Novabiochem®

Innovations 1/10

Total synthesis of the 101 residue d2 domain of VEGF receptor 1

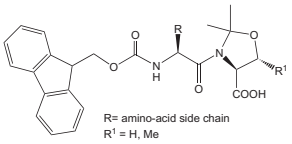
Vascular endothelial growth factor (VEGF) plays a central role in the regulation of angiogenesis through its interaction with VEGF 1 and VEGF 2 receptors [1]. The d2 domain of VEGF receptor 1 binds VEGF only 100-fold less tightly than the intact domain and therefore provides an excellent model for VEGF receptor binding.

Fig. 1: NMR structure of VEGF binding domain [2].

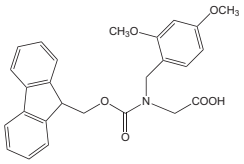
This innovation describes the synthesis of the 101 residue d2 domain of human VEGF receptor 1 (VEGFR1 d2) using data kindly provided by Michel Vidal and Nicolas Inguimbert of the Université Paris Descartes [3]. This protein has eight β-sheet regions, and its synthesis demonstrates the utility of the Novabiochem® Fmoc-Dmb(Gly)-OH and pseudopeptide derivatives in the synthesis of very long aggregation prone sequences.

Using Dmb/pseudoproline derivatives

Pseudoproline dipeptides



Fmoc-Dmb(Gly)-OH



Fmoc-(Dmb)Gly-OH [4, 5] and pseudoproline dipeptides [6] are extremely useful tools for expediting the synthesis of long and complex peptides [3, 7 - 10]. Pseudoproline dipeptides consist of Fmoc-protected dipeptides in which the C-terminal residue is a dimethyloxazolidine derived from Ser/Thr. When coupled into a peptide chain, these derivatives temporarily introduce a structure-breaking secondary acid which helps prevent aggregation during peptide assembly. During deprotection with TFA, the Dmb group and pseudoproline ring are cleaved and the native Gly and Ser/Thr residues are respectively regenerated.

Dmb and pseudoproline derivatives are extremely easy to use, as they can be introduced using any standard coupling methods. Pseudoproline dipeptides introduce two residues at a time, substituting any Aaa-Ser/Thr dipeptide within the peptide chain. Guidelines for selecting the optimum locations of Dmb and pseudoproline dipeptides are given below.

General guidelines for the use of Dmb/pseudoproline derivatives

- Optimal results are obtained if the Dmb/pseudoproline derivatives are spaced 5-6 residues apart throughout the sequence.
- The optimum separation between a Dmb/pseudoproline and a Pro residue is 5-6 amino acid residues.
- The minimum separation between a Dmb/pseudoproline and another Dmb/pseudoproline derivative or Pro residue is 2 residues.
- Aim to insert a Dmb/pseudoproline before regions of hydrophobic residues.

Synthesis of VEGFR1 d2

General synthetic procedures

Peptide synthesis was carried out using an ABI 433A peptide synthesizer on NovaSyn TGA resin (0.23 mmol/g). Couplings were performed using 4-fold excess of Fmoc-amino acids activated with HBTU/HOBt/DIPEA (1:1:2) for 1 hour. A 5 minute capping step was performed after each coupling reaction using 0.5 M acetic anhydride : 0.125 M DIPEA : 0.015 M HOBt in NMP. Fmoc removal was effected by treating the resin with 20% piperidine in NMP for 15 minutes. For certain residues additional extensions to the coupling times were used as indicated in Figure 2.

The peptides were cleaved from the resin by treatment with TFA/EDT/TIPS/water (94:2.5:1:2.5) for 3 h, and isolated by centrifugation followed by precipitation with cold ether.

Peptide assembly

VEGFR1 d2 consists of eight β-sheet strands, making this protein domain an extremely challenging target for chemical synthesis. To overcome the anticipated problems with aggregation during peptide assembly, Goncalves, *et al.* used a low-substitution NovaSyn TGA support to minimize interchain interactions and pseudoproline peptides and (Dmb)Gly to disrupt secondary structure formation. Applying the guidelines for insertion of structure breaking secondary amino acids, four pseudoproline dipeptides and two (Dmb)Gly residues were introduced into the *C*-terminal sequence of the peptide such that these building blocks were spaced 6 - 9 residues apart. For the *N*-terminus, the presence of well spaced Pro residues necessitated only a single insertion of a (Dmb)Gly residue in this region of the peptide.

Couplings were performed with HBTU/HOBt/DIPEA for 1 h. Double couplings were performed for residues indicated in bold in Figure 2, according to findings from an initial synthesis. Capping with acetic anhydride was performed after each coupling step. The progress of the assembly was followed by analyzing products formed after 29, 49, 79 amino acid additions (Figure 3). These results indicate the synthesis proceeded with high efficiency to 50 residues. The product obtained after 79 residues was considerably less pure. UV monitoring of the Fmoc deprotection reactions indicated that difficulties were encountered during the assembly of the sequence Ser-Pro-Asn-Ile-Thr-Val, a region of the peptide where no pseudoproline dipeptides were used. Consequently, the final 22 residues were coupled twice. 1.1 g of crude peptide was obtained following treatment of the peptidyl resin with TFA/EDT/TIPS/water for 3 h.

Fig. 2: Primary sequence of VEGFR d2.

Sites of pseudoproline substitution are highlighted in green; (Dmb)Gly residues are highlighted in red. Bolded residues indicate double coupling.

a) H-**Ser-Asp-Thr-Gly-Arg-Pro-Phe-Val-Glu-Met-Tyr-Ser-Glu-Ile-Pro-Glu-Ile-Ile-His-Met-Thr-Glu-**Gly-Arg-Glu-Leu-Val-Ile-Pro-Cys-Arg-Val-Thr-**Ser**-Pro-**Asn-Ile-Thr**-Val-Thr-Leu-Lys-Lys-**Phe**-Pro-Leu-Asp-Thr-Leu-Ile-**Pro-Asp**-Gly-Lys-**Arg-Ile-**Ile-**Trp**-Asp-Ser-**Arg**-Lys-Gly-Phe-Ile-Ile-Ser-Asn-Ala-Thr-Tyr-**Lys-Glu-Ile**-Gly-Leu-Leu-Thr-Cys-Glu-Ala-Thr-Val-Asn-Gly-His-Leu-Tyr-Lys-Thr-Asn-Tyr-Leu-Thr-His-Arg-Gln-Thr-Asn-Thr-Ile-OH

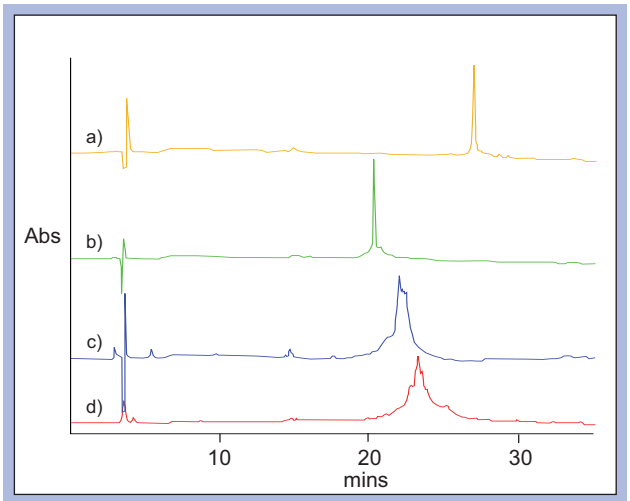


Fig. 3: HPLC elution profiles of a) 29mer, b) 49mer, c) 79mer and d) 101mer.

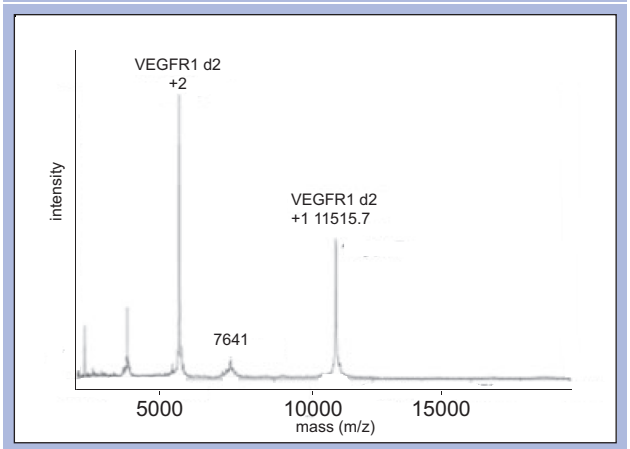


Fig. 4: MALDI-TOF spectrum of reduced VEGFR1 d2 after first HPLC purification.

Purification & folding

The peptide was initially purified by HPLC on a Kromasil C8 column. Fractions containing product were identified by MALDI-TOF and pooled and lyophilized to yield 480 mg of peptide. MALDI-TOF analysis indicated the presence of impurities arising from poor couplings between residues Ser-162 - Val-167. This material was further purified on a C18 Vydac column to yield 25 mg of the peptide (Figure 4).

The reduced linear peptide was allowed to oxidize and refold in 0.1 M Tris, 2.2 M guanidinium chloride, pH 8.0 buffer for 24 h (see Figure 5). The resulting peptide was dialyzed against PBS buffer and then concentrated using a 10 kD cut-off Amicon centrifugation tube. This last step successfully eliminated most of the truncated peptide by-products to yield the folded protein domain in good purity (Figure 5 and Figure 6).

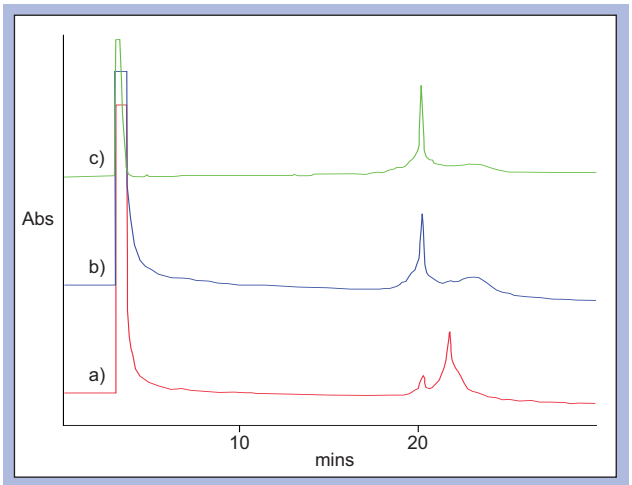


Fig. 5: Refolding and purification of VEGFR1 d2. HPLC elution profiles of recovered after a) 0 h and b) 18 h of air oxidation, and c) dialysis and ultrafiltration.

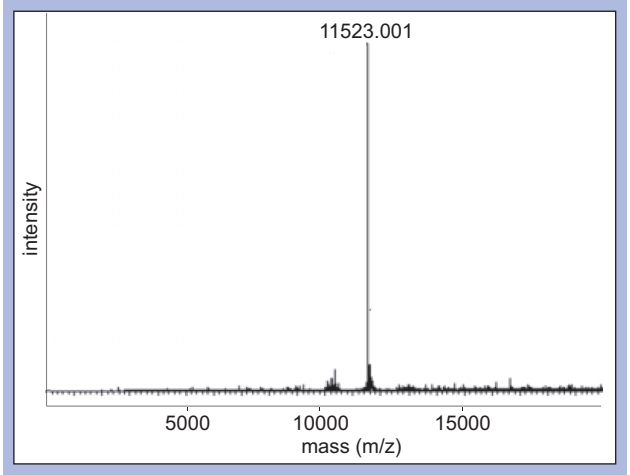


Fig. 6: Deconvoluted MALDI-TOF spectrum of oxidized and purified VEGFR1 d2.

Ordering information

852110	Fmoc-(Dmb)Gly-OH	1 g 5 g
852175	Fmoc-Ala-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852180	Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852185	Fmoc-Asn(Trt)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852183	Fmoc-Asn(Trt)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852186	Fmoc-Asp(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852199	Fmoc-Asp(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852190	Fmoc-Gln(Trt)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852198	Fmoc-Gln(Trt)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852177	Fmoc-Glu(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852196	Fmoc-Glu(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852200	Fmoc-Gly-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852197	Fmoc-Gly-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852194	Fmoc-Ile-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852193	Fmoc-Ile-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852179	Fmoc-Leu-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852184	Fmoc-Leu-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852178	Fmoc-Lys(Boc)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852191	Fmoc-Lys(Boc)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852195	Fmoc-Phe-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852201	Fmoc-Phe-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852187	Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852192	Fmoc-Ser(tBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852202	Fmoc-Trp(Boc)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852188	Fmoc-Trp(Boc)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852189	Fmoc-Tyr(tBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852182	Fmoc-Tyr(tBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852176	Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852181	Fmoc-Val-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
855005	NovaSyn® TGA resin	1 g 5 g 25 g

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