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PREPARATION OF N-CHLORO-5-DIMETHYLAMINONAPHTHALENE-1-SULPHONAMIDE AND ITS APPLICATION AS A FLUORESCENCE REAGENT FOR THE DETECTION OF PHENYLTHIOHYDANTOINAMINO ACIDS*

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SUMMARY

N-Chloro-5-dimethylaminonaphthalene-1-sulphonamide (NCDA) was prepared by the action of sodium hypochlorite on Dns-amide. NCDA is non-fluorescent but developed intense fluorescence on reaction with phenylthiohydantoinamino acid. About 40–60 pmol of phenylthiohydantoinamino acids were detected on thin-layer plates. NCDA was also effective in the determination of phenylthiohydantoinamino acids on thin-layer chromatogram.

INTRODUCTION

In previous papers[1,2] we described fluorimetric methods for the determination of peptides based on their N-chlorination followed by oxidation of thiamine with the chlorinated product to give fluorescent thiochrome. This reaction was applied to the development of fluorescence from phenylthiohydantoinamino acids (PTH-amino acids) on thin-layer plates and showed excellent sensitivity[3]. However, this method requires three steps for the development of fluorescence, *viz.* chlorination of peptides with sodium hypochlorite, removal of excess hypochlorite and reaction of thiamine with the chlorinated product.

This paper describes N-chloro-5-dimethylaminonaphthalene-1-sulphonamide (NCDA), which fluoresces immediately on reaction with peptides and PTH-amino acids. The study was focused on the preparation of NCDA and its application to the detection of PTH-amino acids on thin-layer plates.

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EXPERIMENTAL

Reagents and materials

PTH-amino acids, cycloheptaamylose (C7A) and N,N'-dimethylaminonaphthalene-5-sulphonamide (Dns-amide) were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). C7A was recrystallized from hot water. Each PTH-amino acid was dissolved in ethyl acetate to give a 1 mg/ml solution and the solutions were appropriately diluted with ethyl acetate. PTH-Asp and PTH-Glu were dissolved in hot water and the solutions were appropriately diluted with water.

Kieselgel 60 thin-layer plates (DC-Fertigplatten) were obtained from E. Merck (Darmstadt, G.F.R.). Solvents of analytical-reagent grade were used without further purification for the development of the plates. The developed PTH-amino acids were detected by irradiation with UV light at 365 nm or determined by measuring their fluorescence intensity using a Joko scanning fluorimeter equipped with a low-pressure mercury lamp and 330- and 480-nm filters for excitation and emission, respectively.

Hypochlorite reagent. Commercial 6% Antiformin® (sodium hypochlorite solution) was diluted 2-fold with redistilled water.

NCDA reagent. To a solution of 35 mg (146 μmol) of Dns-amide in 40 ml of methanol was added hypochlorite reagent drop-wise under UV light until the fluorescence of Dns-amide had almost disappeared. Addition of excess of hypochlorite reagent after the disappearance of the fluorescence caused decomposition of the NCDA. To the solution were then added 50 ml of 1.8% (w/v) C7A solution and the resulting mixture was made up to 100 ml with methanol. The ratio of chlorine to Dns-amide in this reagent, measured by titration with 0.1 *N* sodium thiosulphate solution, was 1:1.

Synthesis of NCDA. Dns-amide (7.5 g, 0.03 mol) was dissolved in 6 ml of 5 *N* sodium hydroxide solution and the resulting mixture was filtered through a sintered-glass disk. Chlorine was bubbled through the filtrate at 65–75°C for 1 h and the reaction mixture was further stirred at 84–85°C for 1 h. The reaction mixture was cooled to 25°C to deposit a yellow precipitate. The precipitate was collected by filtration, washed with cold water and dried over phosphorus pentoxide under reduced pressure. The product was then dissolved in 500 ml of acetone and the resulting solution was filtered. The filtrate was evaporated to dryness under reduced pressure, the residue was recrystallized from acetone and dried over phosphorus pentoxide under reduced pressure to give 7.0 g (0.023 mol, 76.7%) of NCDA as a yellow powder, m.p. 209–212°C. Analysis: calculated for $C_{12}H_{12}N_2O_2ClNaS$, C 46.98, H 3.94, N 9.13%; found, C 47.11, H 3.95, N 9.11%. Nuclear magnetic resonance (NMR) results (acetone-d_6): 2.83, $-N(CH_3)_2$; 7.1–8.6, naphthalene ring protons.

Solvents for thin-layer chromatography. Solvent I was chloroform–methanol (9:1) and solvent II chloroform–formic acid (20:1).

Chromatographic procedure

A Kieselgel plate was spotted with PTH-amino acid solution and developed with either solvent I or solvent II. The solvent was removed by blowing cold air from a hair dryer after the development. The plate was sprayed with NCDA reagent (25 μl/cm^2) and the spots were detected by irradiation with UV light. In order to determine the PTH-amino acids, after the development the plate was dried over phosphorus pentoxide under reduced pressure for 60 min, sprayed with NCDA reagent (25 μl/cm^2) and the fluorescence intensity was measured with the scanning fluorimeter.

RESULTS AND DISCUSSION

Micro-detection of PTH-amino acids on thin-layer chromatograms[4] is important in the sequence analysis of peptides by the Edman degradation because the peptide samples available for analysis are often small and difficult to replace. As colorimetric methods[5,6] used for this purpose lack sensitivity, isothiocyanates containing a fluorophore were developed by Tamura[7] and Maeda and co-workers[8,9]. However, these methods require specific conditions for the derivatization of N-terminal amino acids and also for the separation of the derivatives. Although an Edman–Dns derivatization procedure[10,11] has been proposed as a sensitive method for the sequence analysis of peptides, it is time consuming. An improvement in the detection sensitivity for PTH-amino acids is therefore desirable. The use of NCDA resulted in the simple fluorimetric detection of PTH-amino acids separated by conventional chromatographic methods.

The intense fluorescence of a methanolic solution of Dns-amide disappeared completely on addition of hypochlorite reagent, and reappeared on spraying the solution on to PTH-amino acids spotted on thin-layer plates. The excitation and emission maxima of the reappeared fluorescence were around 330 and 480 nm, respectively, as shown in Fig. 1. The non-fluorescent product was the chlorinated sulphonamide. Dns-amide was then chlorinated with chlorine in alkaline media and the product was confirmed as sodium (NCDA) (Fig. 2) by elemental analysis and NMR spectroscopy. This compound had no fluorescence but fluoresced on reaction with PTH-amino acids, giving excitation and fluorescence spectra similar to those in Fig.1. These

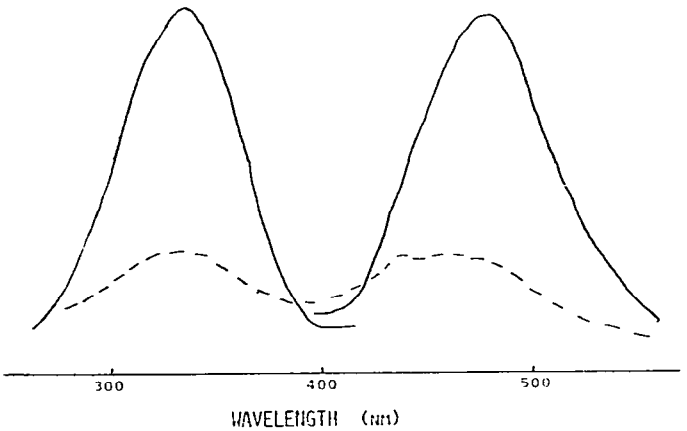

Fig. 1. Excitation and fluorescence spectra (solid line) of PTH-Leu allowed to react with NCDA. A 100-μl volume of 1 m*M* PTH-Leu solution in ethyl acetate was applied to a 1.4 × 1.4 cm square area on a 1.4 × 2 cm Kieselgel plate* and the plate was sprayed with 25 μl/cm^2 of the NCDA reagent. The excitation and fluorescence spectra were taken by inserting the plate into a cell holder attached to a Shimadzu RF 510 spectrofluorophotometer. The blank spectra (broken line) were taken by spraying the same size of Kieselgel plate with 25 μl/cm^2 of the NCDA reagent.

* This plate was prepared by cutting a 20 × 20 cm Kieselgel plate.

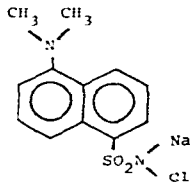

Fig. 2. Structure of NCDA.

result indicates that NCDA is also formed in the reaction mixture of Dns-amide and hypochlorite reagent. NCDA may be converted into Dns-amide by reaction with PTH-amino acid to give fluorescence.

Little difference was observed in the sensitivity between the assay using the synthesized NCDA and that using Dns-amide chlorinated with sodium hypochlorite *in situ*. The latter mixture was used as the NCDA reagent for convenience as the purification of synthetic NCDA required rapid manipulation and was tedious. NCDA is stable for 2 h in methanol at room temperature, whereas it decomposes rapidly in acetone, dioxane or ethylene glycol monoethyl ether.

The conditions for the reaction of NCDA with PTH-amino acids were then examined by using 500 nmol per spot of PTH-Leu and solvent I for development. Fig. 3 shows the fluorescence intensity of PTH-amino acids plotted against the concentration of NCDA reagent. A plateau was reached at 1.4 mg/ml NCDA.

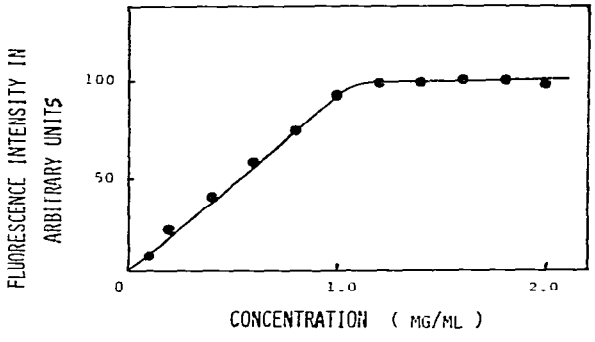

Fig. 3. Fluorescence intensity of PTH-Leu (500 pmol) plotted against the concentration of NCDA in the reagent calculated from the amount of dansylamide used for the preparation of the reagent.

Kinoshita *et al.*[12] have previously reported that the fluorescence intensity of Dns derivatives is greatly enhanced and stabilized in the presence of C7A. Fig. 4 shows the fluorescence intensity of PTH-Leu as a function of C7A concentration in the NCDA reagent. The fluorescence intensity increased with increasing concentration of C7A but precipitation occurred when the C7A concentration was increased above 0.9% (w/v). A concentration of 0.9% (w/v) of C7A was therefore used in the standard procedure. The fluorescence was amplified by a factor of 20 of the intensity at this concentration. The fluorescence developed was stable for 24 h at room temperature when the thin-layer plate was covered with another glass plate. The fluorescence quenched rapidly in the absence of C7A, indicating that C7A stabilizes the fluorescence inten-

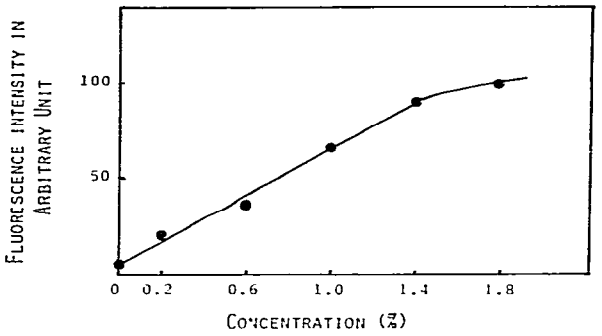

Fig. 4. Fluorescence intensity of PTH-Leu (500 pmol per spot) plotted against the concentration of C7A in the NCDA reagent.

sity. The limit of detection of PTH-amino acids ranged from 40 to 60 pmol, as shown in Table I. This sensitivity is about half to one third of that of the Edman–Dns derivatization method[11]. Table II gives the relative fluorescence intensities of 500 pmol of PTH-amino acids. PTH-Leu, PTH-Phe, PTH-Ala, PTH-Val and PTH-Gly showed similar fluorescence intensities. PTH-Met gave a higher and PTH-Pro, PTH-CysOH and PTH-Asp a lower fluorescence intensity than the other derivatives.

TABLE I

LIMITS OF DETECTION OF PTH-AMINO ACIDS AFTER DEVELOPMENT WITH CHLOROFORM–FORMIC ACID (20:1)

PTH-amino acid	*Limit of detection (pmol)*	*PTH-amino acid*	*Limit of detection (pmol)*
Ala	60	Lys	40
Asp	40	Met	40
Cys	60	Phe	60
Glu	40	Pro	60
Gly	60	Thr	40
Ileu	60	Trp	40
Leu	40	Val	60

TABLE II

RELATIVE FLUORESCENCE INTENSITIES (*F*) OF VARIOUS PTH-AMINO ACIDS (500 PMOL PER SPOT) ON A KIESELGEL G PLATE

The fluorescence intensity of PTH-Leu is expressed as 100.

PTH-amino acid	*F*	*PTH-amino acid*	*F*	*PTH-amino acid*	*F*
Met	130	Leu	100	Thr	80
Lys	126	Phe	101	Ileu	77
		Ala	98	Trp	66
		Gly	97	Pro	55
		Val	95	CysOH	32
				Asp	23

Calibration graphs for PTH-Met, PTH-Leu, PTH-Trp, PTH-Phe and PTH-Ala were linear in the range from 0.08 to 0.6 nmol per spot and passed through the origin. Fig. 5 shows the calibration graphs for PTH-Met, PTH-Leu and PTH-Trp; the slopes of the calibration graphs for PTH-Leu, PTH-Phe and PTH-Ala were almost identical. The coefficient of variation for 500 pmol per spot of PTH-Leu was 8.0% ($n = 6$) when the assay was carried out on different plates.

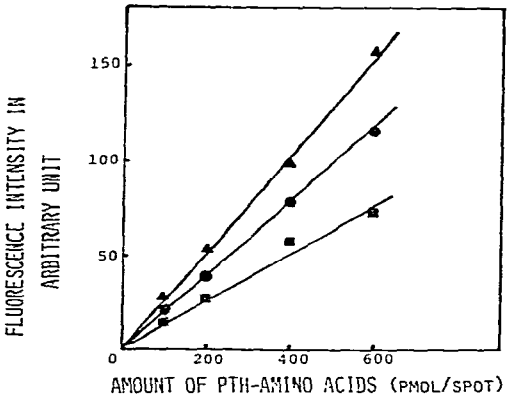

Fig. 5. Calibration graphs for PTH-Met (▲), PTH-Leu (●) and PTH-Trp (■).

NCDA is not only useful for identification of terminal amino acid residues of peptides, but is also applicable to their quantitation, so it is expected to be suitable for the sequence analysis of branched peptides. The conditions for the separation of PTH-amino acids on thin-layers, which have been investigated previously[13,14], can be utilized whithout any modification in conjunction with this reagent. NCDA also reacts with peptides and reducing compounds. The application of this reagent to the analysis of these compounds is now under investigation.

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