Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 597–600

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A FAST AND INEXPENSIVE METHOD FOR N-TERMINAL FLUORESCEIN-LABELING OF PEPTIDES

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Received 14 November 1997; accepted 2 February 1998

Abstract: A new method has been developed to synthesize fluorescein labeled peptides, compounds of increasing importance in bioorganic chemistry, cell biology, pharmacology, drug targeting and medicinal chemistry. We show, that 4(5)-carboxyfluorescein is much more efficient than the hitherto predominantly utilized reagents 4(5)-carboxyfluorescein-N-succinimidylester and 4(5)-fluoresceinisothiocyanate.

Introduction: Fluorescein labeled peptides are tools that are getting increasingly important in life sciences, particularly in the prospering fields of medicinal chemistry, drug targeting, cell biology, immunology, molecular biology and pharmacology. For example, the fluorescein-labeled peptides can be applied to fluorescence microscopy experiments[1], confocal laser scanning microscopy studies[2], collisional fluorescence quenching experiments[3], fluorescence-monitored equilibrium binding studies[4] or flow cytometric studies[5].

There exist two standard methods for the preparation of N-terminally fluorescein-labeled peptides. On the one hand the modification of free peptides is performed in solution with either 4(5)-fluoresceinisothiocyanate (FITC) or 4(5)-carboxyfluorescein-N-succinimidylester (CFSE) (isomeric mixtures), and on the other hand the selective modification can be accomplished with the same reagents using sidechain-protected, polymer-bound peptides.

We show that the use of 4(5)-carboxyfluorescein (CF), which is 150-fold cheaper than its activated analogue, overcomes all the problems outlined above and leads to fluorescein-labeled peptides in an even shorter time. This improvement was achieved by an optimized solid phase coupling procedure using N,N′-diisopropylcarbodiimide (DIC) / 1-hydroxy-benzotriazole (HOBt) activation in N,N-dimethylformamide (DMF).

Chemistry: The model peptide H-YQAAEKEVAQAEAENQALE-NH_2 was synthesized on a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy-resin according to the method described previously[6]. For the side chain protection tert.-butyl (Y,E), trityl (Q,N) and tert -butyloxycarbonyl (K) were used. For the modification reaction, a 0.5 M solution of either (A) CF (9.4 mg, 25 μmol) / DIC (3.91 μl, 25 μmol) / HOBt (3.8 mg, 25 μmol), (B) CFSE (11.8 mg, 25 μmol), (C) FITC (9.7 mg, 25 μmol) or (D) fluorescein (8.3 mg,

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0960-894X/98/$19.00
PII: S0960-894X(98)00084-5

25 μmol) / DIC (3.91 μl, 25 μmol) / HOBt (3.8 mg, 25 μmol) in DMF (50 μl) was added to the resin-linked, side-chain-protected peptide (2.5 μmol) at 25 °C. All reactions were stopped by washing the polymer-bound peptide with DMF after 1h. Post-modification work-up including cleavage, lyophilization, HPLC- and MS-analysis, was carried out according to a procedure published elsewhere[6] (Table 1).

Results and Discussion: First, we verified the assumption that N-terminal modification of peptides with FITC without coupling of a spacer molecule will lead to Edman-degradation, which is still discussed controversially[7,8]. We found a high percentage of peptide missing the N-terminal amino acid but no fluorescein-labeled product (Figure 1C, proven by MS).

CF possesses two carboxyl groups as possible reaction centers, the intrinsic carboxyl group of fluorescein in position 2 and the additional carboxyl group in position 4(5). Since the fluorescence-properties of fluorescein depend on the presence of the free carboxyl-group in position 2, this group has to remain unmodified during reaction. By employment of CFSE this problem is solved elegantly since only the carboxyl group in position 4(5) is activated.

We proposed the hypothesis that DIC/HOBt-activated CF will behave as its preactivated analogue and will couple site-specifically to the N-terminus of the peptide by its supplementary carboxy-group in position 4(5).

Table 1: Analytical data of the fluorescein labeling experiments.

Figure	Reagents	Time [h]		Purity[a] [%]	HPLC[b] [min]	$Mass_{calc}$ [Dalton]	$Mass_{exp}$ [Dalton]	Comment
	–	–	educt:	>90	15.94^{fl-}	2091.2	2090.4	unmodified peptide
			main product:	–				
			side-product:	–				
1A	4(5)-carboxy-fluorescein/ DIC/HOBt	1	educt:	–	–	2091.2	–	–
			main product:	>90	23.09^{fl+}	2449.6	2448.4	fluorescein-labeled peptide
			side-product:	–	–	–	–	
1B	4(5)-carboxy-fluorescein-N-succinimidylester	1	educt:	50	15.81^{fl-}	2091.2	2090.8	unmodified peptide
			main product:	30	23.01^{fl+}	2449.6	2449.2	fluorescein-labeled peptide
			side-product:	–	–	–	–	
1C	4(5)-fluorescein-isothiocyanate	1	educt:	–	–	2091.2	–	–
			main product:	60	14.70^{fl-}	1928.1	1927.4	Edman-degraded peptide (-Y)
			side-product:	20	16.21^{fl-}	n.d.	n.d.	unidentified
1D	fluorescein/ DIC/HOBt	1	educt:	45	15.81^{fl-}	2091.2	2090.8	unmodified peptide
			main product:	20	22.13^{fl-}	2405.5	2405.5	fluoresceinated peptide, but fl-!
			side-product:	25	21.45^{fl-}	–	2216.8	unidentified

[a] according to the HPLC (220 nm).

[b] retention time detected at 220 nm. Additionally, absorbance was measured at 437 nm (the absorption maxima of fluorescein at low pH[9]) to detect dye-containing products (fl+ = absorbtion at 437 nm, fl- = no absorbtion at 437 nm). The solvent system used consisted of 0.1 % TFA in water (A) and 0.1 % TFA in acetonitrile (B). A linear gradient from 5-55 % B in 30 min was applied.

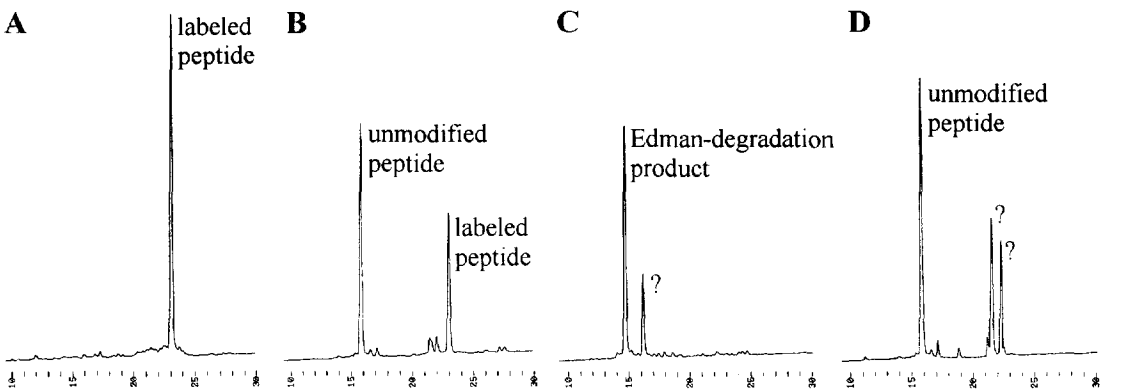

Figure 1: HPLC of the fluorescein labeling experiments after 1h. Labeling was performed with **A** 4(5)-carboxyfluorescein/DIC/HOBt **B** 4(5)-carboxyfluorescein-N-succinimidylester **C** 4(5)-fluoresceinisothiocyanate **D** fluorescein/DIC/HOBt.

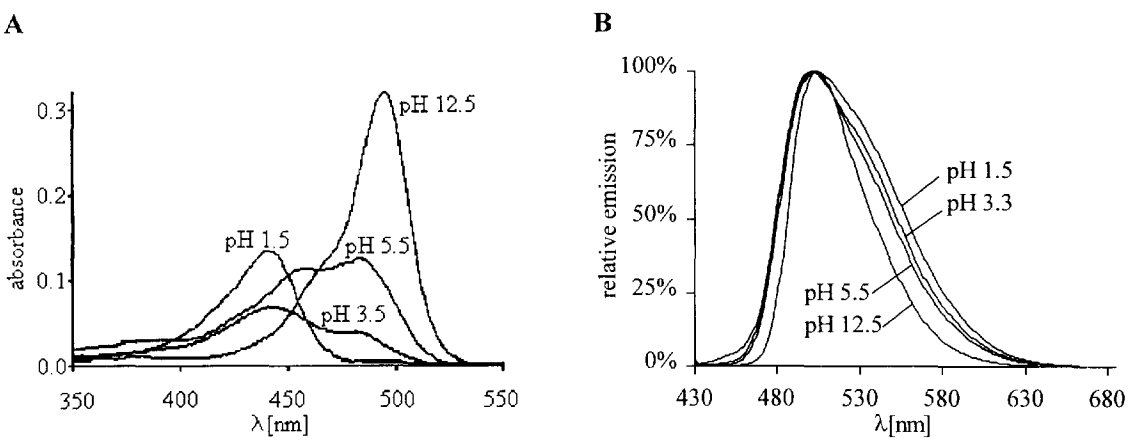

Figure 2: pH-dependent spectrosocopic investigations of the CF-labeled peptide ($c = 10^{-7}$-10^{-6} M) prove the integrity of the fluorescence properties. **A** UV-spectra **B** fluorescence emission spectra (excitation wavelength = 420 nm), both are in agreement with the data for unmodified fluorescein[7].

This is because of the steric hindrance of the carboxyl-group in position 2 and the fact that this carboxyl-group is able to form an intramolecular lactonic bridge, which serves as an auto-protection. The proof of our hypothesis was accomplished by coupling of CF to the peptide applying classical DIC/HOBt-activation. We found complete conversion to the product while surprisingly, the reaction of CFSE showed only a conversion of 30 % to the same product within the same time (Figure 1 A+B, proven by MS). In both cases, the addition of N-ethyldiisopropylamine (Hünigs base) did decrease turnover (data not shown).

To verify the integrity of the fluorescein-inherent carboxyl group in position 2, we performed an analysis of the UV-absorbance- and fluorescence-emission-spectra of the dye-labeled peptide at different pH values. We found that the fluorescein-labeled peptide shows high pH dependence of the absorption and fluorescence in agreement with the different protolytic forms of the dye (Figure 2)[9]. To prove the existence of the unmodified carboxyl group in position 2 we focused on the absorption of the labeled peptide in the visible range of the spectrum at pH = 3.5. We found the weakest absorption there, which is in agreement with the existence of the lactone, because it is the predominant form of the free dye at this pH and the only form which does not absorb in the visible. Therefore, the fluorescein-inherent carboxyl group must have remained unmodified.

Finally we investigated whether the fluorescein-inherent carboxyl group could react with the N-terminus of the peptide at all to form labeled peptides which have the typical properties of fluorescein. Therefore we tried to activate pure fluorescein in the same way as its carboxylated analogue and we found that about 45 % of the peptide remained unmodified after 1h (Figure 1D). The two detected reaction products did not show intact dye moieties as no absorption at 437 nm was found although one showed the expected mass (Table 1). This would raise the question if the single HPLC-product peak of the peptide labeled with CF was contaminated with similar non-absorbing products. Comparing the ratio of the HPLC peak areas detected at 220 nm and 437 nm of the CF-labeled peptide with the ratio of the CFSE-labeled peptide, we found no difference. This further confirms the purity of the peptide labeled with CF.

Conclusion: We demonstrated that the labeling of peptides with DIC/HOBt-activated 4(5)-carboxyfluorescein is an efficient and cheap method to insert fluorescein at the N-terminus of peptides. It further might be an efficient protocol to introduce similar modifications for which activated esters or isothiocyanates have been used so far.

Acknowledgment: This research was supported by grants from the Swiss National Fund (31-050819) and the Swiss Federal Institute of Technology. The authors wish to thank Pål Johansen of the Department of Pharmacy, Swiss Federal Institute of Technology Zürich for recording the fluorescence spectra.

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