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Rapid Communication

Synthesis and Fluorescent Labeling of Beta-Amyloid Peptides

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Abstract: Fluorescent cell analytical techniques require the incorporation of a fluorophore into the target molecule without causing a significant change in the native conformation. Many short peptides have a limited number of reactive groups that can be labeled without affecting the biological activity. In this work we present several methods for labeling β-amyloid peptides (βA[25–35], βA[1–40]) and their derivatives (LPFFD, RIIGL and RVVIA) with different chromophores exclusively at the *N*-terminus. In the case of liquid-phase labeling, fluorescein isothiocyanate was used. The side-chain amino function of Lys, if present in the sequence, was protected with an Fmoc group, whereby the hydrophobic character of the peptide was further increased. The labeling reaction was carried out in an appropriate deaggregating solvent, DMSO. For solid-phase labeling, 5(6)-carboxyfluorescein and 7-amino-4-methyl-3-coumarinylacetic acid were applied. Several cleavage cocktails were tested for removal of the labeled amyloid peptides from the resin in order to completely suppress the oxidation of Met. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Alzheimer's disease; AMCA; β-amyloid; carboxyfluorescein; FITC; fluorescent labeling

THEORETICAL

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of late-life dementia. The major neuropathological feature of AD is the presence of extracellular amyloid deposits, which are aggregates of the 39–42 residue-containing β-amyloid peptides (βA). In addition to the examination of plaque formation by βA, another important field in AD research is the investigation of the binding of βAs to cell membranes in the brain, which results in various primary cellular effects. Earlier studies showed that short peptides are able to inhibit the cytotoxic action of βA peptides (βA[1–42], βA[1–40], βA[25–35]) both *in vitro* [1] and *in vivo* [2]. These results led to the design and synthesis of several functional antagonists in our laboratory. Simultaneously, on the basis of Soto's work [3], we have synthesized a series of new peptides as β-sheet breakers (BSB), which prevent aggregation that seems to be necessary for the neurotoxic action of βA peptides.

The mechanism of action of βA peptides has been studied very intensively, however, a lot of questions remained unanswered. Labeled peptides help to study the cellular action of βA. Radioactive labeling owing to biohazard problems is not preferred, therefore we tried to introduce a fluorophore molecule into the βA peptides. Fluorescent peptides can be used in flow cytometry, fluorescent microscopy, and spectrofluorimetry. Our goal was to carry out the labeling exclusively at the *N*-terminus of the peptide in order to minimize the conformation-modifying

Abbreviations: AD, Alzheimer's disease; ACN, acetonitrile; AMCA, 7-amino-4-methyl-3-coumarinylacetic acid; βA, β-amyloid; DTT, 1,4-dithio-DL-threitol; 5(6)-FAM, a mixture of 5- and 6-carboxyfluorescein; FITC, fluorescein-5-isothiocyanate; FTC-, fluorescein-thiocarbamoyl-; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; MBHA, *para*-methylbenzhydrylamine; TBAI, tetrabutylammonium iodide.

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effect of the fluorophore, which may influence the biological activity. Fluorophore molecules react with side chains of several amino acids, too; leading to polylabeled compounds [4]. As a consequence, the side chain of Lys of βA peptides should be protected during labeling.

For the labeling at the *N*-terminus of peptides, we have performed two series of experiments: in the liquid phase and in the solid phase. Fluorescein isothiocyanate (FITC, $\lambda_{ex} = 494$ nm, $\lambda_{em} = 518$ nm) is a very popular labeling reagent [4,5], however, the fluorescent end product (FTC-peptide) proved to be acid sensitive. The cleavage of fluorescein thiohydantoin from N^{α}-FTC peptides during the last step of the solid-phase synthesis (HF or TFA acidolysis) limits the use of FITC labeling in the liquid phase [6,7].

Three peptides were labeled with FITC in the liquid phase: βA[25–35], the antagonist RIIGL-amide and a new BSB peptide: RVVIA-amide (Table 1). All of these peptides were synthesized in the solid phase using Boc-chemistry and cleaved off with HF from the resin. The ε-amino group of Lys in βA[25–35] was protected with the Fmoc-group which was stable during HF cleavage. The fluorescent labeling process was very simple: the peptide, e.g. [Fmoc-Lys[28]] βA[25–35] was dissolved in DMSO, reacted with FITC overnight under a N_2-blanket at 20°C in darkness (Figure 1). DMSO solubilizes perfectly the highly aggregated βA peptides and stabilizes α-helical or random coiled conformation [8]. Using a 1.2 molar excess of peptide to FITC resulted in much purer crude product than an excess of FITC. The Fmoc-protecting group was removed using 20% piperidine added *in situ* to the reaction mixture in a 20-min reaction. Piperidine was then neutralized with equivalent amounts of TFA and the highly hydrophobic N^{α}-FTC-βA[25–35] was immediately purified by loading the reaction mixture directly onto a semipreparative HPLC-column. The labeling of the two pentapeptides was performed similarly, but without N^{α}-Fmoc protection and cleavage.

Table 1 List of Labeled Peptides

No.	Sequence	Labeling	
		Method	Reagent
1	βA[25–35]-amide	Liquid phase	FITC
		Solid phase	5(6)-FAM
		Solid phase	AMCA
2	RIIGL-amide	Liquid phase	FITC
3	RVVIA-amide	Liquid phase	FITC
4	βA[1–40]-amide	Solid phase	5(6)-FAM
5	LPFFD-amide	Solid phase	5(6)-FAM

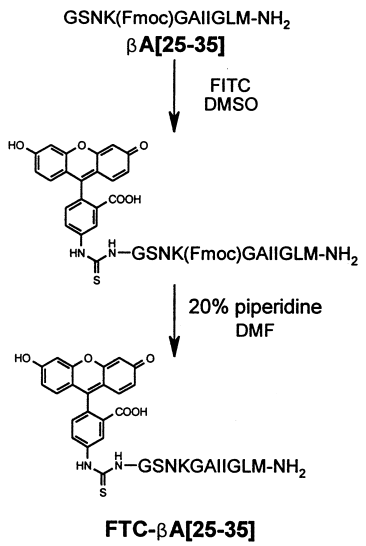

Figure 1 Liquid-phase labeling of -βA[25–35] with FITC.

Fluorescent labeling in the solid phase requires an acid-stable bond between the fluorophore and the peptide. Two fluorophores were used for labeling: carboxyfluorescein (5(6)-FAM, $\lambda_{ex} = 494$ nm, $\lambda_{em} = 518$ nm) and 7-amino-4-methyl-3-coumarinyl-acetic acid (AMCA; $\lambda_{ex} = 349$ nm, $\lambda_{em} = 448$ nm). The labeling reaction is a simple acylation of the *N*-terminal amino group of the peptide resulting in an acid stable amide bond. Regular side-chain protection strategies and Fmoc-chemistry can be used, the last steps include acylation with an excess of fluorophores in the solid phase and subsequent TFA-cleavage from the Rink-resin (Figure 2).

We have synthesized four peptides labeled with fluorescent group at the *N*-terminus by Fmoc-chemistry in the solid phase: 5(6)-FAM-βA[25–35], 5(6)-FAM-βA[1–40], 5(6)-FAM-LPFFD and AMCA-βA[25–35] (Table 1). Cleavage of Met-containing βA peptides from the resin under the usual reaction conditions resulted in the formation of a high amount of Met-sulfoxide as determined in the crude product after isolation and characterization of the by-products with ESI-MS. The ratio of the oxidized and expected peptides depended on the composition of the cleavage cocktail (Figure 3). Several mixtures were applied, which contained: DTT, 0.1–9%; phenol, 0–6%; H_2O, 9–10%; TFA, 82–90%; but even a relatively high DTT content (9%) was not able to suppress the oxidation of Met completely. The following mixture, which is a modification of the

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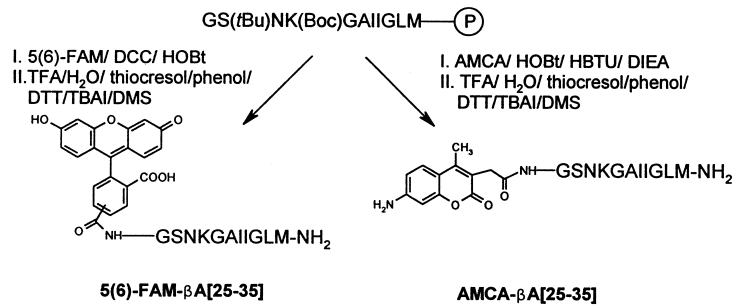

Figure 2 Solid-phase synthesis of 5(6)-FAM-βA[25–35] and AMCA-βA[25–35].

cleavage cocktail of Huang and Rabenstein [9], successfully prevented the oxidation of Met during cleavage from the resin: 81% TFA, 5% phenol, 5% thiocresol, 3% H_2O, 2.5% DTT, 2% DMS and 1.5% TBAI. With the application of this reagent mixture, no Met sulfoxide was detected in the crude peptide.

Summarizing our results, we have worked out effective methods for the fluorescent labeling exclusively at the *N*-terminus of the peptides both in liquid and solid phases with different fluorescent groups. In the liquid phase, N^{α}-Fmoc side-chain protection of Lys during labeling and subsequent removal of the Fmoc-group proved to be a good method for FITC labeling. In the solid phase, practically any kind of acid-stable carboxylated fluorophores (e.g. 5(6)-FAM, AMCA) can be used within the frame of a standard Fmoc-protocol in the solid phase, the resulting amide bond between the fluorophore and the peptide survives the acidolytic cleavage of the peptide from the resin. Met oxidation can be prevented during acidolysis using a cleavage cocktail containing DMS, DTT and tetrabutylammonium iodide.

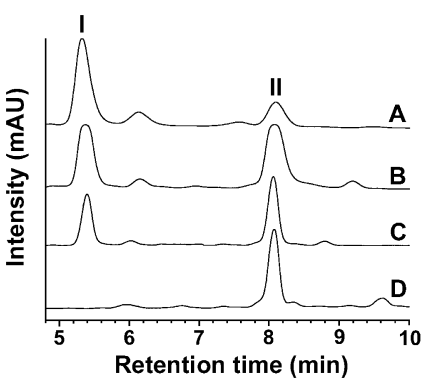

Figure 3 Portions of HPLC profiles of the crude peptide obtained after cleavage with different cleaving cocktails. Peak I (R_t (retention time): 5.3 min) is assigned to [Met(O)^{35}]5(6)-FAM-βA[25–35], while peak II (R_t: 8.0 min) is assigned to 5(6)-FAM-βA[25–35]. Cleaving cocktail (A) consisted of 90% TFA and 10% H_2O; proportion of oxidized peptide (OX) in total peptide amount is 78%. Cocktail (B) contained 85% TFA, 6% phenol, 9% H_2O, and 0.1% DTT; OX in total: 46%. Cocktail (C) contained 82% TFA, 9% DTT and 9% H_2O; OX in total: 43%. Cocktail (D) consisted of 81% TFA, 5% phenol, 5% thiocresol, 3% H_2O, 2.5% DTT, 2% DMS and 1.5% TBAI, no OX was detected in the crude product.

In some cases, the biological activity of the seven new fluorescent labeled peptides has been investigated. 5(6)-FAM-βA[25–35] and 5(6)-FAM-βA[1–40] proved to be almost as neurotoxic as the non-labeled peptides in an *in vitro* experiment using SH-SY5Y cells in a viability test (results will be published elsewhere). These and other results from the literature show that introduction of a fluorescent molecule into the *N*-terminus of the βA peptides may preserve the original biological activity.

EXPERIMENTAL

General Methods

Amino acid analysis of peptides was carried out on HP Amino Quant amino acid analyser after hydrolysis of the samples (6 M HCl, 110°C, 49 h in sealed tubes *in vacuo*). Mass spectra were taken on a FinniganMat TSQ 7000 mass spectrometer in ESI-MS mode. Peptides were purified using a semipreparative HPLC system (HPLC pumps: Knauer type No. 64, programmer: Knauer type No. 50, detector: LKB Bromma 2138 Uvicord S) equipped with a Phenomenex Jupiter column packed with C18 silica gel (300 Å pore size, 10 μm particle size, 250 × 21.20 mm). The following solvent system was used for the elution in a linear gradient mode, at a flow rate of 4.5 ml/min: (A) 0.1% aqueous trifluoroacetic acid (TFA) and (B) 0.1% TFA

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in 80% aqueous acetonitrile (ACN). The fractions were analyzed by analytical HPLC using a Hewlett–Packard Model 1100 liquid chromatograph apparatus, on a Lichrosorb 5 RP-18 (250 × 4 mm, 5 μm, 100 Å pore size) reverse-phase column, and gradient elution with the same solvent system as described above was used at a flow rate of 1.0 ml/min.

(Fmoc-N^{α}-Lys^{28}) β A(25–35)-amide

The peptide was synthesized on an MBHA resin (1.61 g = 0.5 mmol), using a solid phase method with Boc-chemistry by an ABI 430A automated peptide synthesizer. For side-chain protection, Bzl for Ser and Fmoc for Lys were used. Symmetrical anhydride coupling was used in DMF/dichloromethane (DCM) mixture with a twofold excess of reagents, and each coupling cycle was repeated. Boc-Asn was coupled with the DCC/HOBt method. N^{α}-Boc deprotection was performed by TFA/DCM, 1:1 (v/v) mixture. Final deprotection and cleavage of the peptide from the resin were performed with anhydrous hydrogen fluoride in the presence of 8% anisol, 2% dimethyl sulfide (DMS), 2% *p*-cresol and 2% thiocresol at 0°C for 45 min. After the removal of hydrogen fluoride under a stream of N_2 and *in vacuo*, the peptide was precipitated with diethyl ether, filtered, washed with diethyl ether, dissolved in 50% aqueous acetic acid, and lyophilized. The crude peptide (yield: 81%, ca. 80% purity) was used for labeling without any purification.

N^{α}-Fluorescein Thiocarbamoyl-β A(25–35)-amide (FTC-β A(25–35))

(Lys-ε-Fmoc)^{28}βA[25–35]-amide (15.4 mg, 0.012 mmol) was reacted with FITC (3.9 mg, 0.01 mmol) in 0.7 ml of dimethylsulfoxide (DMSO), overnight at room temperature. The reaction mixture was protected from light and kept under N_2. The mixture was then treated with piperidine (175 μl, 20% of the final volume) for 30 min, neutralized with TFA (130 μl), loaded to a semipreparative HPLC column, and purified as described above, using an ACN gradient from 24 to 64%. Yield: 59%; M_w (measured by ESI-MS): 1448.6 (MH^+), mass average; theoretical value: 1448.9 (MH^+).

N^{α}-FTC-RIIGL-amide

The pentapeptide was synthesized on an MBHA resin (1.61 g, 0.5 mmol) using Boc-chemistry and was cleaved from the resin with HF (see above). A total of 33 mg (58 μmol) of crude peptide was reacted with FITC (19 mg, 48 μmol) in DMSO overnight, and purified after completion of the reaction on a semipreparative HPLC column (ACN gradient: 24–64%). Yield: 51%; M_w (measured by ESI-MS): 960.4 (MH^+), mass average; theoretical value: 960.1 (MH^+).

N^{α}-FTC-RVVIA-amide

The synthesis was performed as described above on an MBHA resin (1.11 g, 0.5 mmol). The crude peptide (40 mg, 72 μmol) was reacted with FITC (23 mg, 60 μmol) in DMSO overnight. The ACN gradient for HPLC purification: 24–72%. Yield: 60%; M_w (measured by ESI-MS): 946.2 (MH^+), mass average; theoretical value: 946.1 (MH^+).

N^{α}-5(6)-FAM-β A(25–35)-amide

βA[25–35] was sythesized manually on a Rink-amide resin (1.640 g, 1 mmol) with the use of standard Fmoc-chemistry. Couplings were performed in DMF using a threefold excess of DCC, HOBt and Fmoc amino acids. Fmoc deprotection was performed in a 20% piperidine/DMF mixture for 20 min. The side-chain-protected peptide resin (302 mg, ca. 0.1 mmol) was acylated with 5(6)-FAM (113 mg, 0.3 mmol), DCC (62 mg, 0.3 mmol) and HOBt (41 mg, 0.3 mmol) for 4 h. The peptide was cleaved from the resin with a cleaving cocktail containing 81% TFA, 5% phenol, 5% thiocresol, 3% H_2O, 2.5% DTT, 2% DMS, and 1.5% TBAI for 3 h. The resin was filtered from the mixture, TFA was removed *in vacuo*, the peptide was precipitated in diisopropyl ether and purified as described in general methods. ACN gradient: 24–56%. Yield: 56%; M_w (measured by ESI-MS): 1418.3 (MH^+), mass average; theoretical value: 1417.9 (MH^+).

N^{α}-5(6)-FAM-β A(1–40)-amide (5(6)-FAM-DAEFRHDSGY^{10}EVHHQKLVFF^{20}AEDVGSNKGA^{30}IIGLMV GGVV^{40}-NH_2)

βA[1–40] was sythesized manually on a Tentagel SRAM resin (2.174 g, 0.5 mmol) using a threefold excess of Fmoc amino acids and DCC/HOBt coupling in DMF. After the last acylation and deprotection step, fluorescein labeling was performed on the side-chain protected peptide attached to resin (540 mg, ca. 0.05 mmol) applying a threefold excess of 5(6)-FAM, DCC and HOBt in DMF for 24 h. For Fmoc-deprotection, the resin was treated with 20% piperidine/DMF solution for 20 min. The peptide was cleaved from the resin and precipitated as

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described for βA[25–35], yielding 331 mg of crude product. A total of 165 mg of the crude peptide was purified on a silica HPLC column (BST Si 100S, C-18, ACN gradient: 32–80%). The product of the first peak (40.5 mg) was further purified on a Nucleosil 300 C4 semipreparative HPLC column (ACN gradient: 28–60%) yielding 10 mg of pure end product. M_w (measured by ESI-MS): 4687.9 ($[M-H_5]^{5+} = 938.8$, $[M-H_4]^{4+} = 1172.2$, $[M-H_3]^{3+} = 1563.0$), mass average; theoretical value: 4688.2.

N^{α}-5(6)-FAM-LPFFD-amide

The pentapeptide was synthesized manually on a Rink-amide resin (793 mg, 0.5 mmol) using standard Fmoc-chemistry and DCC/HOBt coupling method with a threefold excess of reagents. 490 mg (ca. 0.2 mmol) resin-linked peptide was reacted with 5(6)-FAM (113 mg, 0.3 mmol), DCC (62 mg, 0.3 mmol) and HOBt (41 mg, 0.3 mmol) for 4 h. The peptide was cleaved from the resin with a mixture of 90% TFA, 5% H_2O and 5% phenol for 4 h. After the filtration of the resin, the peptide was precipitated in diisopryl ether, and purified as described in general methods (ACN gradient: 28–60%). Yield: 51%; M_w (measured by ESI-MS): 996.6 (MH^+), mass average; theoretical value: 996.1 (MH^+).

N^{α}-AMCA-βA(25–35)-amide

AMCA (146.6 mg, 0.2 mmol) was pre-activated with HOBt (27.0 mg, 0.2 mmol) and HBTU (75.8 mg, 0.2 mmol) in DMF in the presence of DIEA (51.7 mg, 0.4 mmol). The resin-linked peptide (302 mg, ca. 0.1 mmol) was suspended in the above mixture which was then shaken overnight. After completion of the coupling reaction, the peptide was cleaved from the resin and purified as given for 5(6)-FAM-βA[25–35]. Yield: 47%; M_w measured by ESI-MS: 1273.6 (MH^+), mass average; theoretical value: 1273.8 (MH^+).

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