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Tetrahedron Letters 45 (2004) 6079–6081
Tetrahedron Letters

An efficient method for the solid-phase synthesis of fluorescently labelled peptides

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Received 3 April 2004; revised 17 May 2004; accepted 17 May 2004
Available online 26 June 2004

Abstract—Fluorescent labelling of peptides is necessary in a wide range of cell biological applications. In the last decade, the application of cell-penetrating molecules has been advanced by the use of peptides, which have proven efficient in aiding nonpermeant molecules to cross the cell membrane. Currently, the development of new cell-penetrating peptides based on the design and synthesis of labelled peptide libraries is becoming critically important. Here we report an improved method for the solid-phase labelling of peptides, mediated by the activation of 5(6)-carboxyfluorescein with PyAOP/ HOAt.
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In the field of drug delivery, fluorescent labels are critically important in that they provide a tool for the observation of pathways followed by a molecule once inside a cell. Thus, spectrofluorimetry, flow cytometry and fluorescent microscopy require fluorescent probes. Our laboratory has recently reported the use of fluorescently labelled peptides for the development of new families of cell-penetrating peptides, including proline-rich dendrimers,[1] amphipathic proline-rich peptides[2,3] and γ-peptides.[4] In some of these studies it became apparent that the fluorescent labelling synthetic step constituted a difficult bottleneck in the processes.

One approach to the preparation of fluorescently labelled peptides is the reaction of the peptide in solution with an activated form of the fluorophore. Thus, reaction of a free amino-containing peptide with fluorescein isothiocyanate[5] and a cysteinyl-peptide with fluorescein-5-maleimide[6] have recently been reported. These approaches suffer from a lack of selectivity in the presence of other functional groups in the amino acid side chains of the peptide and are, in addition, too expensive for medium and large scale synthesis often required for in vivo experiments. A potentially more effective approach would be to assemble the peptide chain on a solid support and incorporate the fluorophore into the peptide whilst still attached to the support. This approach offers the advantage of conveniently protecting amino acid side chains and allows, in principle, good yields to be achieved using repetitive couplings and/or excess reagents. 5(6)-Carboxyfluorescein appears to be an appropriate fluorescent reagent for use in such an approach. It is relatively cheap and its fluorogenic properties are well known[7] and suitable for a variety of cell biology experiments. Moreover, in contrast to the behaviour of the simpler reagent fluorescein,[8] 5(6)-carboxyfluorescein has previously been shown to react in a very reproducible way with amino groups, affording highly fluorescent 5(6)-carboxyfluoresceinamido derivatives.[7,8] However, in spite of the use of improved synthetic procedures, such as those recently reported by Weber et al.,[8] the incorporation of 5(6)-carboxyfluorescein into a solid support-attached peptide chain has proven to be a difficult task.

In this study, hexaproline (P_6), dodecaproline (P_{12}) and 18-proline (P_{18}) peptides were synthesised by solid-phase peptide synthesis using the Fmoc/*t*Bu strategy, as described previously.[2] Incorporation of the 5(6)-carboxyfluorescein label (CF) at the N-terminal position of a series of resin-bound Pro-rich peptides proved to be a synthetic bottleneck. As shown in Table 1, in a first attempt, despite using the improved synthetic protocols of Weber et al.,[8] the yield was modest. Thus, treatment of resin-bound P_6 with 10 equiv of 5(6)-carboxyfluorescein (Acros), 10 equiv of HOBt (Albatros Chem Inc.) and

Keywords: 5(6)-Carboxyfluorescein; Solid-phase peptide synthesis; Pro-rich peptides; Drug delivery.
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doi:10.1016/j.tetlet.2004.05.081

Table 1. Conditions tested for labelling with 5(6)-carboxyfluorescein

Peptidyl-resin	Reaction conditions[a]	Reaction time	Product	Yield[b] (%)
P_6-resin	CF 10 equiv, HOBt 10 equiv, DIC 10 equiv, DMF/DCM 9/1	1 h 30 min	CF-P_6	41
		5 h	CF-P_6	62
		10 h	CF-P_6	90
P_{12}-resin	CF 10 equiv, HOBt 10 equiv, DIC 10 equiv, DMF/DCM 9/1	10 h	CF-P_{12}	42
P_{18}-resin	CF 10 equiv, HOBt 10 equiv, DIC 10 equiv, DMF/DCM 9/1	10 h	CF-P_{18}	8

[a] 5(6)-Carboxyfluorescein (CF), 1-hydroxybenzotriazole (HOBt), diisopropylcarbodiimide (DIC), dimethylformamide (DMF), dichloromethane (DCM).
[b] Yield obtained by HPLC.

DIC (Fluka) for 1 h 30 min resulted in a yield of only 41% in the carboxyfluoresceination reaction. In this hexaproline labelling reaction, the yield was improved by longer reaction times, which afforded 90% yield after 10 h. Increasing peptide chain length resulted in even poorer yields. Thus, for the longer peptides, P_{12}- and P_{18}-resin, yields were only 42% and 8%, respectively, even with a reaction time of 10 h. We therefore decided to study different coupling reagents and experimental conditions.

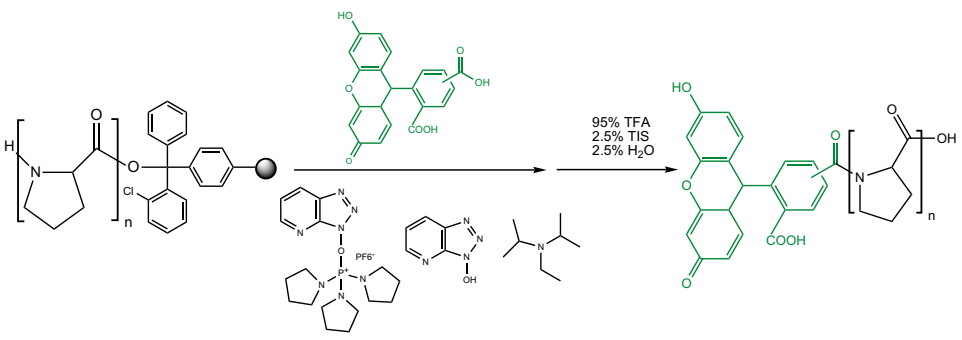
Firstly, activation of the carboxylic group of 5(6)-carboxyfluorescein was attempted with TBTU (Albatros Chem Inc.)[9,10] or HATU (GL Biochem)[11] as coupling reagents. Aza-derivatives of phosphonium, uronium or guanidinium salts have been proved to be very effective coupling reagents either used alone[12,13] or in pairs.[14] Unfortunately, in our case, guanilidated species[15] were confirmed by HPLC-MS as representing the major products obtained in the reaction with HATU; probably due to the high reactivity of the activated coupling reagent compared with the sterically hindered 5(6)-carboxyfluorescein. Consequently, we moved from the use of uronium salts to phosphonium derivatives. Thus, PyBOP (Novabiochem) was used as an activating reagent for 5(6)-carboxyfluorescein, resulting in less than 50% CF-P_{12}. After several attempts, a new protocol, based on the combined use of PyAOP and HOAt,[14] provided the best conditions for the introduction of the fluorescent label (see Table 2).[2] In this new method, depicted in Scheme 1, 5 equiv of 5(6)-carboxyfluorescein were preactivated with 5 equiv of PyAOP (Applied Biosystems), 5 equiv of HOAt (GL Biochem) and 10 equiv of DIEA in DMF/DCM 9/1 for 10 min and then added to the peptide-resin and reacted for 1 h 30 min. Notably, under these conditions the yield of the labelled product CF-Pro_{12} was improved, generating up to 85% labelling. It was also noticeable that the equivalents of 5(6)-carboxyfluorescein used were reduced by half, from 10 to 5 equiv. The influence of the length of the peptide was apparent when the fluorescent marker was reacted with the P_{18} peptide-resin. Even using the optimised protocol with PyAOP/HOAt, the percentage yield of CF-P_{18} peptide was only 60%.

The labelled polyproline peptides were cleaved from the resin by treatment with 1% TFA in DCM solution for 5 min and the crude peptides were purified by reversed phase high performance liquid chromatography (Waters) and characterised by Maldi-TOF (Applied Biosystems): CF-P_6 ($M_{calculated} = 959$, $M_{found} =$

Table 2. Optimised conditions for labelling with 5(6)-carboxyfluorescein

Peptidyl-resin	Reaction conditions[a]	Reaction time	Product	Yield[b] (%)
P_{12}-resin	CF 5 equiv, PyAOP 5 equiv, HOAt 5 equiv, 10 equiv DIEA, DMF/DCM 9/1	1 h 30 min	CF-P_{12}	85
P_{18}-resin	CF 5 equiv, PyAOP 5 equiv, HOAt 5 equiv, 10 equiv DIEA, DMF/DCM 9/1	1 h 30 min	CF-P_{18}	60

[a] 7-Azabenzotriazolyoxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP), 1-hydroxy-7-azabenzotriazole (HOAt), *N*,*N*-ethyldiisopropylamine (DIEA).
[b] Yield obtained by HPLC.



Scheme 1. Conditions for the 5(6)-carboxyfluoresceination solid-phase reaction.

982 [M+Na^+]), CF-P_{12} ($M_{calculated} = 1542$, $M_{found} = 1565$ [M+Na^+]), CF-P_{18} ($M_{calculated} = 2124$, $M_{found} = 2147$ [M+Na^+]). In contrast to the results reported by Fisher et al.[7] polyfluorescein incorporation was not detected.

In conclusion, this new fluoresceination protocol has proved to be the most efficient method for N-terminal, on-resin labelling of a series of peptides. Among others, this protocol offers the following advantages: (i) the use of a total solid-phase synthesis approach, avoiding the purification step prior to labelling; (ii) the lack of a requirement for the extra Cys residue necessary for reaction with fluorescein-5-maleimide;[6] (iii) the elimination of secondary reactions to deal with an over-incorporation of the fluorescent probe (since during the labelling reaction the peptide is anchored on the resin and the side-chains are protected).

The difficulty of labelling has been observed to increase with peptide length, at least for those peptides that adopt a polyproline (PPII) conformation in water, as shown in Table 2.

Even though in the present study the synthesis of Pro_n was used as a proof of concept, it should be remembered that: (i) Pro_n is relevant, since it is internalised in eukaryote NRK cells;[1] (ii) Pro-rich compounds have important properties as enzymatic inhibitors;[16] (iii) compounds with a 50% Pro-content that maintain the same PPII conformation as Pro_n, have recently been reported by our laboratory as potential peptide carriers for cellular drug delivery.[2,3]

The improved protocol described in this article could find application in other fields such as solid-phase combinatorial synthesis of natural product-like molecules, polyamides or PNAs, where fluorescent labelling for cell biology studies are also desirable.

Acknowledgements

The authors acknowledge financial support from the European Union (QLK2-CT-2001-01451), MCYT-FEDER (Bio2002-2301) and the Generalitat de Catalunya (CeRBa and SGR).

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