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Parallel solid-phase synthesis of a small library of linear and hydrocarbon-bridged analogues of $VEGF_{81-91}$: Potential biological tools for studying the VEGF/VEGFR-1 interaction

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ABSTRACT

The design, synthesis and binding affinity for VEGFR-1 receptors of a small library of linear and cyclic analogues of the $VEGF_{81-91}$ fragment are described. Cyclic 11- and 10-mer peptide derivatives were prepared using parallel solid-phase protocols. The formation of hydrocarbon alkene-bridged cyclic peptides was achieved through optimized ring-closing metathesis reactions from linear derivatives with conveniently located allylGly residues. Alkane-bridged analogues were successfully obtained by ulterior on-resin hydrogenation. Binding assays showed that some of these compounds were able to compete with labeled VEGF for interaction with the VEGFR-1 receptor. Several peptide derivatives, **2**, **7** and **8**, showed modest but significant binding affinity, indicating that the designed peptide could mimic the $VEGF_{81-91}$ fragment and therefore disrupt the VEGF/VEGFR-1 interaction. This fact opens the way for using these peptides as the starting point for biological/pharmacological tools to deeply investigate this protein–protein system.

1. Introduction

An attractive approach for the modulation of protein–protein interactions is the use of short peptides that correspond to fragments identified as important for the target interaction. The pro-angiogenic action of the vascular endothelial growth factor (VEGF) is mainly mediated through its binding to specific receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR).[1–4] Considering that one of the best validated signalling pathways in angiogenesis is represented by VEGF and its receptors (VEGFRs),[5] disruption of these protein–protein interactions could be a valid approach for the search of inhibitors of angiogenesis, the process of generation of new blood vessels from preexisting ones. The relevant role of pathologic angiogenesis in a number of diseases,[6,7] as cancer, rheumatoid arthritis and diabetic retinopathy[8,9] justifies the interest of inhibiting this process.

Two main hot-spots have been identified at the interaction interface between VEGF and its receptors Flt-1 and KDR. These are fragments $VEGF_{81-91}$, located at loop 3, and $VEGF_{17-25}$, located at the N-terminal.[10–14] The first hot-spot, fragment $VEGF_{81-91}$ (MRIKPHQGQHI), constitutes part of a β-hairpin structure, slightly distorted at the β-turn, according to the X-ray structure.[15] The first site-directed mutagenesis studies of VEGF, performed by triple mutation, pointed to residues Arg82, Ile83, Lys84 and His86 as essential for the VEGF interaction with KDR.[10] Further mutagenesis studies together with X-ray crystallographic data highlighted residues Ile83, Lys84 and Pro85 as critical for the interaction with VEGF receptors.[11,12] Moreover, most of the amino acid residues of loop 3 are also located in the interaction interface of VEGF and the Fab fragment of the monoclonal antibody Bevacizumab,[16] as well as some other antibodies and peptides.[14,17,18] These studies pointed to residues Met81 and Gln89 of loop 3 as key for the VEGFA-VEGFR interaction.

The only previous work related to VEGF loop 3 mimetics has been reported by Zilberberg et al. that prepared a 17-mer head-to-tail cyclic peptide analogue of $VEGF_{79-93}$ fragment (c[f^1PQIMRIK^{8}PHQGQHIGE]-NH_2).[19] This analogue showed anti-angiogenic properties, and was able to inhibit VEGF binding to its VEGFR-1 and VEGFR-2 receptors. In water, NMR structural studies

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showed no defined structure while, in TFE/water mixtures the chemical shift deviations of Hα and Cα supported the formation of a helical conformation in segment 1–8. No stable β-turns are formed either by D-Phe1-Pro2, or by Pro9-His10.

Based on the above mutagenesis and structural data, a series of analogues of $VEGF_{81-91}$ was designed with the aim of mimicking the biological properties of the native protein with smaller compounds. Peptide sequences normally show high conformational flexibility when excised away from the folded protein, requiring some element of stabilization to retain their native conformation. In this case, hydrocarbon-bridged cyclic peptides were envisaged, taking advantage of the ring closing metathesis reaction (RCM). In recent years, this reaction has been widely applied to stabilize secondary structure elements present in protein hot-spots.[20] The resulting RCM-cyclic peptides have the additional advantage that the new generated hydrocarbon bridge confers the compound extra metabolic resistance. Good examples of this strategy are the hydrogen bond surrogate,[21] and the stapled approach,[22,23] both designed to stabilize α-helical structures in bioactive peptides. Grubbs and co-workers, proved also the usefulness of hydrocarbon bridges to fix the β-turn structure of Glutaredoxine active-site,[24] and Leucocin A.[25] However, to the best of our knowledge, the application of RCM to stabilize β-hairpin structures in bioactive peptides has not been reported yet.

To design peptides that could mimic the native $VEGF_{81-91}$ fragment (**1**), hydrocarbon-bridges were used as tethers to link the two strands of the β-hairpin. Preferred positions for cyclization were 2–10, to keep as much as possible the residues identified as key for the VEGF/VEGFR interaction. First, the alkene-bridged cyclic peptide **3**, analogue of the native $VEGF_{81-91}$ fragment (MRIKPHQGQHI, **1**), might be prepared from the corresponding linear sequence **2**, in which allylGly residues were incorporated at positions 2 and 10, occupied by Arg82 and His90 in the natural sequence (Fig. 1). On the other hand, considering that the β-hairpin structure of the native fragment $VEGF_{81-91}$ was somewhat distorted because of the presence of an extra amino acid after the β-turn, truncated analogues were also designed by deleting Gln87 (**5**, **6**) or Gly88 (**8**, **9**). To explore a wider conformational space, more flexible alkane derivatives **4**, **7** and **10** were also considered, since they would be easily obtained by reduction of the corresponding alkene analogues **3**, **6** and **9**. To study the importance of ring size and of certain residues for receptor recognition, cyclic peptide derivatives **12** and **14**, joining positions 3–9 and 1–11, were also planned.

We report herein the results of the solid-phase synthesis and the preliminary biological studies (binding affinity to VEGFR-1) of this small library of VEGF analogues, taking $VEGF_{81-91}$ fragment as a model.

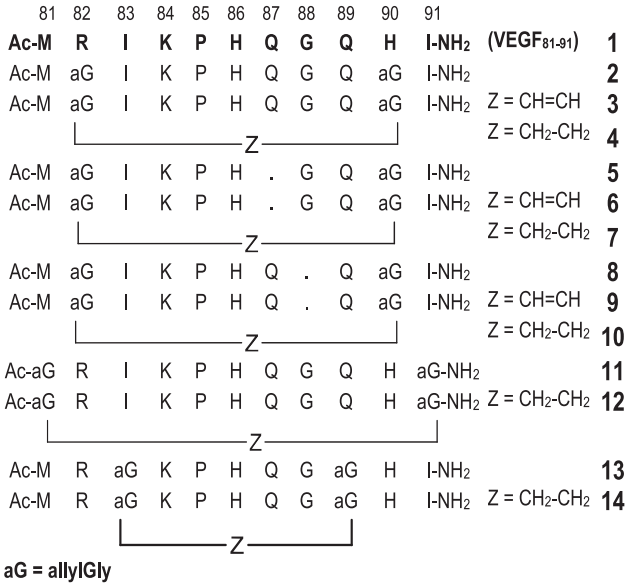


Figure 1. Sequences of the designed $VEGF_{81-91}$ analogues.

2. Results and discussion

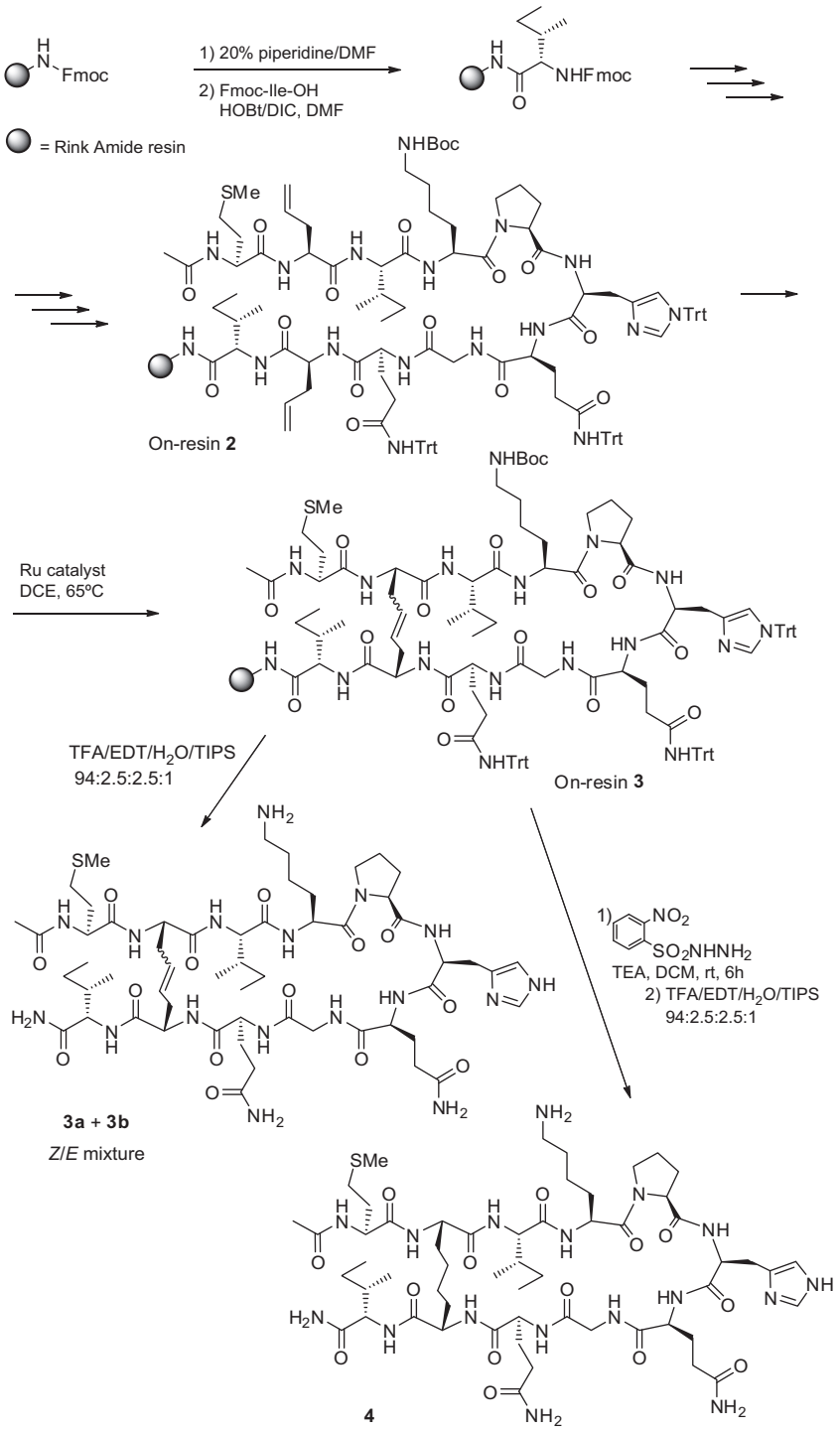
2.1. Synthesis

Designed $VEGF_{81-91}$ analogues were prepared following solid-phase synthesis protocols, performed manually in parallel, using Rink amide MBHA polystyrene resin and a Fmoc/*t*Bu strategy. All compounds were isolated as amide at the C-terminal, and acetylated at the N-terminal. A low load resin (0.34 mmol/g) was chosen to prevent aggregation and intermolecular reactions in the cyclization step (Scheme 1). Cyclic analogues were prepared from the corresponding solid-supported linear peptides, in which the appropriate alkene-containing residues were included at the suitable positions.

The RCM reaction was chosen for the preparation of the designed alkene-bridged cyclic compounds. Although on-resin RCM has been widely described in the literature,[26–28] normally the reaction conditions have to be optimized for each particular substrate. In our case, attempts of cyclization were carried out using second generation Grubbs and Hoveyda-Grubbs catalysts, with linear allylGly-containing peptide **2** anchored to the polymer support. Using conventional heating, several rounds of optimization of the reaction conditions regarding solvent, temperature, reaction time, as well as amount and type of catalyst were performed (Table 1). The final choice was 1,2-dichloroethane (DCE) as solvent at 65 °C, and a second generation of Hoveyda-Grubbs catalyst. The best results were obtained when three portions of 30 mol % of catalyst were added every 24 h. Preliminary treatment of the linear precursor anchored to the resin with a solution of 0.8 M LiCl in DMF, as chaotropic agent, proved to increase yield and purity of cyclic isolated derivatives.[29] After the RCM reaction and washings, a further treatment of the resin with anhydrous DMSO was performed to further eliminate catalyst residues.[30] These conditions permitted the isolation of cyclic compounds **3** and **9** in good yields. However, the preparation of compound **6** required more drastic conditions (85 °C, DMF). In all cases, the linear compound was not detected after RCM, but the formation of the cyclic analogue with the oxidized Met81 side-chain was observed in a 20–30% range. Fortunately, the separation of this by-product by HPLC was easily achieved.

To increase the structural flexibility of cyclic peptides **3**, **6** and **9**, the reduction of the double-bond bridges was attempted. Although very few examples of hydrogenation in solid phase have hitherto been reported, to facilitate the purification step we tried to achieve the olefin reduction while the compounds remained attached to the solid support. For this purpose, a solid-phase olefine reduction by using diimine generated from 2-nitrobenzenesulfonohydrazide was successfully adapted to peptide derivatives.[31] Alkane-bridged compounds **4**, **7** and **10** were satisfactorily obtained by this method (Scheme 1).

The above described RCM cyclization conditions allowed the preparation of 29- and 26-membered medium size cycles (2-10-cyclic peptide **3**, and 2-9-bridged truncated derivatives **6**, **9**) with good overall yields, while failed for the synthesis of the 1-11- and 3-9-bridged cyclic analogues of the native sequence, with 35 and 23 total atoms in the cyclic skeleton, respectively. To try to overcome this problem, we started a new round of optimization of the cyclization conditions by using microwave. On-resin linear



Scheme 1. Representative solid-phase synthetic procedure for cyclic peptides **3** and **4**.

compound **8** was chosen as the prototype for this study. After several attempts, a significant decrease in the total reaction time, from 72 to 1 h, as well as in the amount of catalyst, from 30% to 20% (Table 2) was achieved. The best results were obtained when using DCE as solvent, and three portions of 20 mol % of catalyst were added every 20 min (Table 2, entry 16). Moreover, the overall yield of the cyclic compound **9** was slightly improved by this method, from 5% to 12% (Table 3), and the sulfoxide byproduct formation was also minimized. The use of LiCl in this case did not really make any improvement in the yield (Table 2, entries 9–11, and 15). This is in agreement with the anti-aggregation effect of the microwave irradiation, which makes unnecessary the addition of any chaotropic agent. Assays to increase the reaction temperature failed (entries 10 and 12), leading to low yields and complex mixtures.

However, the results obtained by applying these conditions to the preparation of cycles **12** and **14** were not as expected, and further optimization of the RCM microwave reaction was necessary. Thus, compound **12** was prepared by cyclization of the corresponding on-resin derivative **11** followed by solid-phase reduction of the alkene-bridged cyclic analogue. The conditions finally used for cyclization were similar to that described in entry 16 of Table 2,

Table 1
Optimization of the metathesis reaction to obtain compound **3** by conventional heating

Assay	Catalyst[a]	Solvent	T (°C)	t (h)	LiCl	DMSO	% Cyclic[b]		% Linear[b]	
							3[c]	**3**M(O)[d]	**2**[c]	**2**M(O)[d]
1	15% G	DCM	40	48	—	—	18	—	70	8
2	15% HG	DCM	40	48	—	—	20	—	63	16
3	30% G	DCE	65	72	—	Yes	8	16	12	61
4	30% HG	DCE	65	72	—	Yes	28	28	10	31
5	30% HG	DCE	65	72	Yes	Yes	70	28	—	—
6	30% HG	DCE	65	48	Yes	Yes	70	14	14	—
7	30% HG	DCE	65	24	Yes	Yes	64	9	22	—

[a] G refers to Grubbs, and HG to Hoveyda-Grubbs catalyst (see formula in Supplementary data).
[b] Percentages determined by HPLC-MS; Column: Waters X-Bridge; A = CH_3CN + 0.08% formic acid, B = H_2O + 1% formic acid, gradient: from 5% to 80% of A in 15 min.
[c] Compound with a Met residue.
[d] Compound with sulfoxide of Met side-chain.

Table 2
Optimization of the metathesis reaction to obtain compound **9** through microwave irradiation

Assay	Catalyst	%[a]	No. of additions	t (min)	T	LiCl	% **8**[b]	% **9**[b]	**8**:**9**[b]
1	G	10	1	20	120	––	61	3	20:1
2	HG	10	1	20	120	––	52	40	4:3
3	G	20	1	20	120	––	81	10	8:1
4	HG	20	1	20	120	––	40	52	3:4
5	G	30	1	20	120	––	88	5	17:1
6	HG	30	1	20	120	––	50	50	1:1
7	HG	10	1	40	120	—	65	28	7:3
8	HG	10	1	60	120	—	61	27	2:1
9	HG	10	1	40	120	Yes	81	11	8:1
10	HG	10	1	40	200[c]	Yes	46	4	10:1
11	HG	20	1	40	120	Yes	83	7	12:1
12	HG	20	1	40	200[c]	Yes	72	25	3:1
13	HG	20	2	20	120	—	63	26	5:2
14	HG	20	2	20	120	—	59	38	3:2
15	HG	20	2	20	120	Yes	52	36	3:2
16	HG	20	3	20	120	—	0	100	0:1

[a] Amount of catalyst used.
[b] Percentages and ratio determined by HPLC-MS; Column: Waters X-Bridge; A = CH_3CN + 0.08% formic acid, B=H_2O + 1% formic acid, Gradient: from 5% to 80% of A in 15 min.
[c] Using dichlorobenzene (DCB) as solvent instead of dichloroethane (DCE).

Table 3
Sequence and global yield of compounds **1**–**13** and **15**

Compd	Sequence	Yield (%)[a]
1	Ac-MRIKPHQGQHI-NH_2	—
2	Ac-MAllGIKPHQGQAllGI-NH_2	12
3a[b,c]	Ac-M-c(CH_2-*E*-CH=CH–CH_2)^{2,10} [GIKPHQGQG] I-NH_2	3
3b[b,c]	Ac-M-c(CH_2-*Z*-CH=CH–CH_2)^{2,10} [GIKPHQGQG] I-NH_2	5
4	Ac-M-c[$(CH_2)_4$] ^{2,10} [GIKPHQGQG] I-NH_2	12
5	Ac-MAllGIKPHQGQAllGI-NH_2	12
6a[b, c]	Ac-M-c(CH_2-*E*-CH=CH–CH_2)^{2,9} [GIKPHQGQG]I-NH_2	3
6b[b, c]	Ac-M-c(CH_2-*Z*-CH=CH–CH_2)^{2,9} [GIKPHQGQG]I-NH_2	5
7	Ac-M-c[$(CH_2)_4$]^{2,9} [GIKPHQGQG]I-NH_2	8
8	Ac-MAllGIKPHQQAllGI-NH_2	18
9[d]	Ac-M-c(CH_2–CH=CH–CH_2)^{2,9} [GIKPHQQG]I-NH_2	12(5)[c]
10	Ac-M-c[$(CH_2)_4$] ^{2,9} [GIKPHQQG]I-NH_2	7
11	Ac-AllGRIKPHQGQHAllG-NH_2	17
12[d]	Ac-c[$(CH_2)_4$]^{1,11}[GRIKPHQGQHG]-NH_2	3
13	Ac-MRAllGKPHQGAllGHI-NH_2	36
15	Ac-MRNvaKPHQGNvaHI-NH_2	6

[a] Yield of isolated pure compounds.
[b] *E* and *Z* isomers might be swapped.
[c] Conventional heating.
[d] Microwave assisted RCM.

except that the total amount of catalyst was added in smaller amounts, 10% each time, for a total of six additions. Although the formation of this latter analogue was satisfactory, the subsequent reduction of the double bond was not complete, remaining a 30% of the unsaturated cyclic compound (HPLC-MS data). Regarding compound **14**, the combination of the microwave-assisted RCM protocol and the diimide reduction was not successful. The only isolated compound was the linear peptide **15**, resulting from the hydrogenation of the allylGly side-chains of precursor **13**. In this case, although the addition of catalyst was repeated for nine more times, neither alkane nor alkene cyclic materials were detected in the crude reaction mixture.

All final compounds were purified first by reverse-phase cartridges, and followed by semi-preparative HPLC, leading to the isolation of linear and cyclic peptide derivatives in moderate/good yields and high purity ($\geqslant$99%, Table 3).

For alkene-bridged peptides, mixtures of *E*/*Z* isomers were observed in all cases (**3**, **6** and **9**). The *E*/*Z* mixture could be separated by HPLC in the case of compounds **3** and **6**, permitting the isolation and further biological evaluation of both isomers (Fig. 2). Preliminary NMR experiments did not allow the assignment of the *E*/*Z* geometry because of spectral overlapping with the water signal that interferes with critical cross-peaks in the NOE spectra.

2.2. Binding affinity for VEGFR-1

Synthesized peptides were evaluated for their ability to displace biotinylated $VEGF_{165}$, in a chemiluminescent assay relying on competition between tested compounds and biotinylated $VEGF_{165}$ for binding to the extracellular domain of recombinant VEGFR-1[32,33] (Table 4).

In general, linear compounds **2**, **5** and **8** showed better displacement values than the corresponding cyclic analogues, with

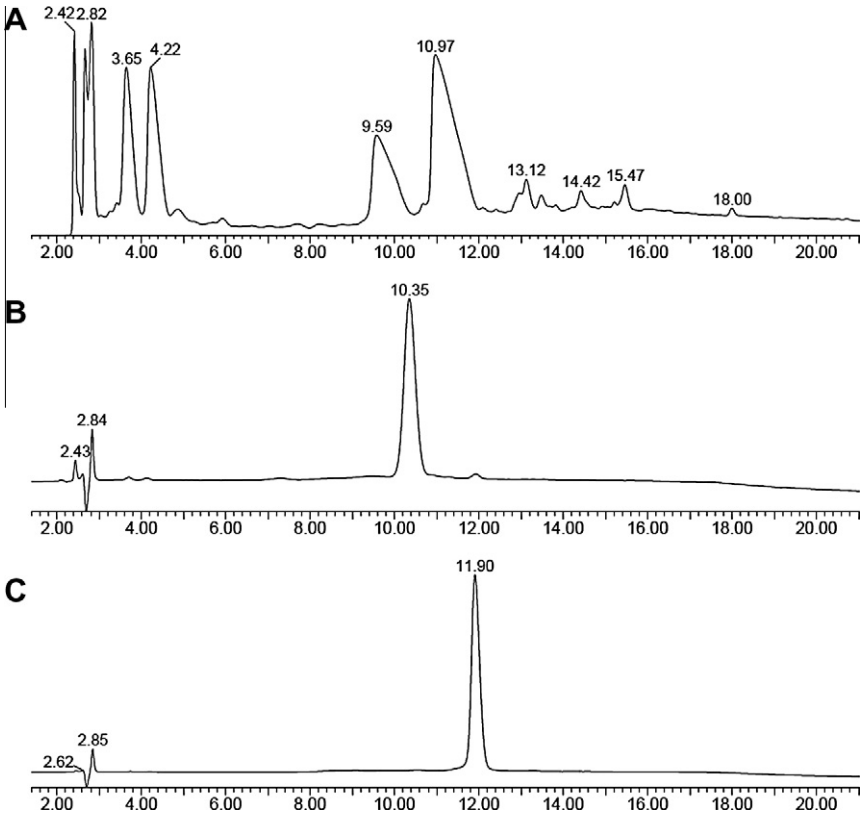


Figure 2. Example of the semi-preparative HPLC purification corresponding to derivative **3** (A) HPLC chromatogram of row material **3**; (B) and (C) HPLC chromatograms of each isomer, **3a** and **3b**, after purification.

Table 4
Inhibitory potency of peptides **1**–**3** and **15** on VEGFR-1

Compd	Sequence	% Inhibition[b]	IC_{50}[c] (μM)	
		D1–D7	D1–D7	D1–D3
1	H-MRIKPHQGQHI-OH	45.1 ± 11.2	102.7 ± 9.3	160.0 ± 20.7
2	Ac-MAllGIKPHQGQAllGI-NH_2	66.0 ± 12.4	61.2 ± 10.1	61.8 ± 8.3
3a[a]	Ac-M-*c*(CH_2-*E*-CH=CH-CH_2)^{2,10}[GIKPHQGQG]I-NH_2	23.0 ± 7.6	N/D[d]	N/D
3b[a]	Ac-M-*c*(CH_2-*Z*-CH=CH-CH_2)^{2,10}[GIKPHQGQG]I-NH_2	24.0 ± 7.1	N/D	N/D
4	Ac-M-*c*[$(CH_2)_4$]^{2,10} [GIKPHQGQG]I-NH_2	10.9 ± 6.3	N/D	N/D
5	Ac-MAllGIKPHGQAllGI-NH_2	47.4 ± 12.7	90.3 ± 8.1	109.5 ± 13.6
6b[a]	Ac-M-*c*(CH_2-*E*-CH=CH-CH_2)^{2,9}[GIKPHGQG]I-NH_2	32.0 ± 12.2	N/D	N/D
7	Ac-M-*c*[$(CH_2)_4$]^{2,9}[GIKPHGQG]I-NH_2	44.3 ± 2.9	105.5 ± 2.4	91.6 ± 5.6
8	Ac-MAllGIKPHQQAllGI-NH_2	71.8 ± 15.3	42.3 ± 17.1	36.5 ± 3.2
9	Ac-M-*c*(CH_2-CH=CH-CH_2)^{2,9}[GIKPHQQG]I-NH_2	33.6 ± 6.8	N/D	N/D
10	Ac-M-*c*[$(CH_2)_4$]^{2,9}[GIKPHQQG]I-NH_2	23.6 ± 13.4	N/D	N/D
11	Ac-AllGRIKPHQGQHAllG-NH_2	54.2 ± 7.3	98.8 ± 8.7	76.8 ± 12.3
12	Ac-*c*[$(CH_2)_4$]^{1,11}[GRIKPHQGQHG]-NH_2	8.8 ± 10.3	N/D	N/D
13	Ac-MRAllGKPHQGAllGHI-NH_2	2.2 ± 1.6	N/D	N/D
15	Ac-MRNvaKPHQGNvaHI-NH_2	4.7 ± 4.9	N/D	N/D

Displacement assays.
[a] *E* and *Z* isomers might be swapped.
[b] Activity corresponds to the percentage of biotinylated VEGF165 displaced by a concentration of peptide of 100 μM on the whole extracellular domain (ECD, D1–D7) of VEGFR-1.
[c] Inhibitory concentration able to displace 50% the binding of biotinylated VEGF165 on VEGFR-1 either at the whole extracellular domain (D1–D7), or at the D1–D3 domains.
[d] N/D = not determined.

affinities in the same order, or even higher (**2** and **8**), than that of the native $VEGF_{81-91}$ fragment, **1**. The lack of affinity found for linear peptides **13** and **15** could be due to the substitution of Ile83 and Gln89 residues, identified as crucial for binding of VEGF to its receptors. The best results correspond to that of the linear truncated peptide, **8**, which exhibit an IC_{50} of about 40 μM, and to the saturated cyclic analogue, **7**, showing a percentage of inhibition comparable to the value observed for the $VEGF_{81-91}$ native fragment, but probably endowed with better metabolic stability.

The possible existence of stabilizing hydrophobic interactions between the side-chains of the two allylGly residues, which could favour the correct conformation for receptor binding, could explain

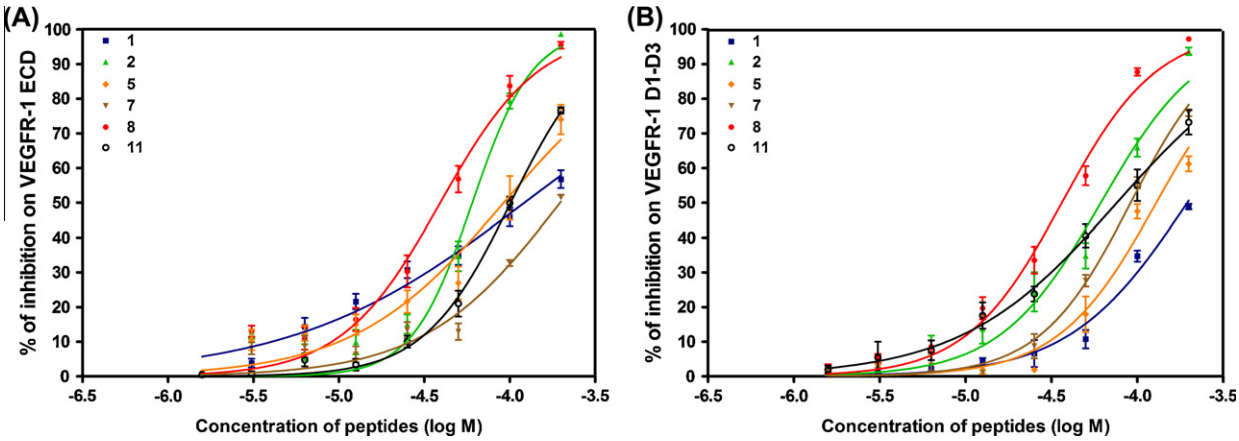


Figure 3. Effects of peptides on VEGFR-1 displacement assay. (A) With VEGFR-1 extracellular domain (ECD); (B) With VEGFR-1 D1–D3 domains.

the increase in affinity of linear peptides **2** and **8** when compared to model **1**. This was also suggested by Vederas and co-workers to rationalize the results obtained for antimicrobial hydrocarbon-bridged cyclic peptides and their parent bis-allylGly linear precursors.[25,34] Conformational studies on both linear and cyclic compounds would be required to correlate the biological binding data with the tendency of the compounds to adopt the three-dimensional structure of the $VEGF_{81-91}$ fragment within the native protein.

Compounds with the higher IC_{50} values for the whole extracellular domain of VEGFR-1 (ECD, D1–D7) were selected for evaluation on the D1–D3 immunoglobulin domains of this receptor, the specific domains for VEGF binding (Table 4, Fig.3). The affinities found for the D1–D7 (ECD) and D1–D3 domains were very similar. Therefore, it can be concluded that these $VEGF_{81-91}$ analogues are probably interacting with the receptor through the same region than the native ligand VEGF.

Comparing the obtained IC_{50} values with those reported by Gautier and co-workers,[35] for peptide mimics that cover a larger epitope than the one presented in this work, they have comparable IC_{50} values in the same binding test. Therefore, the peptides reported here could be considered promising tools for the development of new VEGF mimetics.

3. Conclusions

To study the implication of the $VEGF_{81-91}$ fragment in the VEGF/VEGFR-1 interaction, a small set of linear and cyclic peptide analogues of this fragment have been designed and prepared by parallel solid-phase synthesis. Appropriately placed allylGly residues were used as cyclization-points to restricted peptidomimetics, by ring closing metathesis reaction (RCM). Optimization of the RCM reaction conditions, both using conventional heating and microwave technology, was required for the synthesis of the alkene-bridged derivatives. Ulterior on-resin hydrogenation, using a modified diimine-based method, gave the alkane cyclic analogues. Some of these compounds showed good affinities when evaluated for binding to VEGFR-1 receptor, with similar or even better values than the native $VEGF_{81-91}$ fragment. This work supports the involvement of the VEGF fragment 81-91 in the direct interaction with VEGFR-1. Linear peptides, like **2** and **8**, and the alkane-bridged analogue **7** could be considered as easy-to-use biological/pharmacological tools to study the VEGF/VEGFR interaction. This corroborates the usefulness of the $VEGF_{81-91}$ fragment as a template for the design of peptidomimetics to disrupt or modulate the angiogenesis process. Further work in this direction is being performed with the aim of improving the biological profile of this series of peptide derivatives.

4. Experimental section

4.1. Materials

All reagents were of commercial quality. Fmoc amino acids and HOBt were provided by NeoMPS, DIC by FLUKA, 2nd generation Hoveyda-Grubbs catalyst, *o*-nitrobenzenesulfonyl chloride and hydrazine hydrate by ALDRICH and Rink Amide resin by Novabiochem. Solvents were dried and purified by standard methods. Analytical RP-HPLC was performed on a Waters 600 HPLC apparatus equipped with a reverse-phase ACE-5 C18-300 column (4.6 × 250 mm), at a flow rate of 1.3 mL/min, and a Waters 2487 detector, monitorized at 214 nm. Mixtures of CH_3CN (solvent A) and H_2O + 0.05% trifluoroacetic acid (solvent B) were used as mobile phase. For purification of compounds, a semipreparative column ACE 5 C18-300 (10 × 250 mm) was used at a flow rate of 6 mL/min, with the same solvents as the mobile phase. Solid phase extractions were carried out using reverse phase C18 Discovery SPE cartridges from SUPELCO. Electrospray mass spectra (ES-MS) were performed, in positive mode, in a Waters HPLC-MS ZQ 2000 equipment. HRMS (EI+) were performed in an Agilent 6520 Accurate-Mass Q-TOF LC/MS equipment. When other conditions were used it will be indicated in the description of the product.

Resins were swollen in DCM/DMF/DCM/DMF (4 × 0.5 min). All compounds were synthesized manually in parallel on resin, following the Fmoc/*t*Bu strategy, using Fmoc-Ile-OH, Fmoc-Met-OH, Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH o Fmoc-allylGly-OH as required.

Peptide Met^1-Arg^2-Ile^3-Lys^4-Pro^5-His^6-Gln^7-Gly^8-Gln^9-His^{10}-Ile^{11} ($VEGF_{81-91}$, **1**) was purchased from NeoMPS (Strasbourg, France). Data for cyclic peptide Ac-Met^1-cycle[$(CH_2)_4$]^{2,10}[Gly^2-Ile^3-Lys^4-Pro^5-His^6-Gln^7-Gly^8-Gln^9-Gly^{10}]-Ile^{11}-NH_2 (**4**) is described in Ref. 31.

4.2. General synthetic procedures

4.2.1. Coupling procedure

MBHA-Rink amide resin (100 mg, 0.034 mmol) previously swollen, was treated with 2 mL of 20% piperidine in DMF (1 × 1 min) and (3 × 10 min) and washed with DMF/DCM/DMF/DCM/DMF (4 × 0.5 min). Then, a solution of the corresponding Fmoc-amino acid (0.051 mmol) in anhydrous DMF (1 mL), HOBt (7.8 mg,

0.051 mmol) and DIC (7.8 μL, 0.051 mmol) were successively added. Couplings were allowed to proceed at room temperature overnight. When necessary, the coupling was repeated with a fresh portion of Fmoc-amino acid and the indicated coupling reagents. After complete couplings, the resins were drained and washed with DMF/DCM/DMF/DCM (5 × 0.5 min).

Coupling reactions to primary and secondary amines were monitored by the ninhydrin and the chloranil tests, respectively.

4.2.2. Acetylation procedure

The final Fmoc-resin-bounded peptide derivative, previously swollen, was treated with 2 mL of 20% piperidine in DMF (1 × 1 min) and (3 × 10 min) and washed with DMF/DCM/DMF/DCM/DMF (4 × 0.5 min). Then a mixture of acetic anhydride/DIEA/DMF (2 mL, 1:1:1) was added (1 × 1 min) and (4 × 10 min) and washed with DMF/DCM/DMF/DCM/DMF (4 × 0.5 min).

4.2.3. Ring-closing metathesis reaction

Method A: The acetylated peptide anchored to the resin (0.034 mmol), previously swollen, was treated with a 0.8 M solution of LiCl in DMF for 2 min and washed with DMF/DCM (4 × 0.5 min). This process was repeated once. Then a solution of Hoveyda-Grubbs 2nd generation catalyst (6 mg, 0.01 mmol) in DCE (2 mL) was added under Ar. The reaction was heated at 65°C and smoothly stirred for 24 h. The addition was repeated twice (24 h each time). The reaction mixture was washed with DCE/DMF (3 × 0.5 min) and a solution of 0.73 mL (300 equiv) of anhydrous DMSO in DMF was added and stirred for 12 h. After that, the resin was washed with DMF/DCM/MeOH (3 × 0.5 min).

Method B: The same procedure described in method A, but using DMF at 85 °C instead of DCE at 65 °C.

Method C: A sample of the acetylated peptide anchored to the resin (0.034 mmol), previously purged with Ar in a microwave tube, was treated with a solution of Hoveyda-Grubbs 2nd generation catalyst (4 mg, 0.007 mmol) in DCE (2 mL). The reaction was heated in a microwave synthesizer at 120 °C for 20 min. The addition of the catalyst was repeated twice (20 min each time). The reaction mixture was washed with DCE/DMF (3 × 0.5 min) and a solution of 0.73 mL (300 equiv) of anhydrous DMSO in DMF was added and stirred for 12 h. After that, the resin was washed with DMF/DCM/MeOH (3 × 0.5 min).

Method D: The same procedure described in method C but using 2 mg (0.003 mmol) of Hoveyda-Grubbs 2nd generation catalyst and repeating the addition five more times (20 min each time) to complete 0.6 equiv of catalyst.

Method E: The same procedure described in method C but using 2 mg (0.003 mmol) of Hoveyda-Grubbs 2nd generation catalyst and repeating the addition nine more times (20 min each time) to complete 1 equiv of catalyst.

4.2.4. On-resin hydrogenation procedure

To a solution of 2-nitrobenzenesulfonohydrazine (147 mg, 0.68 mmol) in anhydrous DCM (2 mL), Et_3N (0.188 mL, 1.36 mmol) was added. The mixture was immediately added to the resin under Ar atmosphere. The reduction was completed after 6 h at room temperature. Then the resin was drained and washed with DCM/DMF/DMF:H_2O(1:1)/THF/DCM (4 × 0.5 min).

4.2.5. Cleavage procedure

The resin-bounded derivative was treated with 1 mL of TFA/EDT/H_2O/TIS (94:2.5:2.5:1) for 5 h at room temperature. The filtrates were precipitated from diethylether and centrifuged three times at 5000 rpm for 10 min. After the cleavage, a prepurification of the resulting residue was carried out using reverse-phase cartridges [H_2O/CH_3CN (9:1)] to remove catalyst residues, when applicable, followed by semipreparative HPLC.

4.3. Compound characterization

4.3.1. Ac-Met[1]-AllylGly[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gly[8]-Gln[9]-AllylGly[10]-Ile[11]-NH_2 (2)

After the elongation of the peptide, the resin-bound derivative was cleaved and purified by semipreparative HPLC (15% A 2 min, 15% A to 55% A in 25 min, 55% A to 100% A in 3 min) obtaining 5.2 mg of a white solid (12% total yield). NMR: See Table S1. HPLC: t_R = 12.60 min. ESMS *m/z* 644 $[M+2H]^{2+}$, calcd for $[C_{58}H_{95}N_{17}O_{14}S+2H]^+$, 1288.

4.3.2. Ac-Met[1]-cycle(CH_2-CH=CH-CH_2)[2,10][Gly[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gly[8]-Gln[9]-Gly[10]]-Ile[11]-NH_2 (3)

From **2** anchored to the resin, through the metathesis method A. This derivative was obtained as a mixture of *Z/E* isomers (~7/3), which could be separated by semipreparative HPLC (15% A 2 min, 15% A to 45% A in 25 min, 45% A to 100% A in 3 min). **3a:** white solid (1.3 mg, 3% total yield). HPLC: t_R = 10.35 min. ESIMS *m/z* 630.0 $[M+2H]^{2+}$, calcd for $C_{56}H_{91}N_{17}O_{14}S$, 1258;. HRMS *m/z* 1257.6652 $[M]^+$, calcd for $C_{56}H_{91}N_{17}O_{14}S$, 1257.6645. Compound **3b**: white solid (2.1 mg, 5% total yield). NMR: cis:trans Lys-Pro rotamer ratio (1:4), see Table S1. HPLC: t_R = 11.90 min. ESIMS *m/z* 630.0 $[M+2H]^{2+}$, calcd for $C_{56}H_{91}N_{17}O_{14}S$, 1258. HRMS *m/z* 1257.6652 $[M]^+$, calcd for 1257.6645.

4.3.3. Ac-Met[1]-AllylGly[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gly[7]-Gln[8]-AllylGly[9]-Ile[10]-NH_2 (5)

After the elongation of the peptide, the resin-bound derivative was cleaved and purified by semipreparative HPLC (15% A 2 min, 15% A to 55% A in 25 min, 55% A to 100% A in 3 min) obtaining 4.7 mg of a white solid (12% total yield). NMR: See Table S2. HPLC: t_R = 12.53 min. ESIMS *m/z* 580.0 $[M+2H]^{2+}$, calcd for $C_{53}H_{87}N_{15}O_{12}S$, 1158. HRMS *m/z* 1157.6379 $[M]^+$, calcd for 1157.6379.

4.3.4. Ac-Met[1]-cycle(CH_2-CH=CH-CH_2)[2,9][Gly[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gly[7]-Gln[8]-Gly[9]]-Ile[10]-NH_2 (6)

From **5** anchored to the resin, through the metathesis method B. This derivative was obtained as a mixture of *Z/E* isomers (~6.5/3.5), which could be separated by semipreparative HPLC (15% A 2 min, 15% A to 25% A in 25 min, 25% A to 100% A in 3 min). **6a:** white solid (1.2 mg, 3% total yield). NMR: cis:trans Lys-Pro rotamer ratio (2:3) mixture, see Table S2. HPLC: t_R = 13.52 min. ESIMS *m/z* 566.0 $[M+2H]^{2+}$. HRMS *m/z* 1129.6088 $[M]^+$, calcd for $C_{51}H_{83}N_{15}O_{12}S$, 1129.6066. Compound **6b**: white solid (2.0 mg, 5% total yield). HPLC: t_R = 14.70 min. ESIMS *m/z* 566 $[M+2H]^{2+}$, calcd for $C_{51}H_{83}N_{15}O_{12}S$ 1130. HRMS *m/z* 1129.6088 $[M]^+$, calcd 1129.6066.

4.3.5. Ac-Met[1]-cycle[(CH_2)$_4$][2,9][Gly[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gly[7]-Gln[8]-Gly[9]]-Ile[10]-NH_2 (7)

After the synthesis of the corresponding metathesis-derivative **6**, anchored to the resin, it was hydrogenated according to the method previously described. Then the product was cleaved and purified by semipreparative HPLC (15% A 2 min, 15% A to 25% A in 25 min, 25% A to 100% A in 3 min), and compound **7** was isolated as a white solid (3.1 mg, 8% total yield). NMR: cis:trans Lys-Pro rotamer ratio (1:3), see Table S2. HPLC: t_R = 14.34 min. ESIMS *m/z* 567 $[M+2H]^{2+}$, calcd for $C_{51}H_{85}N_{15}O_{12}S$ 1132. HRMS *m/z* 1131.6216 $[M]^+$, calcd 1131.6223.

4.3.6. Ac-Met[1]-AllylGly[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gln[8]-AllylGly[9]-Ile[10]-NH_2 (8)

After the elongation of the peptide, the resin-bound derivative was cleaved and purified by semipreparative HPLC (15% A 2 min, 15% A to 55% A in 25 min, 55% A to 100% A in 3 min) obtaining 7.5 mg of a white solid (18% total yield). NMR: see Table S2. HPLC:

t_R = 12.09 min. ESIMS *m/z* 616 $[M+2H]^{2+}$, calcd for $C_{56}H_{92}N_{16}O_{13}S$ 1230. HRMS *m/z* 1228.6741 $[M]^+$, calcd 1228.6750.

4.3.7. Ac-Met[1]-cycle(CH_2-CH=CH-CH_2)[2,9][Gly[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gln[8]-Gly[9]]-Ile[10]-NH_2 (9)

From **8** anchored to the resin, through the metathesis method C. After reverse-phase cartridge and purification by semipreparative HPLC (15% A 2 min, 15% A to 45% A in 25 min, 45% A to 100% A in 3 min, compound **9** was isolated as a white solid (4.9 mg, 12% total yield). NMR: 7:3 Z/E mixture, cis:trans Lys-Pro rotamer ratio (1:4/1:3), see Table S2. HPLC: t_R = 11.20 min. ESIMS *m/z* 602 $[M+2H]^{2+}$, calcd for $C_{54}H_{88}N_{16}O_{13}S$ 1202. HRMS *m/z* 1200.6429 $[M]^+$, calcd 1200.6437.

4.3.8. Ac-Met[1]-cycle[$(CH_2)_4$][2,9][Gly[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gln[8]-Gly[9]]-Ile[10]-NH_2 (10)

After the synthesis of the corresponding metathesis-derivative **9**, anchored to the resin, it was hydrogenated according to the method previously described. Then the product was cleaved and purified by semipreparative HPLC (15% A 2 min, 15% A to 35% A in 25 min, 35% A to 100% A in 3 min), and compound **10** was isolated as a white solid (2.8 mg, 7% total yield). NMR: cis:trans Lys-Pro rotamer ratio (1:4), see Table S2. HPLC: t_R = 11.53 min. ESIMS *m/z* 603 $[M+2H]^{2+}$, calcd for $C_{54}H_{90}N_{16}O_{13}S$ 1204. HR-MS *m/z* 1202.6627 $[M]^+$, calcd 1202.6594.

4.3.9. Ac-AllylGly[1]-Arg[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gly[8]-Gln[9]-His[10]-AllylGly[11]-NH_2 (11)

After the elongation of the peptide, the resin-bound derivative was cleaved and purified by semipreparative HPLC (10% A 2 min, 10% A to 50% A in 25 min, 50% A to 100% A in 3 min) obtaining 7.7 mg of a white solid (17% total yield). NMR: See Table S3. HPLC: t_R = 9.62 min. ESIMS *m/z* 1336 $[M+H]^+$, 668.7 $[M+2H]^{2+}$, 446.1 $[M+3H]^{3+}$, calcd for $C_{59}H_{94}N_{22}O_{14}$ 1335. HRMS *m/z* 1334.7310 $[M]^+$, calcd 1334.7320.

4.3.10. Ac-cycle[$(CH_2)_4$][1,11][Gly[1]-Arg[2]-Ile[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gly[8]-Gln[9]-His[10]-Gly[11]]-NH_2 (12)

After the corresponding standard couplings to resin-bound linear peptide **11**, metathesis reaction was carried out following method E. The resulting resin was reduced, according to the general hydrogenation procedure above described. After the cleavage, the row material was a (7:3) mixture of the cyclic alkane/alkene derivatives, from which the title compound could be separated in pure form by semipreparative HPLC (5% A 2 min, 5% A to 15% A in 25 min, 15% A to 100% A in 3 min) as a white solid (1.3 mg, 3% total yield). NMR: cis:trans Lys-Pro rotamer ratio (1:9), see Table S3. HPLC: t_R = 15.90 min. ESIMS *m/z*, 1310 $[M+H]^+$, 655.8 $[M+2H]^{2+}$, 437.5 $[M+3H]^{3+}$, calcd $C_{57}H_{92}N_{22}O_{14}$ 1309.

4.3.11. Ac-Met[1]-Arg[2]-AllylGly[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gly[8]-AllylGly[9]-His[10]-Ile[11]-NH_2 (13)

After the elongation of the peptide, the resin-bound derivative was cleaved and purified by semipreparative HPLC (10% A 2 min, 10% A to 50% A in 25 min, 50% A to 100% A in 3 min) obtaining 22 mg of a white solid (36% total yield). NMR: see Table S3. HPLC: t_R = 13.23 min. ESIMS *m/z*, 1339 $[M+H]^+$, 670.2 $[M+2H]^{2+}$, 447.1 $[M+3H]^{3+}$, calcd for $C_{59}H_{95}N_{21}O_{13}S$ 1338. HRMS *m/z* 1337.7134 $[M]^+$, calcd 1337.7139.

4.3.12. Ac-Met[1]-Arg[2]-Nva[3]-Lys[4]-Pro[5]-His[6]-Gln[7]-Gly[8]-Nva[9]-His[10]-Ile[11]-NH_2 (15)

After the corresponding standard couplings to resin-bound linear peptide **13**, the metathesis reaction was carried out following method E, and the obtained resin was reduced according to the general hydrogenation procedure above described. After the cleavage and prepurification on a reverse-phase cartridge, this derivative was purified by HPLC semipreparative (10% A 2 min, 10% A to 30% A in 25 min, 30% A to 100% A in 3 min) obtaining 2.7 mg of a white solid, corresponding to the titled linear peptide **15** (6% total yield). NMR: See Table S3. HPLC: t_R = 17.37 min. ESIMS *m/z* 1343.2 $[M+H]^+$, 672.2 $[M+2H]^{2+}$, 448.5 $[M+3H]^{3+}$, calcd for $C_{57}H_{91}N_{21}O_{15}S$ 1342. HRMS *m/z* 1341.7410 $[M]^+$, calcd 1341.6724.

4.4. Chemiluminescent competition assay

High-binding 96-well microplates were from Corning Life Sciences, Netherlands. Recombinant human VEGFR-1 ECD/Fc Chimera, VEGFR-1 D1–D3/Fc chimera and $btVEGF_{165}$ obtained as part of a Fluorokine biotinylated VEGF kit, were from R&D Systems, UK. Bovine serum albumin fraction V, BSA was from Sigma-Aldrich, France. AMDEX streptavidin-horseradish peroxidase was from Amersham Biosciences, UK, SuperSignal West Pico Chemiluminescent Substrate from Pierce, USA. Luminescence was quantified with an EnVision 2101 Multilabel Reader from PerkinElmer, USA. Data were analyzed using GraphPad Prism software version 4.03, USA.

The surface of white high-binding 96-well microplate was coated with 100 μL of phosphate-buffered saline solution (PBS, pH 7.4) containing either VEGFR-1 ECD/Fc Chimera (20 ng/well) or VEGFR-1 D1–D3/Fc chimera (15 ng/well) and incubated at 4 °C overnight. After three washes with 250 μL of PBS 0.1 %, (v/v) Tween 20 (buffer A), and the plate was blocked by 200 μL of PBS with 3 % (w/v) of BSA and stirred at 37 °C for 2 h. The plate was washed three times with buffer A. Then, 100 μL of a solution containing 131 pM of $btVEGF_{165}$ and the tested compounds at various concentration diluted in PBS containing 1%DMSO were added in each well. After 3 h stirring at 37 °C, the plate was washed four times with buffer A and 100 μL of streptavidin-horseradish peroxidase diluted at 1:8000 in PBS containing 0.1% (v/v) Tween 20 and 0.3% (w/v) BSA were added per well. After 1 h of incubation at 37 °C under obscurity and stirring, the plate was washed five times with 250 μL of buffer A and 100 μL of the chemiluminescent substrate were added. The remaining bt-$VEGF_{165}$ was detected by chemiluminescence, which was quantified. The percentages of $btVEGF_{165}$ displacement were calculated by the following formula: percentage of displacement = $100 \times [1 - (S - NS)/(MS - NS)]$, where S is the signal measured, NS is the nonspecific binding signal and MS is the maximum binding signal observed with $btVEGF_{165}$ without compounds tested. All the inhibition values are the average results of at least three experiments performed in triplicate.

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Supplementary data

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