic acid; Mes(O₂), methionine sulphone; CmCys, *S*-carboxymethylcysteine; Bis-Lys, 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin-(*s*-4-*NN*-dimethylaminoazobenzene-4'-thiocarbonyl)-L-lysine. The dotted outlines are four blue by-products. The hatched spot is unchanged 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate.

solvent 1, if care is taken to apply the sample as a very small spot.

The possibility of applying 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate to the direct sequencing of peptides and proteins was investigated on dipeptides and the first two or three amino acids of peptides or proteins. No extractions were performed in this simple method. In the first cycle the molar ratio of 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate/peptide or protein was 8:1, and in the second and subsequent cycles it was decreased to 2:1, since the unchanged 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate in the first cycle could be re-used. This non-extraction method comprised a cycle of coupling, drying, cleavage, drying, removal of sample for t.l.c. and repetition. It was found that the application of anhydrous trifluoroacetic acid in the cleavage step

and then heating the cleaved thiozolinone derivatives to obtain thiohydantoin derivatives resulted in very low recoveries of 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoins of amino acids compared with the one-step degradation, i.e. the application of acetic acid saturated with HCl. Besides the five major by-products shown in Fig. 2, only a few faint bluish-purple spots appeared, which do not confuse identification of the distinct cleaved red 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives, even after the second cycle. However, the accumulated by-products substantially affected the identification in the third cycle, and precluded identification of the fourth amino acid of hexapeptide (arginine), insulin A chain (glutamate) and third amino acid of glucagon (glutamine). Alternatively, as reported by Smyth & Elliott (1964), the non-detection

Fig. 2. Identification of the cleaved 4-*NN*-dimethylaminoazobenzene-4'-thiohydantoin amino acid derivatives of hexapeptide (a), insulin A chain (b) and glucagon (c) after two cycles of degradation

Solvents used were the same as in Fig. 1. The blue-purple (dotted) and red (dashed) by-products are indicated. The hatched spot is unchanged 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate.

of glutamic acid and glutamine might be caused by the blockage of the free *N*-terminal. By this method, the sequence of His-Gly, Pro-Hyp, Ala-Leu, Ala-Gly, Gly-Leu, Phe-Gly, Gly-Ala, the first two amino acids of glucagon (His-Ser), the first three amino acids of hexapeptide (Leu-Trp-Met) and insulin A chain (Gly-Ile-Val) were correctly identified. Moreover, the starting peptides could be as little as 0.1–0.5 nmol provided that patience and care were observed, since only 0.02–0.03 nmol was necessarily removed for t.l.c. at each stage.

We consider this reagent to be as sensitive as dansyl chloride for *N*-terminal determination, but without damaging the acid-labile amino acids, such as asparagine, glutamine and tryptophan (second of hexapeptide), without the necessity of examination under u.v. light, and with considerable time-saving (about 3–4 h from coupling to t.l.c. identification). It is believed that the combination of this new *N*-terminal reagent and phenylisothiocyanate could be a very convenient tool in the extended manual sequence

analysis, and is expected to overcome the inevitable shortcomings of the Edman-dansyl method, i.e. the destruction of acid-labile amino acids.

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