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Total chemical synthesis of the D2 domain of human VEGF receptor 1

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The interaction of the vascular endothelial growth factor (VEGF) with its cellular receptors exerts a central role in the regulation of angiogenesis. Among these receptors, the VEGF receptor 1 may be implicated in pathological angiogenesis. Here, we report the first total chemical synthesis of the VEGF-binding domain of the VEGF receptor 1. Aggregation issues were overcome by the use of a low-substituted resin and the stepwise introduction of pseudoproline dipeptides and Dmb-glycines. The folding of the protein was achieved by air oxidation and its biological activity was verified on ELISA-based assays. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: angiogenesis; vascular endothelial growth factor; VEGF receptor 1; chemical synthesis of proteins; pseudoproline; dimethyloxazolidine dipeptide; folding

Introduction

Angiogenesis is defined as the formation of new blood vessels from an already established vasculature. This process is finely tuned by several regulators, among which the VEGF exerts a predominant role [1]. In recent years, it has become evident that dysregulated angiogenesis is a key component in several pathologies such as cancer, age-related macular degeneration and inflammatory disorders [2]. Consequently, modulating angiogenesis represents an important pharmaceutical objective.

VEGF pro-angiogenic activity is principally mediated by its binding to two tyrosine-kinase receptors, VEGF receptor 1 and 2 (VEGFR1 and VEGFR2). VEGF binds VEGF receptor 3 also but this receptor is rather implicated in the lymphangiogenesis process [3]. Among these receptors, VEGFR1 has the strongest affinity for the VEGF ($K_D = 10-30$ pM) and a few reports tend to indicate that this receptor may be more specifically implicated in pathological angiogenesis [4].

Human mature VEGFR1 is a glycosylated protein, member of the tyrosine-kinase receptors superfamily and belongs to the subgroup of the platelet-derived growth factor receptors family. VEGFR1 is constituted by an extracellular domain organized into seven immunoglobulin-like folds, followed by a single transmembrane region, a juxtamembrane domain, a split tyrosine-kinase domain and a C-terminal tail (Figure 1) [5]. Domain deletion experiments carried out on the VEGFR1 extracellular portion by Wiesmann *et al*. [6] have shown that the second immunoglobulin-like domain, from the N-terminus (VEGFR1 d2), is necessary and sufficient to bind VEGF. Indeed, VEGFR1 d2 alone binds VEGF only 100-fold less tightly than the full-length protein. The structure of this domain ($VEGFR1_{129-229}$) has been solved free in solution by NMR [7]. Its structure has also been determined by X-ray crystallography in complex with VEGF and the placental growth factor and it does not exhibit significant differences with the free form [6–8]. A few peptides and small molecules have been identified as ligands of VEGFR1 and were able to modulate VEGFR1 activity [9,10]. Because the previously described ligands are likely to interact with the 101-amino acid polypeptide chain of VEGFR1 d2, we have investigated the possibility of synthesizing this domain by solid phase peptide synthesis, which constituted an alternative to its expression in *Escherichia coli* [6,7].

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Abbreviations used: *$btVEGF_{165}$, biotinylated $VEGF_{165}$; DIPEA, N,N′-diisopropylethylamine; Dmb, dimethoxybenzyl; ELISA, Enzyme linked immunosorbent assay; ESI–Q–Tof, Electrospray ionization–Quadripole–Time of flight mass spectroscopy; Fmoc, fluorenylmethyloxycarbonyl; HBTU, O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate; HOBt, N-hydroxybenzotriazole; MALDI–Tof, Matrix assisted laser desorption ionization–Time of flight; NMP, 1-methyl-2-pyrrolidinone; RP-HPLC, Reverse-phase high performance liquid chromatography; t_R, retention time; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VEGFR1 d2, vascular endothelial growth factor receptor 1 immunoglobulin-like domain 2; VEGFR1 ECD, VEGFR1 extracellular domain.*

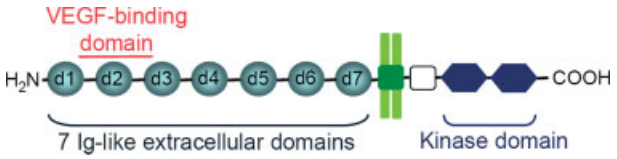

Figure 1. Schematic of the structure of the vascular endothelial growth factor receptor 1. The VEGF-binding domain is essentially located on the extracellular immunoglobulin-like (Ig) d2. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

Herein, we report our preliminary results on the stepwise chemical synthesis of VEGFR1 d2 by using N-(Dmb) amino acid and pseudoproline (dimethyloxazolidine) dipeptide building blocks, which allows us to overcome aggregation problems that could be expected for proteins bearing a high content of β-sheet. The peptide was purified by RP-HPLC, refolded and its affinity for VEGF was verified on ELISA-type assays.

Results

Stepwise Synthesis of VEGFR1 D2

In our first attempt we encountered difficulties that could be attributed to the low solvatation of the growing protected peptide. Indeed, the domain VEGFR1 d2 is characterized by the presence of eight β-sheet strands, which are well-known elements that favor peptide aggregation (Figure 2A) [11].

In order to solve this problem, different strategies were simultaneously employed to perform an efficient synthesis of VEGFR1 d2. First, the synthesis was conducted using Fmoc/tBu chemistry with a low-substituted (0.25 mmol/mg) Novasyn-TGA resin to limit interchain interactions [12]. Then, we searched to limit aggregation by introducing pseudoproline dipeptides and N-(Dmb)-protected glycines [N-(Dmb)-glycines] in the peptide sequence [13,14]. N-(Dmb)-glycine exploits the natural propensity of sterically hindered N_α-alkyl amino acids to disrupt the formation of secondary structure whereas the cyclic structure of pseudoproline dipeptides permits to introduce a bent in the peptide backbone during its assembly, in the same manner as proline [15]. Based on the primary sequence of VEGFR1 d2, we introduced four pseudoproline dipeptides and two N-(Dmb)-glycines in the C-terminal part of the peptide so that these building blocks were spaced by 6–9 residues apart throughout this region (Figure 2B).

In addition, the N-terminal region of VEGFR1 d2 contained by itself six prolines, distant by five to nine residues from one another, except for the fragment Pro(143)-Pro(167) separated by 13 residues. Therefore, we chose to introduce an N-(Dmb)-glycine in this last fragment and to conserve the rest of the N-terminal sequence unchanged.

Couplings were performed with HBTU/DIPEA reagents in NMP for 1 h. A double-coupling was realized for some residues, selected according to the results of our first synthesis and a systematic capping was performed after each coupling by treatment with acetic anhydride for 5 min. Figure 3 represents the RP-HPLC profile of crude peptides cleaved from small samples of peptidyl-resin corresponding to the fragments Glu(201)-Ile(229), Gly(181)-Ile(229), Gly(151)-Ile(229), Ser(129)-Ile(229). The elongation proceeded smoothly for the coupling of the first 50 residues but proved to be rather difficult for the last part. This result was confirmed by the UV monitoring of the Fmoc cleavage (data not shown), which indicated that the loss of purity was caused by the difficult coupling/deprotection steps of the sequence ^{162}Ser-Pro-Asn-Ile-Thr-^{167}Val, which is rich in β-branched aminoacids, thus encouraging us to perform a systematic double coupling for the last 22 residues of the sequence. At the end of the synthesis, the peptide was cleaved from the resin and deprotected by treatment with TFA 95% in the presence of ethanedithiol and triisopropylsilane as scavengers. 1.1 g of crude peptide, in its reduced form, was obtained after lyophilization.

Protein Purification and Folding

The crude peptide was first purified by semi-preparative RP-HPLC on a C8 column. Runs were performed with 80 mg of crude, and similar fractions, according to their retention time, were collected and pooled with an automated collector. These fractions were then analyzed by MALDI–Tof spectroscopy to identify the ones containing reduced VEGFR1 d2. At the end of this step, 480 mg of crude VEGFR1 d2 was obtained. Impurities were characterized by a mass comprising between 7300 and 7800 Da (Figure 4). Therefore, we assume that they corresponded to the truncated forms of VEGFR1 d2 arising from the weak coupling of the residues ^{162}Ser-Pro-Asn-Ile-Thr-^{167}Val. The crude was submitted to a second purification by RP-HPLC on a C18 column. The fractions containing the desired peptide were pooled and freeze-dried to give 25 mg VEGFR1 d2 corresponding to 2.2% of the crude peptide. ESI–Q–Tof analysis permitted to confirm the nature of the peptide with a measured mass of 11 523.001 $\pm$ 0.0362 Da (calculated mass: 11 523.17 Da) (Figure 5A).

Then, the peptide was dissolved at 0.2 mg/ml in a 0.1 M Tris, 2.2 M guanidine, pH 8.0 buffer for 1 h and submitted to air oxidation at 4 °C for 24 h. A minimum concentration of 1.8 M of guanidine was necessary to avoid aggregation of the unfolded peptide. The

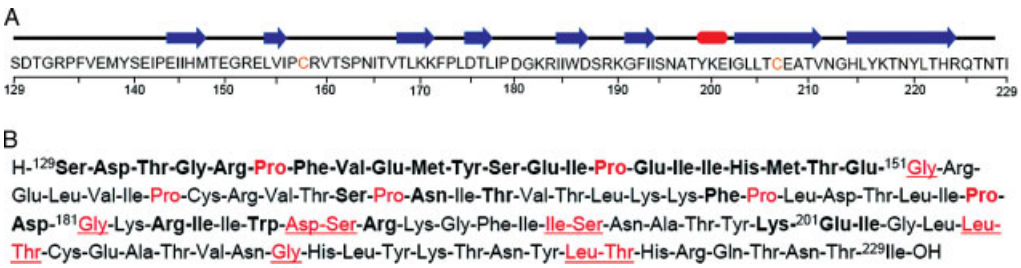

Figure 2. (A) Amino acids sequence of VEGFR1 d2. Corresponding secondary structure elements are labeled as follows: β strands are represented with arrows and the α-helix is indicated by a rectangle. (B) Synthesis sequence of the 101-residue VEGFR1 d2. Sites substituted by dimethyloxazolidine dipeptides and (Dmb)-glycines are and underlined. Proline residues are written in grey. Double-coupled residues are in bold. Superscript numbers indicate when resin samples were removed for analysis. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

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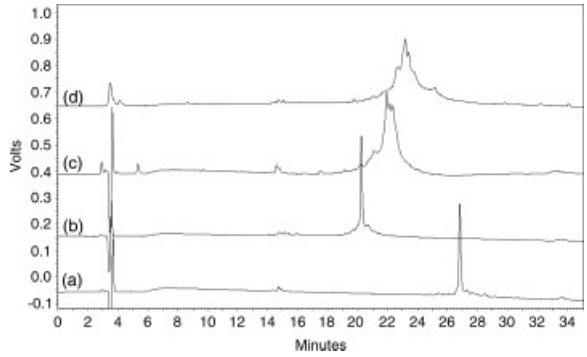

Figure 3. RP-HPLC analytical chromatograms of peptide samples obtained after the coupling of 29-mer (a), 49-mer (b), 79-mer (c) and 101-mer (d) peptides. Conditions were as described in Material and Methods.

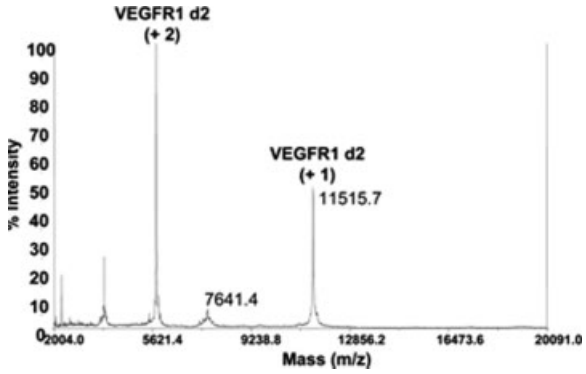

Figure 4. MALDI–Tof analysis after one purification step. Reduced VEGFR1 d2 is contaminated by the presence of truncated peptides with a mass of 7300–7800 Da.

folding process was monitored by analytical RP-HPLC (Figure 6). Interestingly, even before bubbling air into the solution ($t = 0$ h), an evolution of the solution was observed with the apparition of a peak with a shorter retention time (20.2 min) compared to the reduced form of VEGFR1 d2 (21.8 min). This peak may arise from a partial oxidation of the protein. After 18 h, the peak corresponding to reduced VEGFR1 d2 had almost completely disappeared and was replaced by the peak at 20.2 min, confirming that it corresponded to the oxidized form of VEGFR1 d2. In addition, a broad peak was concomitantly formed (retention time: 23 min) that may correspond to misfolded forms of VEGFR1 d2. No evolution of the HPLC profile was observed between 18 h and 24 h. The solution was then dialyzed against a 50 mM Na_2HPO_4, 150 mM NaCl buffer at pH 6.5 for 24 h at 4 °C. The peptide was concentrated and filtered with an Amicon Ultra centrifugation tube equipped with a molecular-weight cut-off filter of 10 000 Da. RP-HPLC analysis indicated that the desired peptide was retained and that most of the impurities had been eliminated.

The mass of the folded peptide, determined by ESI–Q–Tof mass spectroscopy after deconvolution, was of 11 521.001 ± 0.0326 Da with a maximal intensity for the 9th charged state (Figure 5B). The loss of 2 Da, compared to the reduced form, is in agreement with the formation of a disulfide bridge during the folding process. In

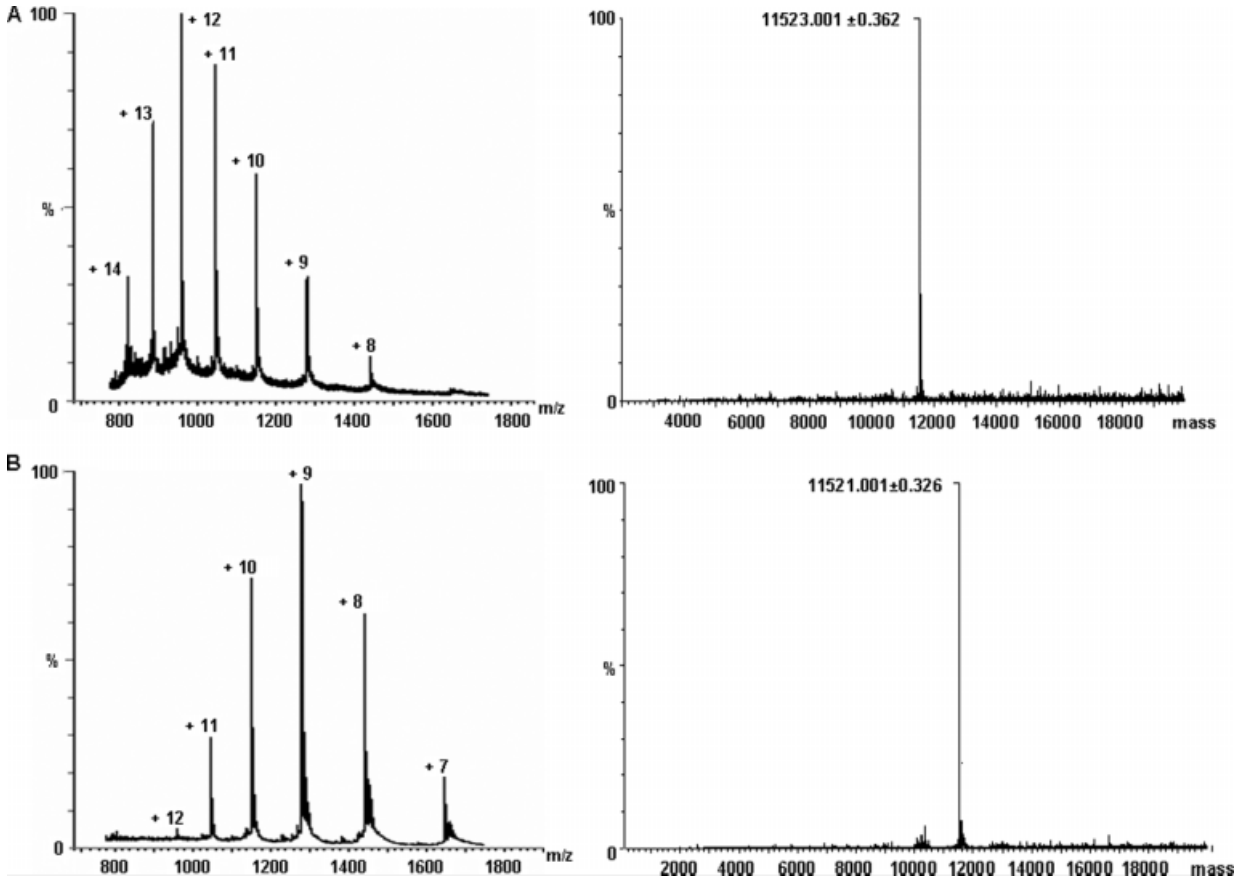

Figure 5. Multi-charged ESI–MS spectra (left) and corresponding deconvoluted spectra (right) of (A) the reduced and (B) folded forms of VEGFR1 d2.

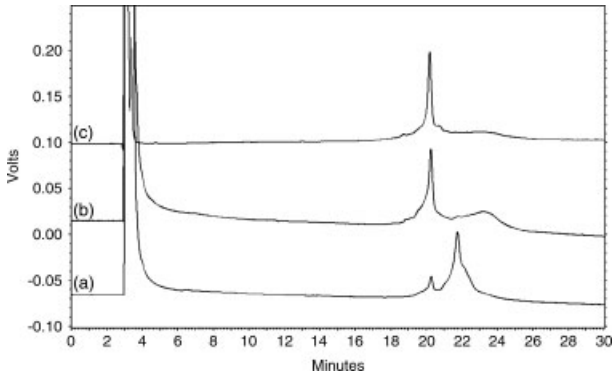

Figure 6. Folding and purification of VEGFR1 d2. RP-HPLC elution profiles of analytical samples recovered after (a) 0 h and (b) 18 h of air oxidation and (c) after dialysis and ultrafiltration.

addition, the shift of the *m/z* pattern of VEGFR1 d2, compared to its reduced form, confirms its folded structure.

The concentration of the solution was determined by UV absorbance spectroscopy at 280 nm indicating that 8.6 mg of folded and pure VEGFR1 d2 were obtained (folding yield 35%).

ELISA-Based Assays

While the mass spectra confirmed the formation of the disulfide bridge, we next verified if VEGFR1 d2 has adopted its bioactive conformation during the folding process. Therefore, we employed an ELISA-based assay to confirm the ability of VEGFR1 d2 to bind $VEGF_{165}$ [16].

First, a fixed amount of VEGFR1 d2 was coated on a high-binding microplate and was put in presence of different doses of $btVEGF_{165}$. After wash steps, the remaining $btVEGF_{165}$, bound to VEGFR1 d2, was detected by chemiluminescence thanks to a streptavidin–horseradish peroxidase conjugate. Thereby a saturation curve of VEGFR1 d2 by $btVEGF_{165}$ was obtained with a K_D of 1.62 nM for 250 ng of immobilized VEGFR1 d2 (Figure 7A). The binding of $btVEGF_{165}$ on this receptor domain could be inhibited by the addition of unlabeled $VEGF_{165}$ with an IC_{50} of 0.52 nM (Figure 7B).

Alternatively, VEGFR1 d2 was tested as an inhibitor of $btVEGF_{165}$ interaction with the full length extracellular domain of VEGFR1 (VEGFR1 ECD). In this case, VEGFR1 ECD was adsorbed on the microplate and put in presence of a fixed quantity of biotinylated $VEGF_{165}$ and different amounts of VEGFR1 d2. A dose-dependent inhibition of the VEGF–VEGFR1 ECD interaction was obtained with an IC_{50} of 0.6 μM in the conditions of the experiment (Figure 7C).

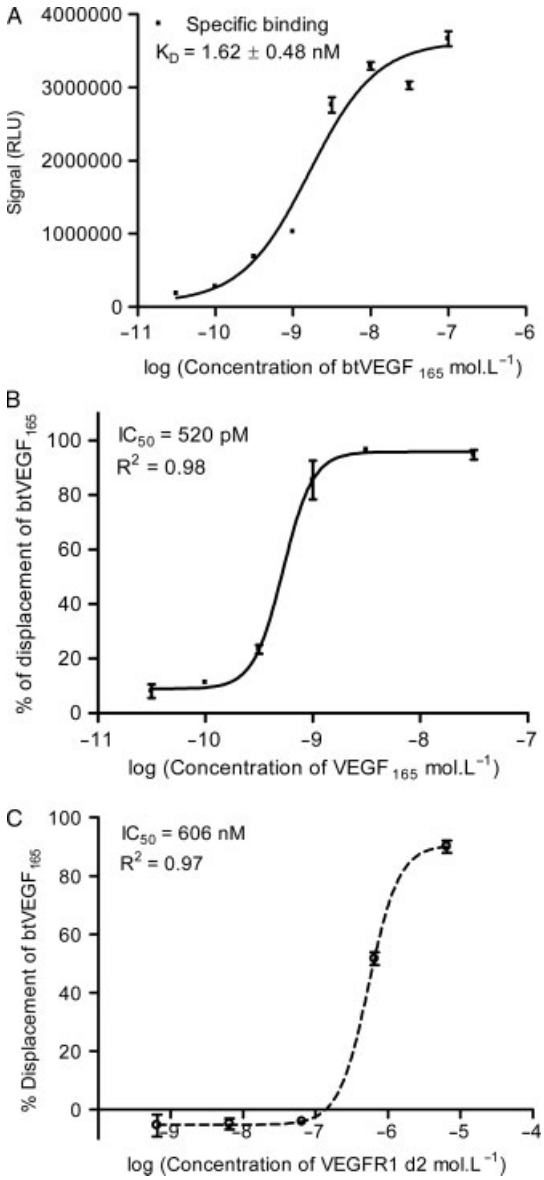

Figure 7. Biological activity of VEGFR1 d2. (A) Saturation assay of VEGFR1 d2 by biotinylated $VEGF_{165}$. (B) Inhibition of biotinylated $VEGF_{165}$ binding to VEGFR1 d2 by $VEGF_{165}$. (C) Inhibition of biotinylated $VEGF_{165}$ binding to VEGFR1 ECD by VEGFR1 d2.

Discussion

In this study, we have synthesized a protein domain of 101 residues corresponding to the VEGF-binding region of the VEGF receptor 1. This domain constitutes a valuable target for the development of protein–protein inhibitors displaying anti-angiogenic properties. Although its synthesis by conventional SPPS appeared as extremely difficult because of aggregation issues, the introduction of constrained amino acids during peptide elongation allowed us to circumvent most of these difficulties. However, despite these precautions, the coupling of the small sequence Ser(162)-Val(167) of the peptide proved to be weak and diminished the efficiency of the synthesis. Hopefully, we expect that introducing a double coupling for these residues will overcome this difficulty and render the synthesis of VEGFR1 d2 a fast and reliable task.

In addition, these results have confirmed the common observation that the folding of proteins obtained through SPPS is not more difficult than the folding of proteins obtained as inclusion bodies in bacteria [17,18]. Another interesting point is the interest of employing molecular-weight-based separation methods in combination with RP-HPLC purification. The use of centrifugation tubes equipped with molecular-weight cut-off filters, in the last purification step, allowed us to eliminate most of the by-products corresponding to truncated peptides. Alternatively, this method could be of considerable interest at the beginning of the purification process, as it permits to purify rapidly significant amounts of peptides.

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The biological evaluation of VEGFR1 d2 activity, in combination with ESI–Q–Tof mass spectroscopy, allowed us to confirm the correct folding of the protein domain. Based on the saturation assay, the K_D of VEGFR1 d2 for biotinylated $VEGF_{165}$ was 1.62 nM. This result is in good agreement with previous studies indicating that this region of VEGFR1 was responsible for the binding of VEGF. Indeed, for comparison, the K_D of $btVEGF_{165}$ for the full-length VEGFR1 ECD is estimated at 757 pM [16].

To conclude, we expect that the flexibility of the SPPS will facilitate the incorporation of unnatural amino acids in the peptide sequence. These modifications should represent a considerable advantage in the development of potent small-molecule ligands of VEGFR-1. And, we expect that this strategy could be adapted to the production of VEGFR2 domain 2, whose tridimensional structure has never been solved.

Materials and Methods

General

Novasyn TGA resin (substitution: 0.23 mmol/g) was purchased from Novabiochem. HBTU, HOBt and DIPEA were from Applied Biosystems (Courtaboeuf, France). All amino acids, from Novabiochem (Meudon, France) or Bachem (Weil am Rhein, Germany) were N^{α}-terminal protected by Fmoc and their side chains were protected as follows: Arg(*N*-Pmc); Asn(*N*-Trt); Asp(*O*-tBu); Cys(*S*-Trt); Gln(*N*-Trt); Glu(*O*-tBu); His(*N*-Trt); Lys(*N*-Boc); Ser(*O*-tBu); Thr(*O*-tBu); Trp(*N*-Boc) and Tyr(*O*-*t*Bu). N^{α}-Fmoc-N^{α}-Dmb-Glycine-OH and the pseudoproline dipeptides employed [Fmoc-Leu-Thr($\psi^{Me,Me}$pro)-OH, Fmoc-Ile-Ser($\psi^{Me,Me}$pro)-OH, Fmoc-Asp(OtBu)-Ser($\psi^{Me,Me}$pro)-OH] were purchased from Novabiochem. Peptide synthesis solvents and acetonitrile for HPLC were analytical grade and were acquired from commercial sources and used without further purification.

Peptide Synthesis, Purification and Analysis

The peptide was synthesized by Merrifield stepwise solid phase synthesis on an Applied Biosystems 433A automated peptide synthesizer using standard scale (0.25 mmol) *FastMoc* chemistry. Coupling reactions were performed using Fmoc amino acids (4 eq.), activated with HBTU (4 eq.) and HOBt (4 eq.) in the presence of DIPEA (8 eq.), for 1 h. A capping was performed after each coupling by treatment with acetic anhydride capping solution (0.5 M acetic anhydride, 0.125 M DIEA, 0.015 M HOBt in NMP) for 5 min. Fmoc removal was realized by treating the resin with 20% piperidine in NMP for 15 min.

Final peptide and samples were cleaved from the resin with simultaneous removal of side-chain protecting groups by treatment with 15 ml TFA/water/ethanedithiol/triisopropylsilane (94/2.5/2.5/1 v/v) for 3 h at room temperature. The filtrate from the cleavage reaction was evaporated, precipitated in cold diethyl oxide, collected by centrifugation and lyophilized.

The crude peptide (1.1 g) was first purified by semi-preparative RP-HPLC on a C8 column (Kromasil, 10 µm, 20 × 250 mm) with a gradient program (solvent A is water with 0.1% TFA and solvent B is a mixture of 70% acetonitrile and 30% water with 0.09% TFA) at a flow rate of 8 ml/min with UV detection at 214 nm and 254 nm. Fractions were analyzed by RP-HPLC on a Symmetry 300 C18 column (Vydac, 5 µm, 4.6 × 250 mm) at a flow rate of 1 ml/min with a gradient program (10% to 100% solvent B in 30 min) and the fractions containing the peptide were collected and lyophilized to yield the peptide as a white solid. The peptide identity was checked by MALDI–Tof mass spectroscopy on a 4700 Proteomics Analyzer (Applied Biosystems) with α-cyano-4-hydroxycinnamic acid or sinapinic acid matrix. Spectra were recorded in positive and linear modes.

The peptide (240 mg) was submitted to a second purification by RP-HPLC on a C18 column (Vydac, 218TP54, 5 µm, 10 × 250 mm) at a flow rate of 2 ml/min and fractions were analyzed as previously. The ESI–Q–Tof mass spectrum of the unfolded VEGFR1 d2 was recorded on a Q–Tof-1 mass spectrometer (Micromass, UK) and deconvoluted with MassLynx 4.0 software (Micromass, UK). ESI–MS, *m/z* calculated for $C_{516}H_{826}N_{138}O_{152}S_4$: 11 523.17, was found to be 11 523.001 ± 0.0362; t_R = 21.8 min (40–70% of solvent B in 30 min).

Unfolded peptide was then dissolved at 0.2 mg/ml in a 2.2 M guanidinium, 0.1 M Tris, pH 8.0 buffer. Air was bubbled in the solution for 24 h at 4 °C with gentle mixing. Folding was monitored by analytical RP-HPLC as previously. Next, the folded peptide was purified and concentrated on an Amicon Ultra-15 centrifugal filter device equipped with a 10 000 Da cutoff membrane. The peptide was then dialyzed against 3 × 1 l of a 50 mM Na_2HPO_4, 150 mM NaCl buffer at pH 6.5 for 24 h. The solution was finally concentrated to 1.8 mg/ml (4.3 mg, yield: 0.15%) and analyzed by ESI–MS. *m/z* calculated for $C_{516}H_{824}N_{138}O_{152}S_4$: 11 521.16, was found to be 11 521.001 ± 0.0326; t_R = 20.2 min (40–70% of solvent B in 30 min, purity >95%).

Chemiluminescent ELISA-Type Assays

The assays were performed as previously described [9].

Supporting information

Supporting information may be found in the online version of this article.

Acknowledgements

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References

1 Ferrara N, Gerber HP. The role of vascular endothelial growth factor in angiogenesis. *Acta Haematol.* 2001; **106**: 148–156.
2 Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; **407**: 249–257.
3 Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat. Med.* 2003; **9**: 669–676.
4 Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling – in control of vascular function. *Nat. Rev. Mol. Cell Biol.* 2006; **7**: 359–371.
5 Shibuya M. Structure and dual function of vascular endothelial growth factor receptor-1 (Flt-1). *Int. J. Biochem. Cell Biol.* 2001; **33**: 409–420.
6 Wiesmann C, Fuh G, Christinger HW, Eigenbrot C, Wells JA, de Vos AM. Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor. *Cell* 1997; **91**: 695–704.
7 Starovasnik MA, Christinger HW, Wiesmann C, Champe MA, de Vos AM, Skelton NJ. Solution structure of the VEGF-binding domain of Flt-1: comparison of its free and bound states. *J. Mol. Biol.* 1997; **293**: 531–544.

8 Christinger HW, Fuh G, de Vos AM, Wiesmann C. The crystal structure of placental growth factor in complex with domain 2 of vascular endothelial growth factor receptor-1. *J. Biol. Chem.* 2004; **279**: 10382–10388.
9 Goncalves V, Gautier B, Coric P, Bouaziz S, Lenoir C, Garbay C, Vidal M, Inguimbert N. Rational design, structure, and biological evaluation of cyclic peptides mimicking the vascular endothelial growth factor. *J. Med. Chem.* 2007; **50**: 5135–5146.
10 D'Andrea LD, Del Gatto A, Pedone C, Benedetti E. Peptide-based molecules in angiogenesis. *Chem. Biol. Drug Des.* 2006; **67**: 115–126.
11 Bedford J, Hyde C, Johnson T, Jun W, Owen D, Quibell M, Sheppard RC. Amino acid structure and ''difficult sequences'' in solid phase peptide synthesis. *Int. J. Pept. Protein Res.* 1992; **40**: 300–307.
12 Tam JP, Lu YA. Coupling difficulty associated with interchain clustering and phase-transition in solid-phase peptide-synthesis. *J. Am. Chem. Soc.* 1995; **117**: 12058–12063.
13 Johnson T, Quibell M, Owen D, Sheppard RC. A reversible protecting group for the amide bond in peptides – use in the synthesis of difficult sequences. *J. Chem. Soc. Chem. Com.* 1993; **4**: 369–372.
14 Wohr T, Wahl F, Nefzi A, Rohwedder B, Sato T, Sun XC, Mutter M. Pseudo-prolines as a solubilizing, structure-disrupting protection technique in peptide synthesis. *J. Am. Chem. Soc.* 1996; **118**: 9218–9227.
15 Toniolo C, Bonora GM, Mutter M, Pillai VNR. Linear oligopeptides. 78. The effect of the insertion of a proline residue on the solution conformation of host peptides. *Macromol. Chem. Phys.* 1981; **182**: 2007–2014.
16 Goncalves V, Gautier B, Garbay C, Vidal M, Inguimbert N. Development of a chemiluminescent screening assay for detection of vascular endothelial growth factor receptor 1 ligands. *Anal. Biochem.* 2007; **366**: 108–110.
17 Lu WY, Qasim MA, Kent SBH. Comparative total syntheses of turkey ovomucoid third domain by both stepwise solid phase peptide synthesis and native chemical ligation. *J. Am. Chem. Soc.* 1996; **118**: 8518–8523.
18 Dong CZ, Romieu A, Mounier CM, Heymans F, Roques BP, Godfroid JJ. Total direct chemical synthesis and biological activities of human group IIA secretory phospholipase A2. *Biochem. J.* 2002; **365**: 505–511.

