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PROTEIN MICROSEQUENCING WITH POST-COLUMN FLUORESCENT PHENYLISOTHIOCYANATE ANALOGUES*

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SUMMARY

A novel class of isothiocyanates has been introduced for protein microsequencing. These modified phenylisothiocyanates (PITC) are substituted in the 4-position of the phenyl ring with a protected amine and have a general structure *tert.*-Boc-NH-$(CH_2)_n$-PITC. The most important feature of these compounds is that a primary amine is generated during the acid cleavage step of the sequencing process. This primary amine is then available for fluorescent labeling at the time of identification to increase the detectibility of the modified phenylthiohydantoin (PTH)-amino acid.

Preliminary studies were performed on aniline, benzylamine and phenethylamine to evaluate the optimal mode for fluorescent post-column detection. Following synthesis of 4-(Boc-amino)PITC and 4-(Boc-aminomethyl)PITC, these compounds were found to sequence polypeptides with repetitive yields similar to PITC. Preliminary studies on the fluorescence detection of the aminomethylPTH-amino acids showed at least a 2.5-fold increase in sensitivity over the most commonly used detection method, UV detection at 254 nm. These results indicate that this approach may provide a general method for picomole and subpicomole protein sequence analysis using currently available instrumentation.

INTRODUCTION

Recent advances in protein microsequencing have focused on the optimization of sequencer function coupled with improvements in the direct identification of phenylthiohydantoin amino acids (PTHs) by reversed-phase high-performance liquid chromatography (RP-HPLC). These developments have enabled protein sequence analysis to be carried out in the 10–500 pmole range in several laboratories, including our own[1–4]. Further improvements in the $\leqslant 1$ pmole range are necessary, however, to

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perform sequence analysis on the amounts of material that can be purified by such microanalytical techniques as two-dimensional gel electrophoresis.

The most direct approach to this level of protein microsequencing is to enhance the sensitivity of PTH identification for direct analysis by RP-HPLC. Previous attempts to increase the detectibility of PTHs have included the addition of ring structures to the phenylisothiocyanates (PITC). The use of compounds such as 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate (DABITC)[5] has been confounded by problems that include compound instability, poor solubility in the solvents compatible with automated sequencers and perhaps a decreased rate of reaction in the formation of the phenylthiocarbamyl polypeptide derivative. This has resulted in no net enhancement of sensitivity over PITC when these reagents are used in automated sequencers (compare the results in ref. 6 with those in refs. 1–4).

Many of the problems outlined here may be eliminated by not introducing the additional moieties necessary to increase the detectibility until identification of the PTH-amino acids by RP-HPLC. Here we describe some preliminary studies on the use of a novel class of isothiocyanates for protein microsequencing which are based on this concept. These compounds, with the general structure *tert.*-Boc-NH-$(CH_2)_n$-PITC where $n = 0$ or 1, react similarly to PITC in the Edman degradation (Fig. 1). When the new reagent is used, however, the acid cleavage step not only catalyses the removal of the amino-terminal amino acid but also deprotects the amino

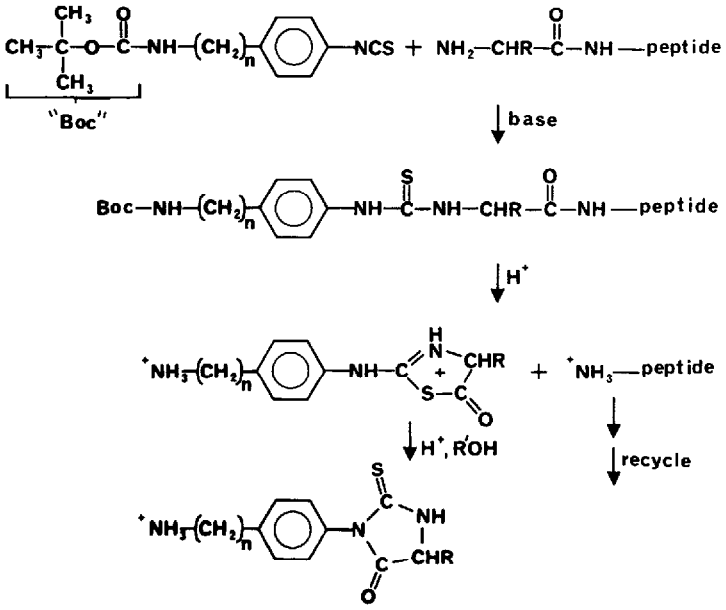

Fig. 1. General scheme of the Edman degradation using the modified PITC reagents. The first step of this two-step process is the coupling of the PITC analog to the α-amino group of the polypeptide to form a Boc-amino-substituted phenylthiocarbamoyl peptide. In a subsequent step, an acid-catalyzed cyclization reaction takes place, causing cleavage at the first peptide bond. At the same time, the acid removes the Boc group giving an amino-substituted thiazolinone and generating a new amino terminal group on the peptide. Following conversion of the amino-substituted thiazolinone to a thiohydantoin (PTH), the primary amine is available for reaction with fluorogenic reagents to increase the detectability of the PTH for identification.

group on the phenyl ring. This amino group is then available for fluorescent labeling following completion of the sequencing cycle to increase the sensitivity of identification of the PTH-amino acids.

EXPERIMENTAL

Apparatus

The various PTH amino acid derivatives were identified using a Waters Assoc. HPLC system, which consisted of a pump (Model 6000A), an automatic injector (WISP) and an integrator/printer/plotter (Model 730, Data Module). The column effluent was detected with a UV detector (Waters Assoc. Model 440 or 441) prior to post-column fluorescence detection. The post-column system consisted of a reagent delivery pump (Waters Assoc. Model 6000A), a mixing tee which was placed after the UV detector, a delay coil and a flow fluorimeter (Gilson Model 121). The fluorimeter filters used for orthophthalaldehyde (OPA) were a Corning 7-60 for excitation and 450-7C for emission. With fluorescamine, Corning 7-51 and 3-71 filters were used.

Materials

The materials used in this study were of analytical-reagent grade, HPLC grade or the best grade available. Where the choice of supplier may affect the result, the preferred source is listed. The Boc-aminophenylisothiocyanate (BAMPITC) and Boc-aminomethylphenylisothiocyanate (BAPITC) were synthesized in house and will be the subject of another paper.

Methods

Reversed-phase separations of the PTH-amino acid derivatives were performed on a Waters Assoc. μBondapak C_{18} column (30 × 0.39 cm I.D.) under isocratic conditions with 10 m*M* potassium phosphate (at either pH 2.5 or 4.1) that contained 10–30% of either acetonitrile (Baker, HPLC grade) or methanol (HPLC grade). When OPA was used for post-column fluorescent detection, the reagent was prepared by dissolving 400 mg of OPA (Pickering Labs.) in 2 ml of methanol (Baker, reagent grade) that contained 0.5 ml of β-mercaptoethanol. This OPA solution was then mixed into 400 ml of helium-degassed potassium borate buffer (either OPA diluent from Pickering Labs. or 800 m*M* boric acid adjusted to pH 10.5 with potassium hydroxide) and kept under a helium atmosphere while in use. Brij 35 (Pierce, 30% solution) was added (1.5 ml per 400 ml of buffer) in some experiments.

The post-column fluorescamine reagent was freshly prepared each day by dissolving 15 mg of fluorescamine (Pierce) in 100 ml of acetonitrile (Baker, reagent grade) under helium. Following dissolution of the fluorescamine, 150 μl of triethylamine (redistilled off ninhydrin) were added and the solution kept under helium during use.

The solid-phase sequencing methods and reagents were as previously described[4,7].

RESULTS

Detection

A series of model compounds were studied to demonstrate that fluorescence detection of the $NH_2(CH_2)_n$-PTH derivatives would be possible at the low picomole and subpicomole levels. The compounds chosen (aniline, benzylamine and phenethylamine) mimic the deprotected amino group that is generated from the parent compound during sequencing (Fig. 1). The model compounds were separated by RP-HPLC and detected by measuring the absorbance at 214 nm prior to post-column derivatization with either OPA[8] or fluorescamine[9].

When OPA without Brij 35 in the buffer was used for post-column derivatization under the standard conditions, aniline failed to show any appreciable fluorescence while benzylamine and phenethylamine gave a strong response (Fig. 2A). When Brij 35 was added to the post-column reagent and all other conditions were kept the same, the aniline again showed no fluorescence, but the benzylamine peak was reduced in size while the phenethylamine peak remained unchanged (Fig. 2B).

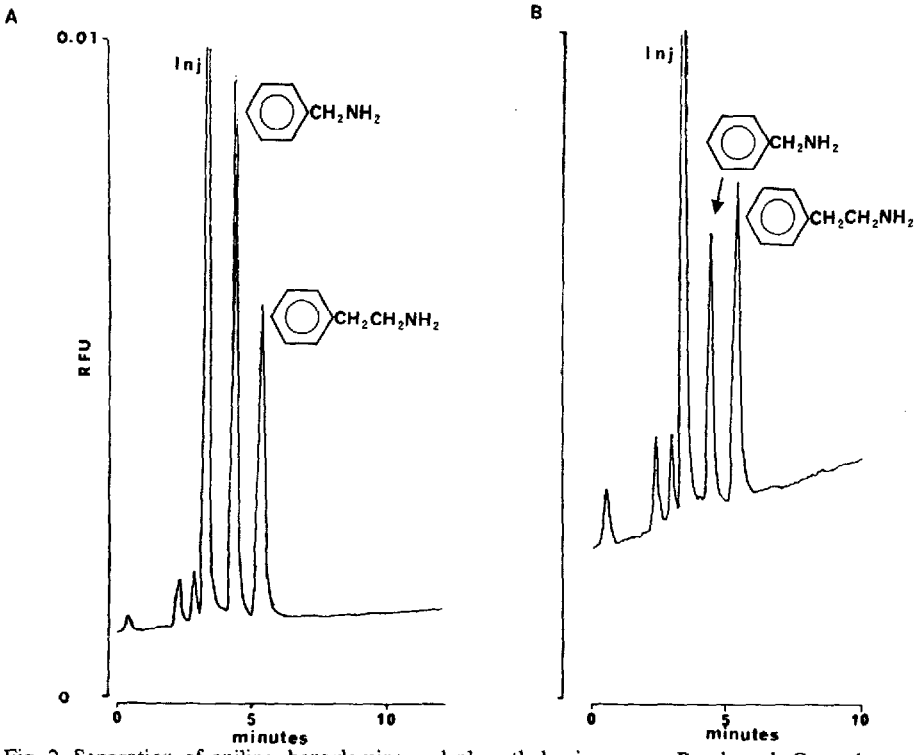

Fig. 2. Separation of aniline, benzylamine and phenethylamine on a μBondapack C_{18} column using a mobile phase of 20% methanol in 10 m*M* potassium phosphate (pH 2.5) at a flow-rate of 1 ml/min (column pressure 2000 p.s.i.). Following UV detection at 214 nm (trace not shown), the column effluent was mixed with OPA reagent (1.0 ml/min) prior to fluorescence detection [OPA filters, 0.01 Relative Fluorescence Units (RFU)]. In (A), OPA without Brij 35 was prepared as described, whereas in (B) Brij 35 was added to the OPA reagent. Aniline (which eluted between the injection peak and benzylamine) did not react with OPA under these conditions to give a fluorescent product detectable using the OPA filters. Benzylamine showed a decreased fluorescence yield under these conditions when Brij 35 was added to the OPA reagent.

Post-column derivatization with fluorescamine was then investigated to determine its feasibility with the same model compounds. It was found that a single tertiary amine-containing reagent solution could be used in place of the normal two-reagent mixing method. Over a period of days, a large increase in background fluorescence was observed. This was readily accommodated by the fluorescence detector and integrator/printer/plotter used and caused no decrease in the signal-to-noise ratio or the sensitivity of detection at the picomole level.

The pH of the aqueous phase of the column elution buffer was increased to be compatible with the single-reagent fluorescamine detection system. This caused

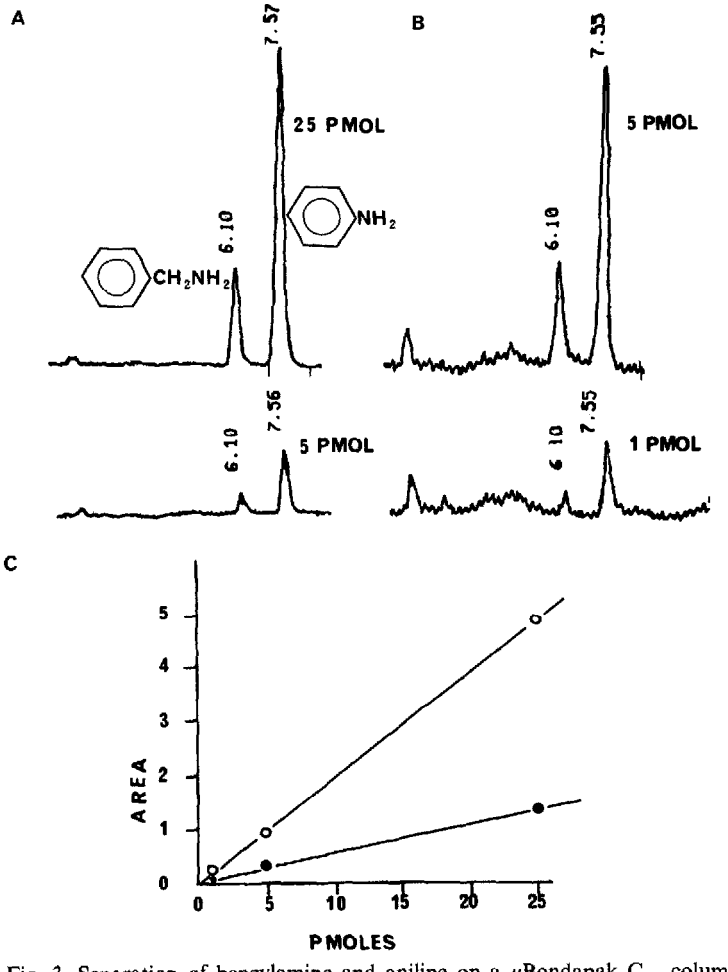

Fig. 3. Separation of benzylamine and aniline on a μBondapak C_{18} column using 10 m*M* potassium phosphate (pH 4.1) with 10% acetonitrile as the mobile phase at a flow-rate of 0.8 ml/min. Following UV detection (trace not shown), the column eluate was mixed with a fluorescamine solution (as described in the text) which was pumped at a flow-rate of 0.4 ml/min. Under these buffer conditions the order of elution of the compounds was altered from that described in Fig. 2, with benzylamine eluting before aniline. In (A) the fluorimeter was set at 1.0 RFU and in (B) at 0.2 RFU. In (C) the integrated peak areas from (A) and (B) were plotted against the concentration injected. Both benzylamine (●) and aniline (○) show a linear response which passes through the origin.

changes in the order of elution of the model compounds: benzylamine eluted before aniline, which co-eluted with phenethylamine. Thus only benzylamine and aniline were used in these experiments. As can be seen in Fig. 3A and B, both benzylamine and aniline react with fluorescamine to give strongly fluorescent derivatives at the 25, 5 and 1 pmole levels. Further, the fluorescence yields as a function of concentration of these compounds are linear and pass through the origin (Fig. 3C).

Sequencing

After demonstrating that both aniline and benzylamine were capable of fluorescent derivatization and detection at the picomole level, the corresponding isothiocyanates were synthesized. The sequencing efficiency of *tert.*-BOC-aminomethylphenylisothiocyanate (BAMPITC) was then studied by simply substituting a 5% solution of this compound in acetonitrile into the PITC reservoir of an unmodified solid-phase sequencer. A simple model peptide was sequenced with PITC and then with BAMPITC in a separate run. The PTH derivatives resulting from these runs were separated by RP-HPLC as described under *Methods*, using an isocratic eluent. The aminomethyl-PTH derivatives eluted earlier than the corresponding PTH derivatives owing to the influence of the protonated 4-aminomethyl moiety. A comparison of the repetitive yields from these two consecutive sequencer runs is made in Table I. These results show that the BAMPITC sequenced with the same efficiency as PITC under the conditions used here. In separate experiments, BAPITC showed identical sequencing efficiency to BAMPITC and PITC (data not shown). The BAMPITC was as stable as PITC under the conditions employed in the automated sequencer. This was determined by measuring repetitive yields *versus* time at room temperature for solutions of both compounds.

The sensitivities of detection of the aminomethyl-PTH amino acids by UV absorbance and by fluorescence from post-column derivatization were compared. As can be seen in Fig. 4, the fluorescence trace gave increased sensitivity over the ab-

TABLE I

YIELDS OF PTH-AMINO ACIDS FROM SEQUENCING

Identical amounts of the peptide Leu–Ala–Gly–Val–Leu–Ala–Gly–Val–Phe covalently attached to polystyrene beads were sequenced as described in the text. PTH derivatives were detected and quantitated by their UV absorbance at 254 nm. The repetitive stepwise yield given is the average calculated by comparing the yields in cycles 1 and 5, 2 and 6, 3 and 7, and 4 and 8.

Amino acid (cycle)	*PITC*		*BAM-PITC*	
	Elution time (min)	*Amount (area units)*	*Elution time (min)*	*Amount (area units)*
Leu (1)	30.13	384	16.20	432
Ala (2)	9.65	426	6.45	479
Gly (3)	7.52	500	5.70	326
Repetitive stepwise yield	92.5%		91.5%	

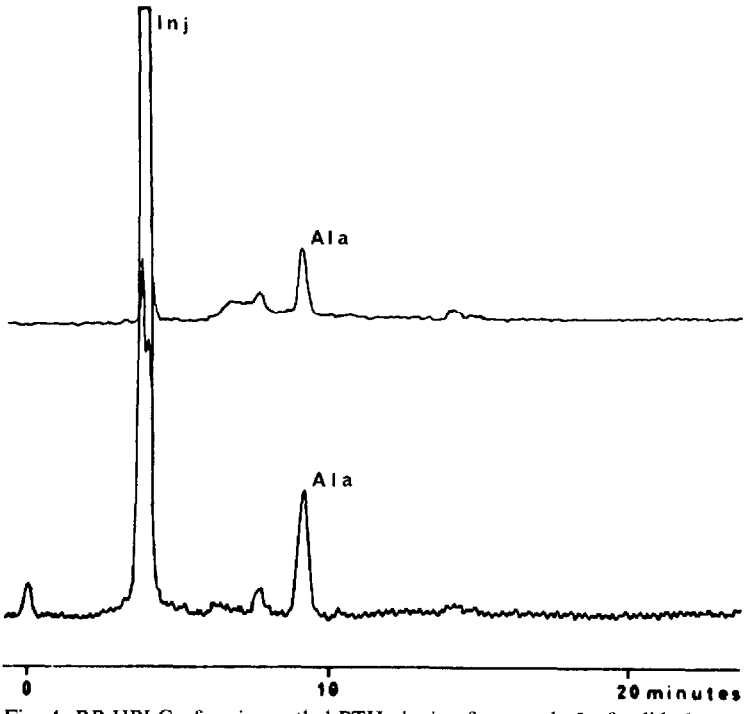

Fig. 4. RP-HPLC of aminomethyl-PTH-alanine from cycle 2 of solid-phase sequencing of the peptide Leu–Ala–Gly–Val–Leu–Ala–Gly–Val–Phe covalently attached to polystyrene beads. The mobile phase of 10 m*M* potassium phosphate (pH 4.12) with 10% acetonitrile at 0.8 ml/min was used to separate the aminomethyl-PTHs on a μBondapak C_{18} column. Detection of the column effluent was by UV absorbance at 254 nm (upper trace, 0.005 a.u.f.s.) prior to mixing with fluorescamine reagent at 0.4 ml/min and fluorescence detection (lower trace, 0.2 RFU). The fluorescence trace showed a 2.5-fold increase in sensitivity over the UV trace.

sorbance at 254 nm. Under these conditions, the fluorescence represented a 2.5-fold increase in sensitivity over the UV trace. In addition, examination of other cycles of this and other sequencer runs showed that the strongly UV-absorbing contaminants, which commonly occur in PTH samples and which can interfere with PTH identification, were not detected by fluorescence.

DISCUSSION

The new class of isothiocyanates introduced in this report represent a novel approach to increasing the sensitivity of detection of phenylthiohydantoins resulting from the sequencing of peptides and proteins. These new compounds take advantage of the acid cleavage step in the Edman degradation to deprotect a functional group on the derivatized amino acid generated during polypeptide sequencing (Fig. 1). In a subsequent step, this functional group forms the basis for improved detectability via fluorescence following separation by RP-HPLC.

The results presented here demonstrate that Boc-aminomethylphenylisothiocyanate (BAMPITC) incorporates the most favorable aspects of PITC, such as solubility in the solvents used in automated sequencers, stability in these solvents over

time periods compatible with use in such instruments and high reactivity with polypeptide amino groups. The feasibility of PTH identification by post-column reaction with fluorogenic reagents and fluorescence detection has also been demonstrated. A significant advantage of this approach is that it allows increased detectibility of the PTH derivatives while decreasing the background due to UV-absorbing artifacts resulting from buffers and/or solvents used in automated sequencers.

In particular, the simplified fluorescamine procedure represents a significant improvement over the most commonly used methods for post-column fluorescamine detection[10]. Usually, the column effluent is first mixed with buffer to increase the pH and the the fluorescamine, dissolved in a non-hydroxylic solvent to prevent solvolysis, is added to the stream via a second mixing step. This two-step process requires two pumps for the post-column reaction system and generally leads to decreased sensitivity owing to band spreading. The method for post-column fluorescamine detection that we have introduced has several advantages: it is a simpler system to construct and considerably less expensive, and in addition should be more sensitive owing to less mixing noise and band spreading.

These initial experiments show a several-fold increased sensitivity of PTH identification compared with UV absorbance detection. The potential sensitivity of fluorescence detection is such that it is to be expected that further refinement of the approach will lead to substantial improvements beyond the levels reported here.

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