4-(*N-tert*-Butyloxycarbonylaminomethyl)-phenylisothiocyanate: its synthesis and use in microsequencing

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The chemical synthesis of 4-(*N-tert*-butyloxycarbonylaminomethyl)-phenylisothiocyanate starting from 4-nitrobenzylamine is described. This derivative represents an Edman-type reagent with a masked amino group which renders the thiohydantoin upon deblocking susceptible to fluorogenic detection. The coupling efficiency is determined in comparison to degradations with PITC, DABITC and FITC. The detection sensitivity on thin layer chromatograms is compared to the thiohydantoins derived from DABITC.

Edman degradation *Coupling* *Fluorescence* *Isothiocyanate* *Synthesis* *Microsequencing*

1. INTRODUCTION

The Edman degradation of peptides and proteins with phenylisothiocyanate (PITC) represents the classical standard technique for protein sequencing. The rather low sensitivity in detection of the degradation products in thin-layer chromatographic systems as well as recurrent background problems in high-performance liquid chromatography (HPLC) systems based on UV detection at high sensitivity levels has prompted for many years studies for the application of new PITC derivatives. Thus, the attachment of a dimethylaminoazobenzene (DAB) chromophore to the Edman reagent in conjunction with a double-coupling procedure (i.e., first coupling with DABITC followed by a second coupling with standard PITC) expanded the range and reliability of microsequencing considerably in the manual as well as the automated mode [1–3]. Recently, authors in [4] proposed a novel class of phenylisothiocyanates carrying in 4-position a *tert*-butyloxycarbonyl (tBOC) protected aminomethyl group which is unmasked upon cleavage, rendering the resulting aminothiohydantoin susceptible to fluorogenic detection with *o*-phthalaldehyde or fluorescamine. For the 4-(*N-tert*-butyloxycarbonylaminomethyl)-phenylisothiocyanate (BAMPITC) a 2.5-fold increase in sensitivity over UV detection at 254 nm is claimed. The synthesis and chemical characterization of this compound has not been reported. Here we describe a simple 3-step synthetic route to this derivative starting from commercially available chemicals. We present quantitative data for the degradation with this reagent and compare its properties to those of other isothiocyanates.

Abbreviations: BAMPITC, 4-(*N-tert*-butyloxycarbonylaminomethyl)phenylisothiocyanate; tBOC, *tert*-butyloxycarbonyl; DAB, 4-*N*,*N*-dimethylaminoazobenzene; DABITC, 4-*N*,*N*-dimethylaminoazobenzene 4′-isothiocyanate; DABTH, 4-*N*,*N*-dimethylaminoazobenzene 4′-thiohydantoin; DCCI, dicyclohexylcarbodiimide; FITC, fluorescein isothiocyanate; TFA, trifluoroacetic acid; PITC, phenylisothiocyanate

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2. MATERIALS AND METHODS

4-Nitrobenzylamine was purchased from EGA-

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Chemie (Steinheim, FRG). Di-*tert*-butyldicarbonate was obtained from Fluka (Eschborn) and palladium catalyst (10% Pd/C) from Merck (Darmstadt). Infrared spectra were recorded with a Beckman Acculab 6 spectrophotometer. Mass spectra were recorded with a Varian MAT 44S instrument equipped with a SS 200 data system for electron impact ionization and chemical ionization with isobutane. Fast atom bombardment spectra were obtained with a MAT 311 instrument in a methanol/glycerol system.

2.1. *Synthesis of 4-(*N-tert-*butyloxycarbonyl)-nitrobenzylamine (I)*

The tBOC group was introduced with the help of di-*tert*-butyl dicarbonate [5].

5.6 g 4-Nitrobenzylamine (30 mmol) were dissolved in a mixture of 30 ml 1 M NaOH, 15 ml $NaHCO_3$ saturated solution and 20 ml dimethyl formamide. 9 g di-*tert*-butyl dicarbonate (41.3 mmol) in 30 ml *tert*-butanol were added. After 2 h stirring at room temperature the reaction mixture was diluted with water (75 ml) and extracted twice with two portions of 60 ml petrol ether. The aequous phase was acidified with citric acid to pH 2 and extracted with 3 × 50 ml-vols of ethyl acetate. The combined extracts were washed with saturated NaCl-solution and dried over $MgSO_4$. After evaporation a crude residue resulted which was recrystallized from ethyl acetate/petrol ether. Yield: 6.7 g (88.4%), m.p. 102–103°C. R_f(solvent A) = 0.65, R_f(solvent B) = 0.94. In the electron impact mass spectrum the product showed a molecular ion M^+ = 252.

Elemental analysis: $C_{12}H_{16}N_2O_4$	C	H	N
(Mol. mass 252.3) Calculated:	57.12%	6.40%	11.10%
Found:	57.33%	6.60%	11.20%

2.2. *Synthesis of 4-(*N-tert-*butyloxycarbonyl)-aminobenzylamine (II)*

3 g 4-(*N-tert*-butyloxycarbonyl)-nitrobenzylamine (11.9 mmol) were hydrogenated in methanolic solution with palladium as catalyst (10% Pd/C, 0.15 g) at room temperature. The reaction was monitored by thin-layer chromatography and stopped after ~3 h. After filtration the reaction mixture was carefully concentrated by rotary evaporation with the bath temperature kept as low as possible. The amino derivative (II) was obtained as an oil (2.64 g; R_f(solvent A) = 0.12, R_f(solvent B) = 0.88). In the fast atom bombardment mass spectrum the product showed a molecular ion $[MH]^+$ = 223 and the complex $[MH-glycerol]^+$ = 315.

2.3. *Synthesis of 4-(*N-tert-*butyloxycarbonyl-aminomethyl)-phenylisothiocyanate (BAMPITC, III)*

The isothiocyanate group was introduced according to [6]. 2.64 g of 4-(*N-tert*-butyloxycarbonyl)-aminobenzylamine (11.9 mmol) were dissolved in 50 ml anhydrous pyridine and 5.7 ml carbon disulfide (84 mmol) and 2.45 g dicyclohexyl carbodiimide (11.9 mmol) were added. The precipitate of dicyclohexyl thiourea was removed by filtration and the solution concentrated by rotary evaporation. The product was dissolved in anhydrous benzene and passed through a silica gel column (4.5 × 40 cm, silica gel 60, 230–400 mesh, Merck) with benzene as eluent to remove residual traces of dicyclohexyl thiourea and other impurities. On removal of the solvent the isothiocyanate crystallized in pure form. Yield: 1.75 g (56%), m.p. 113–114°C. R_f(solvent A) = 0.66, R_f(solvent B) = 0.97, R_f(solvent C) = 0.94.

Elemental analysis: $C_{13}H_{16}N_2O_2S$	C	H	N
(Mol. mass 264.4) Calculated:	59.06%	6.11%	10.60%
Found:	59.19%	6.11%	10.59%

In the electron impact mass spectrum the product showed a molecular ion peak M^+ = 264 and in the fast atom bombardment mass spectrum the corresponding peaks $[MH]^+$ = 265 and $[MH\text{-glycerol}]^+$ = 357.

2.4. *Edman degradations and coupling yields*

For the determination of coupling yields manual degradations in the liquid phase were performed in the following way: 10 nM insulin B-chain (oxidized) were dissolved in 120 μl 67% pyridine and incubated with 10 μl PITC (83.4 μmol) for 30 min at 55°C. Excess reagent was removed by extraction with benzene. Cleavage was performed with TFA for 10 min at 55°C. The thiazolinones were extracted with ethyl acetate and converted to phenyl thiohydantoins with 40% TFA for 30 min at 55°C. Phenyl thiohydantoins were quantitatively determined by HPLC with an isocratic system [7]. The coupling yields for the degradations with

BAMPITC were determined by replacing PITC by this reagent at the respective cycles and measuring the overlaps in the next cycles by reaction with PITC and analysis of the phenyl thiohydantoins. The coupling step was performed by adding BAMPITC (4 μmol) dissolved in 40 μl pyridine in two portions at a 30 min interval to the insulin B-chain sample contained in 80 μl 50% pyridine, thus arriving at a final pyridine concentration of 75% in the assay. The total incubation time was 1 h.

In automated solid phase sequencing with DABITC/PITC [3] the first cycle was performed with only PITC in order to avoid incorrect coupling yields due to excess amino propyl groups originating from the solid support. In the second cycle the second coupling with PITC was omitted, i.e., coupling was performed exclusively with BAMPITC or DABITC. Overlaps in the third cycle for coupling yield calculation were determined by HPLC analysis of the DAB-thiohydantoins [8].

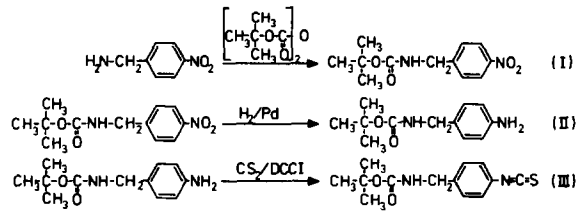
The coupling yield for an amino acid residue was defined as the percentage of thiohydantoin obtained after subtraction of overlap.

2.5. *Thin-layer chromatography*

Synthetic intermediates were analyzed on silica gel plates (Merck 60F 254) in the following solvents: A. Cyclohexane/chloroform/acetic acid (45:45:10, v/v); B. Chloroform/methanol/acetic acid (19:5:1, v/v); C, *n*-Butanol/acetic acid/water (4:1:1, v/v). The derivative that has been released from cycle 4 of the oxidized insulin B-chain (Gln) obtained from automated solid-phase sequencing was subjected to thin-layer chromatography on a silica gel microplate (3.5 × 3.5 cm) in 1.5% formic acid and detected by its fluorescence after dipping of the plate into 10% triethylamine in dichloromethane (v/v) followed by 0.027% fluorescamine in acetone (w/v) and repeated treatment with triethylamine after intermittent drying (R_f = 0.5). The DAB thiohydantoin from cycle 6 (Leu) was separated with 33% acetic acid as solvent (R_f = 0.2).

3. RESULTS AND DISCUSSION

The BAMPITC derivative was synthesized by the following reaction scheme:



The final product was obtained in good yield in chromatographically pure form with an elemental composition approaching theoretical values. The BAMPITC derivative was, in addition, characterized by its infrared (IR) and mass spectra (fig.1,2). In the IR spectrum the characteristic antisymme-

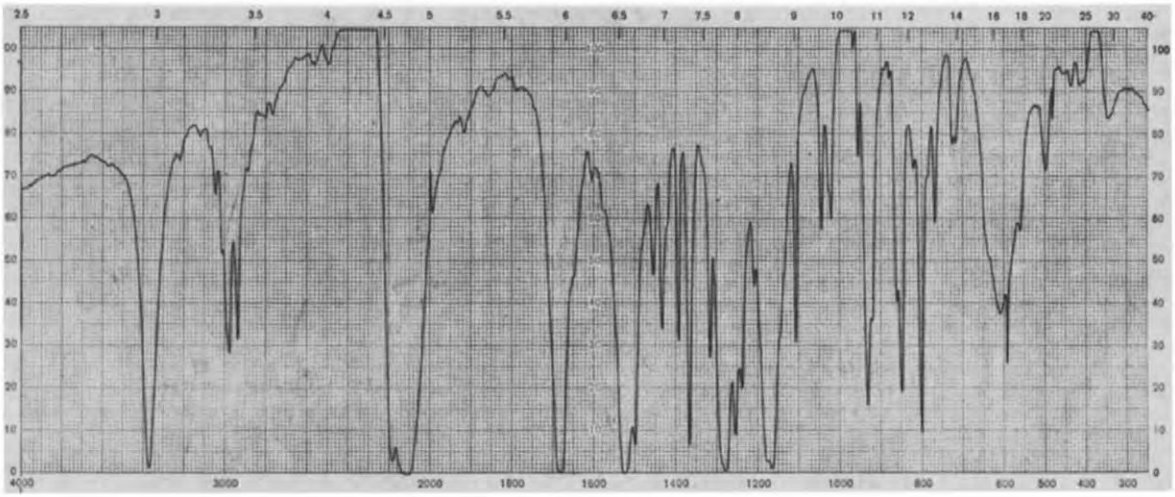


Fig.1. IR spectrum of BAMPITC (KBr).

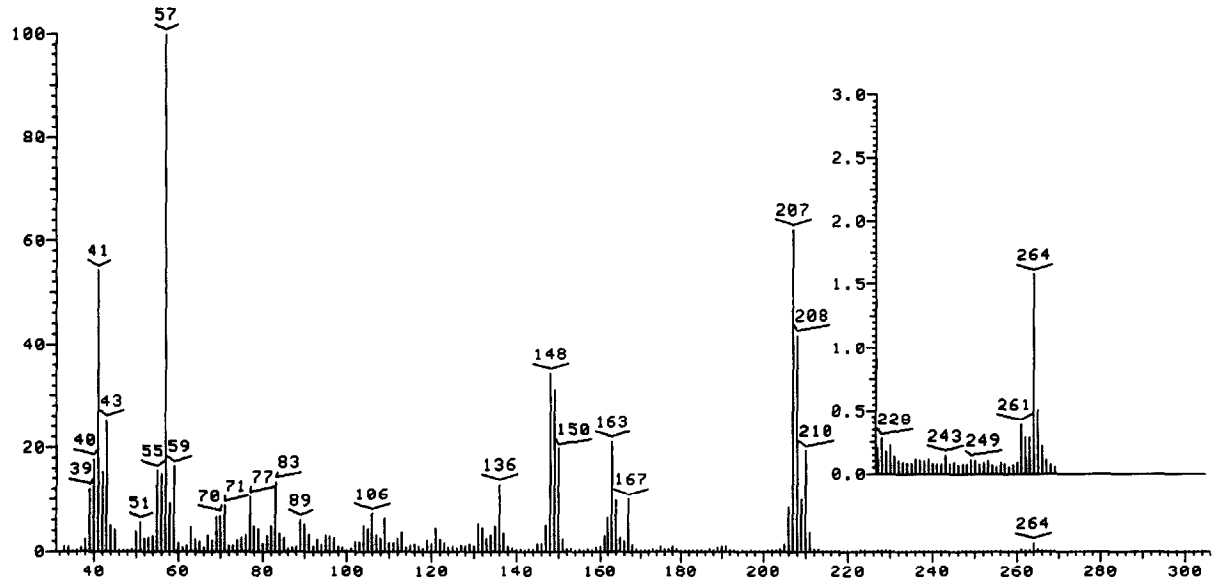


Fig.2. Electron impact mass spectrum of BAMPITC.

trical and symmetrical valence bond resonances of the isothiocyanate group appear as bands at 2120 cm^{-1} and 930 cm^{-1}. The band at 2980 cm^{-1} belongs to the aromatic system, whereas the bands at 1680 and 3370 cm^{-1} are typical for resonances of the amide bond present in this compound.

The electron impact mass spectrum recorded for the BAMPITC derivative shows a molecular ion peak at the mass number 264. The major fragment peak at mass number 207 represents the ion SCN-C_6H_4-CH_2-NH-CO-O^+, accordingly, mass number fragments 163 and 148 can be assigned to degradation products thereof, namely SCN-C_6H_4-CH_2-NH^+ and SCN-C_6H_4-CH_2^+. The strongest peak with the mass number 57 represents the $C_4H_9^+$ fragment. Chemical ionization mass spectra yielded a molecular ion peak $[MH]^+ = 265$ which was also observed in the fast atom bombardment spectrum in addition to the complex $[MH-glycerol]^+ = 357$.

To compare the coupling efficiency of BAMPITC in the Edman degradation to PITC and DABITC, insulin B-chain (oxidized) was sequenced manually in the liquid phase without attachment to a solid support, as well as in the automated solid phase sequencer covalently bound to amino propyl glass via carbodiimide. According to [4] BAMPITC could be used in sequencing with the same efficiency as PITC. In the manual degradation test (table 1) coupling yields for BAMPITC identical to PITC could be obtained for the first (Phe) and third (Asn) residues by doubling of coupling time. Since qualitative stability tests of BAMPITC by thin-layer chromatography have shown that under coupling conditions the reagent is about half decomposed within 1 h, coupling was performed with two portions in two 30-min steps. Valine from the second position, however, showed more overlap in the third cycle compared to PITC.

The coupling efficiency of BAMPITC in automated solid phase sequencing in comparison to DABITC was tested by omitting the second coupling with PITC and measuring the overlap apparent in the following cycle. As can be seen from table 1 DABITC alone yields under reduced coupling time and temperature (20 min, 50°C) about 80% coupling, necessitating complete reaction with PITC in a second step for clean-cut degradations as proposed in the DABITC/PITC double-coupling procedure [2]. Under identical conditions BAMPITC yields only 48% coupling. From these results we conclude that BAMPITC can be useful in automated sequencing only in combination with PITC if extended sequencing is desired. Apparently efficient coupling is sterically hindered by bulky adjuncts like the tBOC- or DAB-groups. Even lower reactivity has been observed for the

Table 1

Coupling yields with the first 3 residues of insulin B-chain (oxidized) for different isothiocyanates

	PITC	BAMPITC	BAMPITC	DABITC
	Manual sequencing, liquid phase		Automated sequencing, solid phase	
1. Cycle: Phe	92%	90%	–	–
2. Cycle: Val	80%	75%	48%	80%
3. Cycle: Asn	89%	92%	not determined	
Coupling conditions	30 min 55°C	60 min 55°C	20 min 50°C	

fluorescein isothiocyanate (FITC) for which complete coupling could not be obtained under any conditions. Moreover, in contrast to PITC, these reagents with reduced coupling efficiency react apparently towards different amino acid residues no longer uniform, as has been observed above for valine in the manual coupling test.

For high sensitivity detection complete removal of the tBOC group is mandatory. Under the cleavage conditions of the Edman degradation deblocking of the amino groups is quantitative as tested by thin-layer chromatography of the reagent itself (not shown).

Authors in [4] report that the 4-aminomethyl phenyl thiohydantoins after HPLC and post-column reaction with fluorescamine or *o*-phthalaldehyde give strong fluorescence down to the 1 pmol level. Since, on the other hand, 1–2 pmol of the DABTH are equally detectable by HPLC [9], it appears that the sensitivity range of the BAMPITC derivative is equal or slightly better compared to the DABTH. We examined the detection limits for both thiohydantoins by thin-layer chromatography in the following way: amino propyl glass-bound insulin B-chain (~2 nmol) was degraded for 4 cycles in the solid phase sequencer with BAMPITC/PITC double coupling followed by DABITC/PITC for the next steps. The derivative that has been released from cycle 4 (Gln) was subjected in concentrations from 1 to 40 pmol (0.05–2% of the sample) to thin-layer chromatography using silica gel plates and visualized by reaction with fluorescamine. In parallel, the DABTH from cycle 6 (Leu) was examined for comparison at the same dilutions. Whereas 1–2 pmol of the fluorescent derivative were still clearly visible, the limit of detection for the DABTH as estimated by visual inspection was 10–20 pmol, about one magnitude of order lower. The DABTH microchromatography is, however, 5 times more sensitive if polyamide microplates are used [1,2]. Attempts to adapt this technique for the 4-aminomethyl phenyl thiohydantoins failed because of generation of highly fluorescent background on spraying the polyamide plates with fluorescamine.

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