A new sensitive Edman-type reagent: 4-(*N*-1-dimethylaminonaphthalene-5-sulfonylamino)phenyl isothiocyanate

Its synthesis and application for micro-sequencing of polypeptides

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Received 19 December 1985

A new fluorescent PITC homologue Edman-type reagent, 4-(*N*-1-dimethylaminonaphthalene-5-sulfonylamino)phenyl isothiocyanate (DNSAPITC), was synthesized following a simple three-step synthetic route. The reagent was crystallized and characterized by thin-layer chromatography, IR and electron impact mass spectrometry. Reference DNSAPTH-amino acid derivatives were prepared and a two-dimensional chromatography system on micro-polyamide sheets was developed for separating this mixture. On these sheets the sensitivity was 1–5 pmol, by exposure at 366 nm. Model peptides and proteins were subjected to Edman degradations with this new reagent. A similar coupling efficiency and repetitive degradation yield to those of PITC were found with this reagent. The advantages and limitations of this reagent for sensitive micro-sequencing are discussed.

Edman-type reagent *Dansylaminophenyl isothiocyanate derivative* *Isothiocyanate synthesis* *Micro-sequencing* *Amino acid dansylamino PTH derivative analysis*

1. INTRODUCTION

Sensitive Edman-type reagents have been proposed for the sequential degradation of polypeptides, such as FITC [1,2] and recently the reagent BAMPITC which was introduced by L'Italien and Kent [3]. The latter reagent possesses a cryptic amino group which will be exposed after the cleavage reaction permitting the detection of the phenylthiohydantoin (PTH) derivatives with OPA (*o*-phthaldialdehyde) or other sensitive amino group reagents. The degradation using FITC, however, is hampered by a rather low coupling yield to the amino groups of the peptides and the presence of reactive side groups in the reagent itself, e.g. carboxyl- (or lactone) and phenolic groups, leading to many side reactions during the degradation thereby obscuring the sequence results [4].

We synthesized BAMPITC [5] and tested its use for micro-sequencing [6]. It was found, however, that the BAMPITC-peptide derivatives (*N*-Boc-aminomethyl-substituted phenylthiocarbamoyl peptides) generated by the reaction with BAMPITC unexpectedly eliminate methylamine during the cleavage reaction with anhydrous trifluoroacetic acid. Apparently, with this reagent a novel unusual reaction mechanism has been en-

Abbreviations: FITC, fluorescein isothiocyanate; BAMPITC, 4-(*N*-*t*-butoxycarbonylaminomethyl)phenyl isothiocyanate; DNSAPITC, 4-(*N*-1-dimethylaminonaphthalene-5-sulfonylamino)phenyl isothiocyanate; DNSAPTH-, amino acid derivative released from reaction with DNSAPITC; DCCD, dicyclohexylcarbodiimide

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

countered [6]. Consequently, the BAMPITC reagent produces no specific amino acid derivatives that can be detected upon reaction with OPA, dansyl or dansyl chloride. Therefore, in contrast to the proposed use in Edman chemistry [3], this reagent cannot be employed in Edman degradations [6].

We have now synthesized a new fluorescent PITC homologue Edman-type reagent, namely DNSAPITC, and describe a simple three-step synthetic route for this compound starting from *p*-acetamidoaniline [7] and studied its use in microsequencing.

2. MATERIALS AND METHODS

p-Acetamidoaniline and DCCD were purchased from Fluka (Buchs, Switzerland), dansyl chloride and insulin B chain (oxidized, bovine) from Serva (Heidelberg) and silica gel 60, 230–400 mesh, from Merck (Darmstadt).

Pyridine, pro-analysis grade from Merck, was redistilled from KOH, ninhydrin and KOH, then stored over a molecular sieve. Benzene was filtered through aluminum oxide (neutral I from Woelm, Eschwege, FRG), redistilled and stored over solid sodium.

Infrared (IR) spectra were recorded with a Beckman Acculab 6 spectrophotometer. Mass spectra were obtained with a Varian MAT 44S instrument equipped with an SS200 data system for electron impact ionization.

2.1. *Synthesis of the DNSAPITC reagent*

The DNSAPITC reagent was synthesized by a three-step reaction starting from dansyl chloride and reaction with *p*-acetamidoaniline (fig.1).

2.1.1. Synthesis of 4-(*N*-1-dimethylamino-naphthalene-5-sulfonylamino)phenyl acetamide

1.5 g *p*-acetamidoaniline (10 mmol) were dissolved in 80 ml of 0.1 M $NaHCO_3$, pH 9.0, and 20 ml CH_3CN, then stirred under the drop-wise addition of 3.23 g (12 mmol) dansyl chloride dissolved in 60 ml CH_3CN. The pH should be kept at pH 9.0 by addition of 1 M NaOH. The mixture was stirred at room temperature for 20 min, then at 45°C for 2 h, cooled to room temperature, and extracted with ethyl acetate (3 × 80 ml). The combined extracts were concentrated by rotary evaporation and the crude crystals obtained were recrystallized from boiling methanol (m.p. 201–203°C, amount 3.18 g, yield 83%).

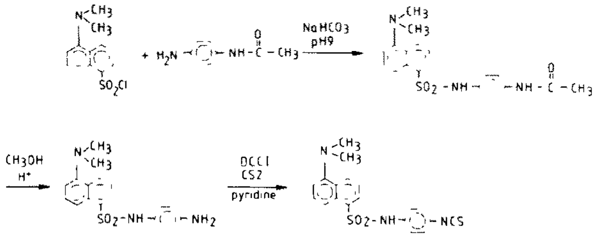

Fig.1. Synthesis of DNSAPITC.

2.1.2. Synthesis of DNSA-phenylamine

3.18 g 4-DNSA-phenyl acetamide were dissolved in 128 ml methanol and 32 ml conc. HCl and refluxed for 1 h. After cooling to 4°C, 4 M NaOH was added to a final pH of 10.0. The solution was extracted with ethyl acetate (3 × 100 ml), concentrated by rotary evaporation and dried in vacuo (over NaOH pellets) overnight. The resultant oil gave a single spot on silica gel thin-layer chromatography (TLC) (solvent system: $CHCl_3$/MeOH, 9:1, v/v); yield 2.96 g.

2.1.3. Preparation of DNSAPITC

The isothiocyanate group was introduced according to Liū et al. [8]. 2.96 g of the amine compound (8.68 mmol) were dissolved in 37 ml anhydrous pyridine (stored over NaOH pellets) and 4.2 ml carbon disulfide (69.6 mmol), and 1.79 g (8.68 mmol) DCCD were added in an ice-salt bath kept at 0°C. The solution was stirred at below 0°C for 4 h and then at ambient temperature for 4 h. The reaction was completed by the addition of 0.44 g DCCD and stirring at room temperature overnight for 14 h. The pyridine was evaporated under reduced pressure and 15 ml ethyl acetate were added. The precipitate of dicyclohexylthiourea was removed by filtration and the filtrate concentrated by rotary evaporation. The residue was dissolved in anhydrous benzene and chromatographed over a silica gel column (2 × 28 cm) 3–4 times. The separation of the isothiocyanate derivative from impurities was achieved by using anhydrous benzene as eluent. The solution was evaporated to dryness under reduced pressure and gave an oily product of

DNSAPITC which was recrystallized from warm *n*-heptane/ethyl acetate (2:1, v/v) and benzene. The crystals formed yellow needles (m.p. 124–127°C, yield 1.27 mg); recrystallization from the same solvents gave crystals of m.p. 125–128°C. The product was pure according to one-dimensional TLC (solvent: benzene/MeOH, 95:5, v/v). The IR spectrum showed the characteristic valence bond of the isothiocyanate group at 2100 nm. In the electron impact mass spectrum the product showed a molecular ion peak $M^+ = 383$.

2.2. *Preparation of reference DNSAPTH-amino acid derivatives*

Amino acids (3 μmol) were dissolved in 80 μl of 50% pyridine in water and 10 μl triethylamine and treated with 1 μmol DNSAPITC dissolved in 40 μl of 100% pyridine. The mixture was heated at 55°C for 50 min and dried in vacuo. Conversion to the PTH derivatives was made in 50 μl of 50% trifluoroacetic acid in water at 55°C for 50 min, and the solution dried in vacuo.

2.3. *Edman degradation with DNSAPITC and coupling yields*

2–4 nmol insulin B chain (oxidized) were dissolved in 80 μl of 50% pyridine in water, treated with 2 μmol DNSAPITC in 40 μl pyridine and incubated at 55°C for 30 min, then a further 2 μmol DNSAPITC in 40 μl pyridine were added and incubated at 55°C for 20 min. After the first coupling, 5 μl PITC were added and incubated at 55°C for 10 min. The excess of reagent and the byproducts were removed by extraction with 4 × 200 μl *n*-butyl acetate/benzene (2:1, v/v). The water phase was dried in vacuo. Cleavage was performed in 50 μl anhydrous trifluoroacetic acid for 10 min at 55°C. After drying in vacuo the remainder was dissolved in 40 μl water and the thiazolinone derivatives extracted with 60 μl *n*-butyl acetate. Conversion to the DNSAPTH-amino acid derivatives was done in 50% trifluoroacetic acid/water for 45 min at 55°C.

The coupling yields for the degradation with DNSAPITC were determined by replacing PITC by the new reagent at the respective cycle and measuring the overlap in the next step by reaction with PITC and quantitative analysis of the PTH amino acid by isocratic HPLC. The conditions for the identification were according to [9]. The HPLC column (250 × 4 mm) used was LiChrosphere Super C8 (4 μm, Merck). The eluent was mixed as follows: 195 ml 2-propanol (LiChrosolv grade, Merck) were made up to 1 l with water (Millipore Mill-Q system equipped with ION-EX and Super-C carbon cartridges) and degassed. Then 10 ml tetrahydrofuran, 10 μl sodium azide (4 mg/ml) and 5 ml of 1 M sodium acetate, pH 5.3, were added and sonicated for 10 min.

3. RESULTS AND DISCUSSION

The synthesis of DNSAPITC as given in fig.1 yielded the reagent in chromatographically pure

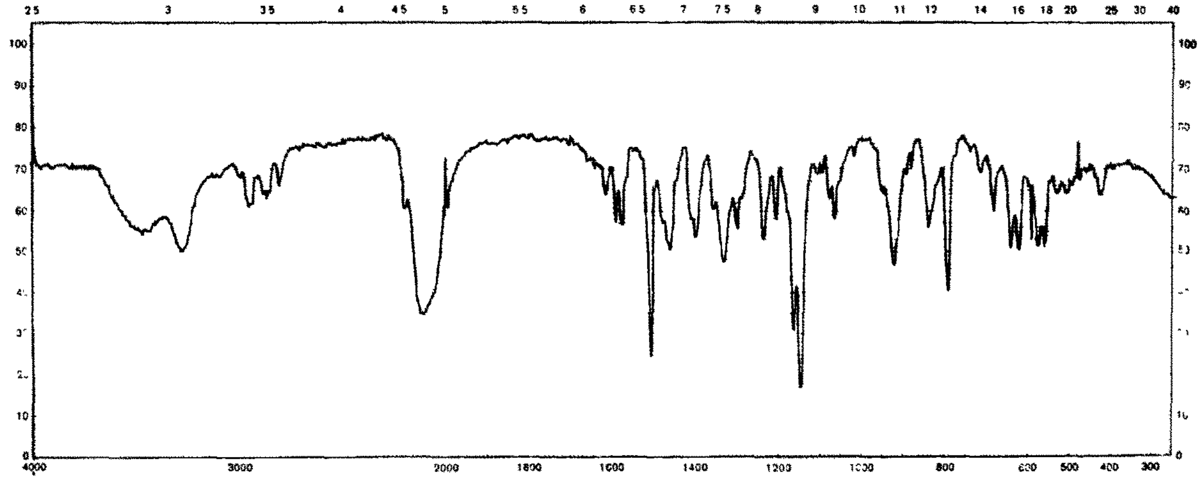

Fig.2. Infrared spectrum of DNSAPITC.

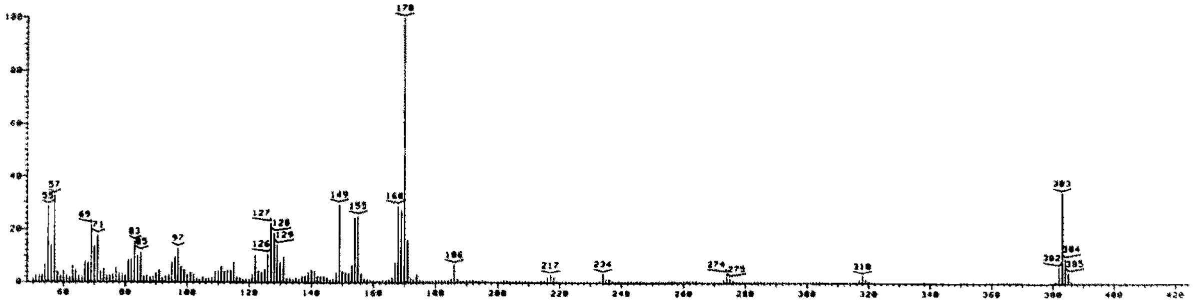

Fig.3. Mass spectrum of DNSAPITC.

form as proved by TLC. In addition, the product was characterized by its IR and mass spectra (see figs 2 and 3).

In the IR spectrum the characteristic antisymmetrical and symmetrical valence bond resonances of the isothiocyanate group appear as bands at 2100 and 920 cm^{-1}.

The electron impact mass spectrum of the DNSAPITC reagent shows a molecular ion peak at mass number 383. The fragment peak at mass number 234 represents $(CH_3)_2N$-naphthyl-SO_2^+ and the mass number 149 that of HN^+-phenyl-NCS.

Table 1

Coupling yields (%) of DNSAPITC and PITC for the degradation of insulin B chain (cycle 1–3) in the liquid and solid phase

		DNSAPITC		PITC (liquid phase) [5]
		Liquid phase	Solid phase	
Cycle 1	Phe	95	–	92
Cycle 2	Val	86	63	80
Cycle 3	Asn	75	63	89

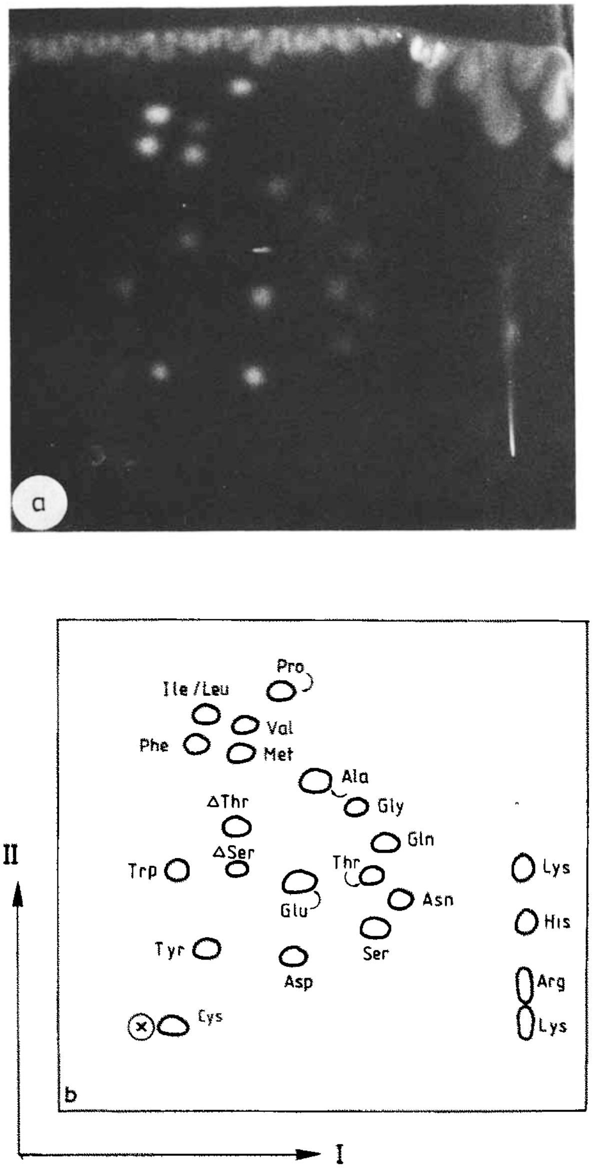

Fig.4. Two-dimensional TLC of DNSAPTH-amino acid derivatives. Reference DNSAPTH-amino acids were separated on 5 × 5 cm polyamide sheets, 50 pmol each, employing the following solvent mixtures: first dimension chromatography, water/acetonitrile/formic acid (5:1:0.5, v/v); second dimension, benzene/acetic acid (2:1, v/v). The lowest detectable amounts were 1–5 pmol of the DNSAPTH derivatives. (a) Two-dimensional chromatogram under exposure to UV light at 366 nm, and (b) correlation of the spots to the individual DNSAPTH-amino acid derivatives.

To compare the coupling efficiency of DNSAPITC in the Edman degradation with that of PITC, insulin B chain (oxidized) was sequenced manually in the liquid- and solid-phase mode. DNSAPITC was found to degrade polypeptides with repetitive yields similar to those of PITC. The results of the degradations are presented in table 1. As can be seen from this table the coupling efficiency in the liquid phase is higher than in the solid mode as tested on insulin B chain coupled to aminopropyl glass [10]. This result might reflect the decreased coupling as a consequence of steric hindrance. Another possibility would be that the reactivity of the polar isothiocyanate homologue with the peptide embedded within a hydrophobic surrounding at the glass surface is reduced.

The DNSAPTH-amino acid derivatives can be well resolved by two-dimensional TLC on polyamide sheets as is demonstrated in fig.4. The following solvent systems gave good separations for these amino acid derivatives (see fig.4): solvent system I, water/acetonitrile/formic acid (5:1:0.5, v/v); solvent system II, benzene/acetic acid (2:1, v/v). Using these solvent mixtures we were able to sequence insulin B chain for 20 cycles, employing the manual liquid-phase mode.

At present, the new DNSA reagent is being tested for its use in automated solid-phase sequencing and other fluorescent Edman-type reagents are being synthesized.

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