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A solid phase linker strategy for the direct synthesis of EDANS-labelled peptide substrates

Joerg Beythien[a] and Peter D. White[b,*]

[a]*Novabiochem, Merck Biosciences AG, Weidenmattweg 4, CH-4448 Laufelfingen, Switzerland*
[b]*Novabiochem, Merck Biosciences Ltd, Padge Road, Nottingham NG9 2JR, UK*

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Abstract—A novel linker strategy for the efficient synthesis of peptides C-terminally labelled with the EDANS fluorophore is described. Using this support, FRET peptide substrates bearing EDANS/Dabcyl fluorescent donor/acceptor groups can be readily prepared using standard Fmoc solid phase methods.

Proteases are essential for all aspects of life and are important therapeutic targets. The sequencing of the genomes of humans and other organisms has led to the discovery of many novel proteases, and considerable efforts are now underway to define their biological functions. These developments have created a requirement for efficient and rapid techniques for determining protein specificity and activity. Of particular interest are solid phase methods as these are easily adapted to high-throughput chemical synthesis and to the production of combinatorial libraries, and hence offer the capability to make and screen large numbers of putative protease substrates quickly and efficiently.[1]

One of the most commonly employed types of protease substrate consists of a polypeptide in which a fluorescent donor chromophore and quenching acceptor chromophore are positioned on either side of the enzyme cleavage site.[2] In such substrates the fluorescence is quenched by intramolecular resonance energy transfer (FRET) between the donor and acceptor until the substrate is cleaved and the donor and acceptor moieties become physically separated.

A particularly effective donor/acceptor pair is EDANS/Dabcyl (Fig. 1) due to the excellent spectral overlap between the emission spectrum of EDANS and the absorbance spectrum of Dabcyl.[2,3] Two approaches have been described for the introduction of EDANS. The first involves coupling of EDANS to a peptide fragment in solution, a method which is cumbersome and not amenable to solid phase synthesis.[3,4] The second approach utilises an N(α)-protected amino acid which has been derivatised with EDANS, such as Fmoc-Glu(EDANS)-OH.[5,6] Whilst such derivatives are compatible with solid phase methods, their synthesis is complex and they introduce a modified amino acid into the peptide sequence. Moreover, because these derivatives exhibit poor solubility and couple slowly, their introduction during automated synthesis usually requires manual intervention.

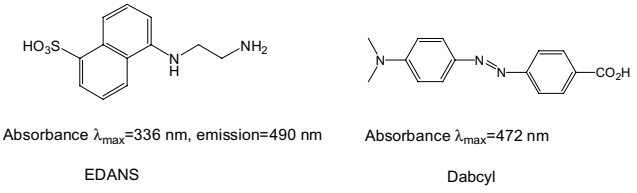

Figure 1. EDANS and Dabcyl acceptor/donor pair.

To overcome these limitations and to meet the requirements for high-throughput synthesis, we developed a strategy in which EDANS is simply reversibly immobilised to a solid support by reductive amination to backbone-amide-type linker.[7] For our purposes, we selected the 4-(3-formyl-2-methoxyphenoxy)-butanoic acid (FMPB) linker[8] as we believed this would confer the desired acid lability to the final linker. The synthesis of

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* Corresponding author. Tel.: +44 115 9430840; fax: +44 115 9430951; e-mail: peter.white@merckbiosciences.co.uk

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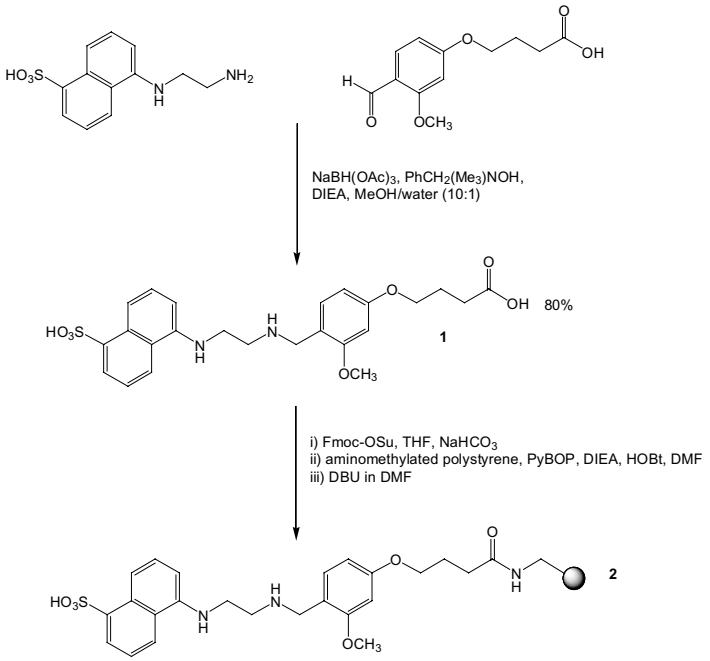
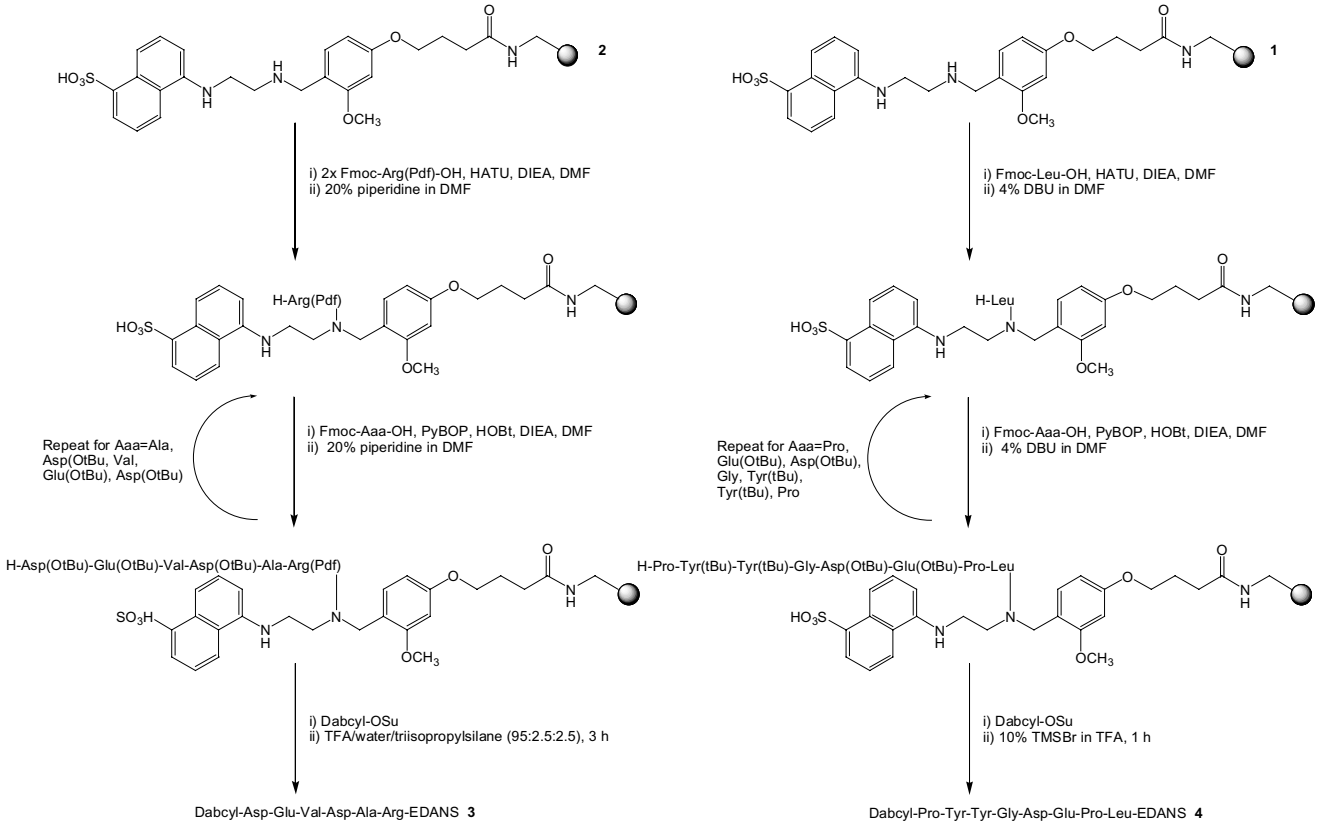
solid-supported EDANS was achieved as described in Scheme 1. The key intermediate **1** was prepared by the reductive amination of FMPB with EDANS and sodium triacetoxyborohydride in the presence of benzyltrimethylammonium hydroxide in MeOH/water. Following completion of the reaction, acidification of the reaction mixture to pH 4.0 afforded **1** as a crystalline red compound in 80% yield.



Scheme 1. Synthesis of EDANS loaded resin **2**.



Scheme 2. Synthesis of Dabcyl/EDANS peptides **3**, **4**.

By making the chromophore part of the linker, the need for additional synthetic steps for chromophore introduction or the use of expensive labelled amino acid derivatives are avoided. In the context of high-throughput synthesis, the assurance that the chromophore is present at the outset is particularly important, as checking for complete chromophore incorporation is not practical when dealing with large numbers of reactions. This procedure provides a support of defined and reproducible substitution (typically ~0.5 mmol/g).

The potential of resin **2** for the synthesis of EDANS-labelled peptides was evaluated using peptides **3** and **4** as examples (Scheme 2). In the case of peptide **3**, the C-terminal residue was attached by double coupling Fmoc-Arg(Pbf)-OH that was activated with HATU in the presence of DIEA. A double coupling was used to ensure complete condensation, as activated arginine derivatives are prone to intramolecular lactamisation to unreactive γ-lactams. For peptide **4**, a single coupling of Fmoc-Leu-OH under identical conditions was used. Satisfactory substitutions have also been obtained using Fmoc-amino acid pre-formed Pfp esters. Under the above coupling conditions, the secondary anilino functionality of EDANS[5] was found to be unreactive and was therefore not protected. The use of the more aggressive coupling reagent such as PyBrOP, however, did lead to the formation of anilide by-products and should therefore be avoided.

Additions of subsequent Fmoc-protected amino acids were carried out using PyBOP/DIEA. 20% piperidine in DMF and 4% DBU in DMF were used for Fmoc removal during the synthesis of peptides **3** and **4**, respectively. Typically, the use of DBU is preferred when coupling methods employed are capable of activating the EDANS sulfonate group, as the use of piperidine in such circumstances could lead to the formation of piperidinylsulfonamides. In both cases the N-terminal Dabcyl group was introduced using Dabcyl-OSu. Peptide **3** was obtained in good purity (Fig. 2) and good yield (76%) following treatment of the corresponding peptidyl resin with 95% TFA cocktail for 3 h. For peptide **4**, the use of the more acidic cleavage cocktail 10% TMSBr in TFA afforded the desired peptide in moderate yield (63%) and excellent purity (Fig. 3) in only 1 h following reprecipitation from AcOH/MeCN with ether/MeCN.[9] The identities of both peptides were confirmed by LC–ESMS.

Figure 2. HPLC profile of crude peptide **3**. (HPLC conditions: Vydac peptide/protein C18 column; gradient: 20–100%B in 20 min, 1 ml/min; A: 0.1% TFA aq; B: MeCN/water/TFA (90:10:0.1).

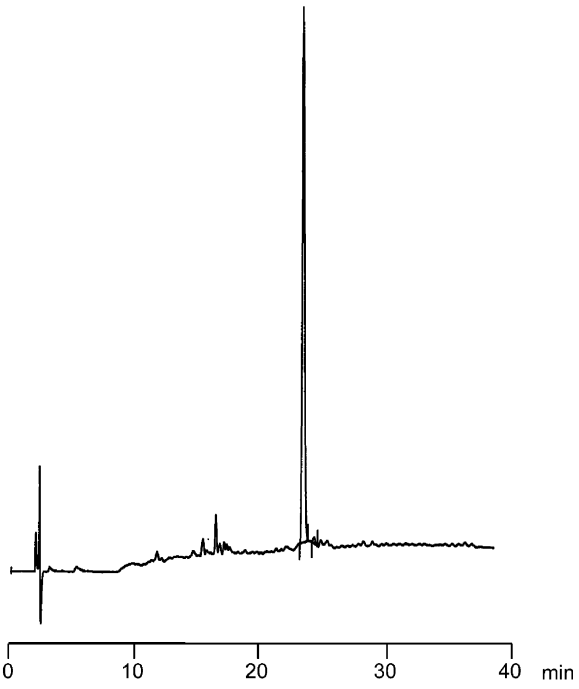


Figure 3. HPLC profile of crude peptide **4**. (HPLC conditions: Nucleosil 33-5 C18 column; gradient: 30–50% B in 30 min, 1 ml/min; A: 0.1% TFA aq; B: 0.1% TFA in MeCN.

In summary, our recently developed EDANS linker **1** provides a simple and effective method for the preparation of EDANS-labelled peptide by solid phase synthesis. The linker attached to aminomethyl polystyrene is now commercially available as EDANS NovaTag resin from Novabiochem, Switzerland.

References and notes

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9. To a suspension of resin **1** (0.5 g; loading 0.53 mmol/g) in DMF was added a solution of Fmoc-Leu-OH (177 mg, 0.5 mmol), HATU (200 mg, 0.53 mmol) and DIEA (350 μl, 2 mmol) in a minimum volume of DMF. The mixture is left to stand with gentle agitation for 1 h, after which time the chloranil test was negative. The resin was removed by filtration, washed with DMF, isopropanol, H_2O, and THF, isopropanol, MeOH, ether, and hexane, and dried overnight. The loading of Fmoc-Leu-resin **2** was determined using the Fmoc UV assay[10] to be 0.43 mmol/g. This resin (300 mg) was suspended in DMF and left to swell for 30 min, and then treated twice for 10 min with 4% DBU in DMF to effect Fmoc removal. The resin was washed with DMF, and a mixture of Fmoc-Pro-OH (110 mg, 0.32 mmol), HOBt (30 mg, 0.2 mmol), PyBOP (180 mg, 0.35 mmol) and DIEA (750 μl, 0.88 mmol) in DMF was added to the resin and gently agitated for 30 min, after which time the TNBS test was negative. The other amino acids were added in an identical manner. Following removal of the final Fmoc group with DBU in DMF, the resin was end-capped by treatment with Dabcyl-OSu (120 mg, 0.32 mmol), HOBt (30 mg, 0.2 mmol) and collidine (500 μl) in DMF. The resin is removed by filtration, washed: with DMF, isopropanol, H_2O, THF, isopropanol, MeOH ether, hexane and dried overnight. The peptide was cleaved from the resin by treatment with 10% TMSBr in TFA (3 ml) for 1 h. After this time, ether (60 ml) was added and the mixture was centrifuged. The supernatant was decanted and the residues were washed twice with fresh ether. The peptide was extracted twice from the resin with AcOH/MeCN (1:1 v/v, 3 ml). The resin was removed by filtration and the peptide precipitated from the filtrate by the addition of ether/MeCN (1:1 v/v, 60 ml). The precipitate was washed with ether and dried under vacuum to afford the desired peptide as a red solid (115 mg, 63%); *m/z* (+ES) 1453.77 (MH^+), calcd 1453.61.
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