Diminished Oligomerization in the Synthesis of New Anti-Angiogenic Cyclic Peptide Using Solution Instead of Solid-Phase Cyclization

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This manuscript is dedicated to Professor Gerard Deleris.

ABSTRACT:

The design and synthesis of novel peptides that inhibit angiogenesis is an important area for anti-angiogenic drug development. Cyclic and small peptides present several advantages for therapeutic application, including stability, solubility, increased bio-availability and lack of immune response in the host cell. We describe here the synthesis and biological evaluations of a new cyclic peptide analog of CBO-P11: cyclo(RIKPHE), designated herein as CBO-P23M, a hexamer peptide encompassing residues 82 to 86 of VEGF which are involved in the interaction with VEGF receptor-2. CBO-P23M was prepared using in solution cyclization, therefore reducing the peptide cyclodimerization occurred during solid-phase cyclization. The cyclic dimer of CBO-P23M, which was obtained as the main side product during synthesis of the corresponding monomer, was also isolated and investigated. Both peptides markedly reduce VEGF-A-induced phosphorylation of VEGFR-2 and Erk1/2. Moreover, they exhibit anti-angiogenic activity in an in vitro morphogenesis study. Therefore CBO-P23M and CBO-P23M dimer appear as attractive candidates for the development of novel angiogenesis inhibitors for the treatment of cancer and other angiogenesis-related diseases. © *2016 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 106: 368–375, 2016.*

Keywords: cyclic peptide; angiogenesis; CBO-P23M; CBO-P11; solid-supported cyclization; in solution cyclization; dimerization

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INTRODUCTION

Cancer is an angiogenesis-dependent disease[1,2] and blocking such angiogenesis is a well-established strategy to markedly reduce tumor growth and metastasis.[3]

Among the numerous factors involved in angiogenesis, vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells that plays a central role in angiogenesis by interaction with its specific receptors (VEGFR-1 or VEGFR-2) and subsequent activation of intracellular MAPK signaling pathways (e.g. Erk1/2).[4] Therefore, VEGF and its receptors VEGFR-1 and VEGFR-2 are prime targets for anti-angiogenic intervention which is thought to be one of the most promising strategies in cancer therapy. Several approaches have been employed to inhibit VEGF function. These include monoclonal neutralizing antibodies directed against VEGF and VEGF receptors, recombinant fusion proteins, recombinant soluble VEGF receptors, peptides that interfere with VEGF/VEGFR interactions and small organic molecules targeting the intracellular receptor kinase domain.[5]

A wide number of agents targeting both VEGF and its receptors have recently become standard treatments for different tumor types.[6] However, as tumor resistance to these agents may arise after a few months of treatment, new alternatives need to be investigated.

Within this context, our group has synthesized, characterized and evaluated as angiogenesis inhibitors several novel peptides derived from 79 to 93 VEGF sequence which is involved in the interaction with VEGFR-2.[7]

Among these analogs, the CBO-P11, a 17-amino acids cyclo peptide: cyclo(fPQIMRIKPHQGQHIGE), exhibits anti-angiogenic activity *in ovo* on the chicken chorioallantoic membrane (CAM) assay. Moreover, this peptide inhibits the growth of both established human intracranial and syngeneic glioma in mice.[8]

These results prompted us to investigate the anti-angiogenetic activity of a new, smaller CBO-P11 analog: cyclo(RIKPHE), designated herein CBO-P23M, which encompasses the shorter 82-86 VEGF sequence with the key residues Arg82, Lys84, and His86.[9] A glutamic acid was added to the C-terminal end of CBO-P23M in order to envisage a solid supported head to tail cyclization strategy and also for further functionalization. The extensive polymerization observed during the solid-supported cyclization led us to develop an alternative synthetic protocol employing cyclization in solution. This synthetic strategy allowed the preparation of CBO-P23M and its dimer as the main products which were isolated and tested in vitro as novel anti-angiogenesis agents.

Both our peptides markedly reduce VEGF-A-induced phosphorylation of VEGFR-2 and Erk1/2 and exhibit anti-angiogenic activity in an *in vitro* morphogenesis assay.

RESULTS AND DISCUSSION

Chemistry

In order to obtain CBO-P23M peptide, we decided to follow the synthetic pathway depicted in Scheme 1. This synthesis is based on the three-dimensional protection strategy Fmoc/*t*Bu/OAllyl and the Glu side chain anchoring to the resin (Scheme 1, step i). This synthetic approach allows the orthogonal deprotection of the C- and N-*termini* for the subsequent on-resin head-to-tail cyclization (Scheme 1, step iv).[10]

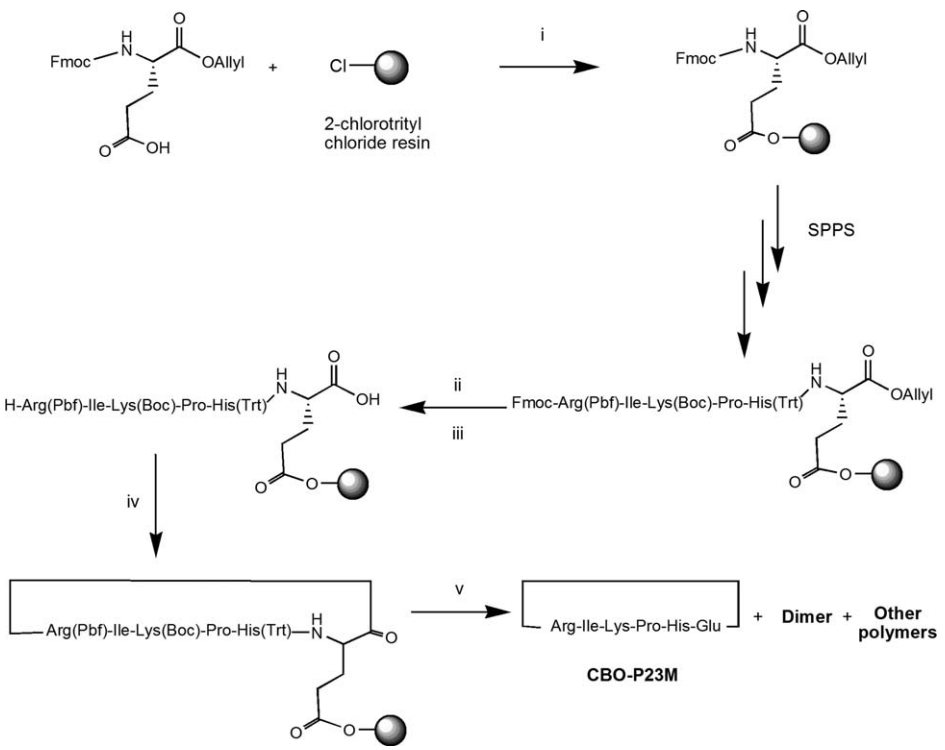
While the solid-phase cyclization usually takes advantage of the pseudo-dilution phenomenon, in our synthesis, the on-resin cyclization favors the formation of the cyclodimerization product and only traces of the desired monomer are produced (Figure 1). Further polymerization by-products are also observed: the cyclic trimer and tetramer. The attempts to isolate these products by RP-HPLC failed due to the small quantity of CBO-P23M formed and the co-elution of dimer, trimer and tetramer under the same HPLC peak at $t_R = 5.7$ min (Figure 1).

In order to avoid the formation of these side-products, the solid supported cyclization was investigated by modifying different parameters. Firstly, we reduced the cyclization time from 18 h to 1.5 h. Under these conditions only the monomeric, dimeric and trimeric linear peptides were obtained. Then, we tried the PyBOP/HOBt/DIEA coupling system. In this case, cyclo-dimerization and trimerization were mainly observed as for DIC/HOBt activation. Finally, we employed a low loaded resin (0.09 mmol/g) which was unfortunately not able to avoid the formation of cyclic polymers.

Under these circumstances, we next envisioned the synthesis of CBO-P23M peptide using the "in solution" cyclization of side-chain protected peptide (Scheme 2). For this purpose, we started the elongation of our linear peptide from the proline residue in order to avoid epimerization of the C-terminal residue during peptide cyclization.[11] Then we performed the head to tail cyclization in solution under high diluted conditions and we obtained the expected CBO-P23M by reducing the cyclodimerization and the formation of the other polymerization side-products (Figure 2). The crude product was purified and the CBO-P23M (Figure 3) and its dimer (Figure 4) isolated with 6% and 4% yield, respectively.

Biological Evaluation

Because MAPK signaling pathway is one of the main target of VEGF-A, we analyzed, by Western Blotting, VEGF receptor 2 (VEGFR-2) and Erk1/2 phosphorylation by VEGF-A in absence or presence of CBO-P11,[8] CBO-P23M or CBO-P23M dimer. As shown in Figure 5, treatment of HUVECs with 20 ng/mL of VEGF-A for 10 min strongly stimulates VEGFR-2 and Erk1/2 phosphorylation. Interestingly, 15 min of treatment (5 min of pretreatment and 10 min of co-treatment) with 20 μ*M* of CBO-P11, CBO-P23M, or CBO-P23M dimer markedly reduces VEGF-A-induced phosphorylation of VEGFR-2 and



SCHEME 1 Synthesis of CBO-P23M by on resin cyclization; Reagents and conditions: (i) DIEA, DCM, 3 h; (ii) $PhSiH_3$, $Pd(PPh_3)_4$, 3 h; (iii) 20% piperidine in DMF, 2 × 20 min; (iv) Method a) DIC/HOBt, DIEA in DMF, 18 h; Method b) DIC/HOBt, DIEA in DMF, 1.5 h; Method c) PyBOP/HOBt, DIEA in DMF, 18 h (v) TFA/H_2O/TIS, 90/5/5, v/v/v, 3 h.

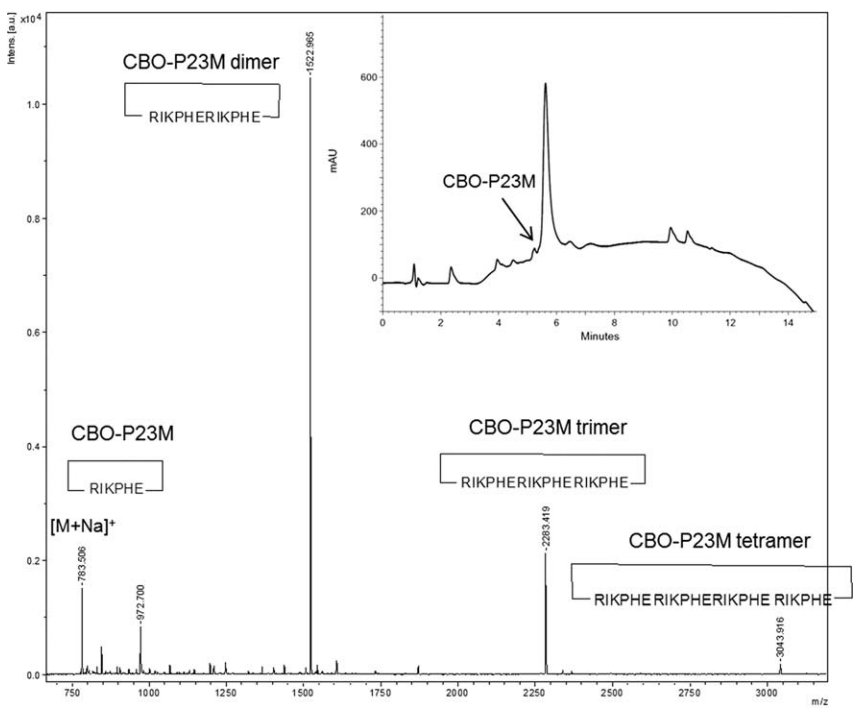
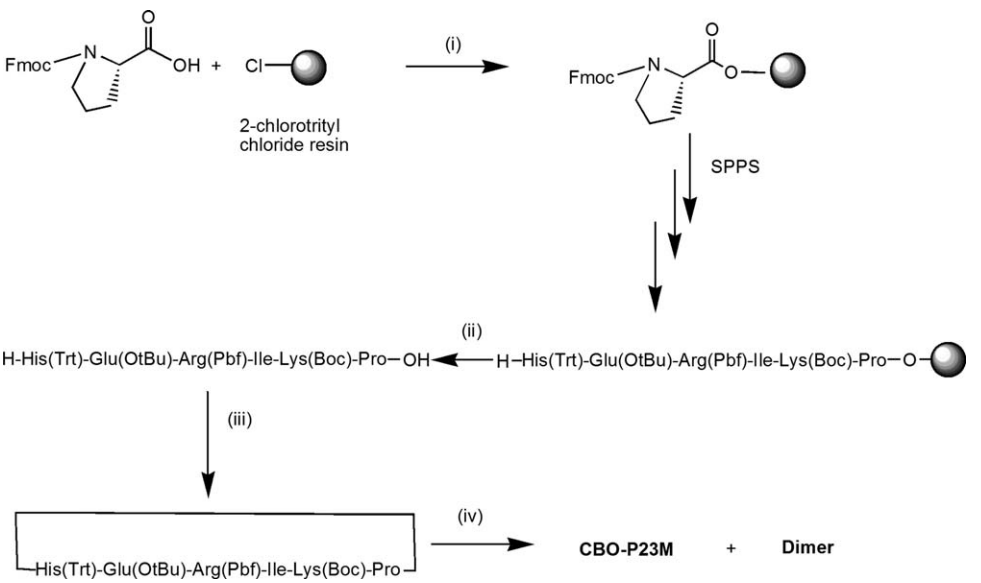


FIGURE 1 MALDI spectrum and HPLC profile of crude CBO-P23M obtained by on solid support cyclization (18 h reaction, DIC/HOBt as coupling system).



SCHEME 2 Synthesis of CBO-P23M by in solution cyclization; reagents and conditions: (i) DIEA, DCM, 3 h, (ii) 1% TFA in DCM, 5 × 1 min, (iii) DIC/HOBt, DIEA in DCM, 18 h, (iv) TFA/H_2O/TIS, 90/5/5, v/v/v, 3 h.

Erk1/2. CBO-P23M and CBO-P23M dimer display almost the same inhibitor effect on VEGFR-2 phosphorylation (69.6% and 79.3%, respectively); higher than that observed with CBO-P11 (46.7%). On the other side, VEGF-induced phosphorylation of Erk1/2 is uniformly inhibited by CBO-P23M, its dimer and CBO-P11.

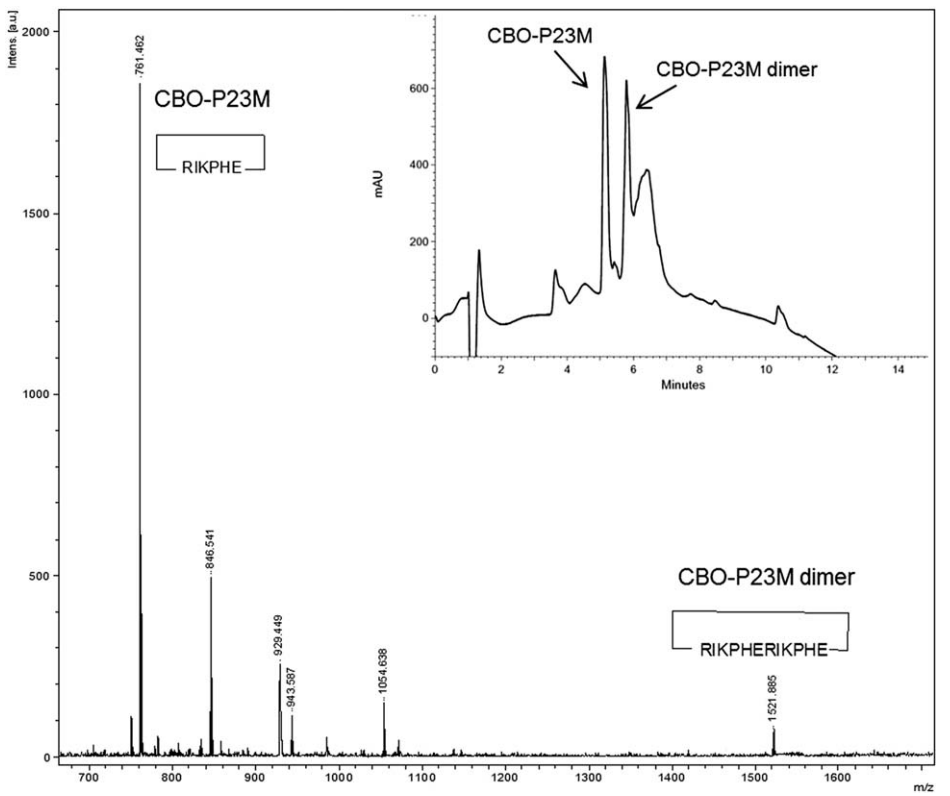


FIGURE 2 MALDI spectrum and HPLC profile of crude CBO-P23M obtained by in solution cyclization.

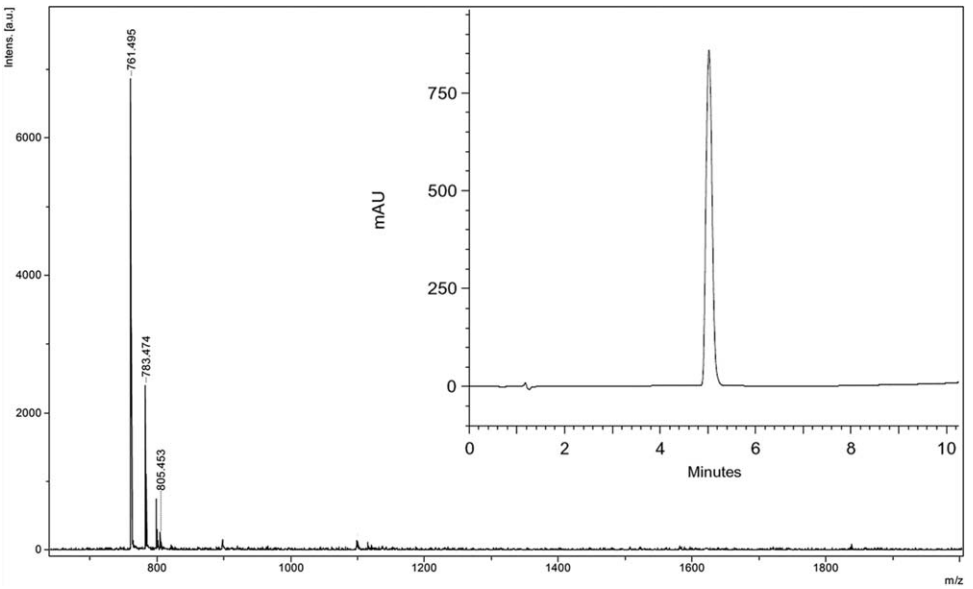


FIGURE 3 MALDI spectrum and HPLC profile of pure CBO-P23M.

Then, we investigated the capacity of HUVECs to form capillary-like structure by in vitro morphogenesis assay (Figure 6). This experiment is considered as the closest to *in vivo* angiogenesis[12] as it recapitulates several cellular events leading to vessel formation when HUVECs are cultured on extracellular matrix such as Matrigel®. Typical capillary-like structures were observed in basal conditions (EGM-2 containing less than 2.5 ng/mL of VEGF-A) whereas, when cultured in presence of additional 20 ng/mL VEGF-A, the formation of the structures is stimulated by 44%. In contrast, treatments with 10 μ*M* of CBO-P11, CBO-P23M, or CBO-P23M dimer drastically reduced this network. Taken together, these *in vitro* morphogenesis results highlight an anti-angiogenic capacity of CBO-P23M and CBO-P23M dimer comparable to that of CBO-P11.

MATERIALS AND METHODS

Chemicals

2-Chlorotrityl chloride resin, Fmoc-protected amino acids (Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Hys(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Glu-OAllyl), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), Hydroxybenzotriazole (HOBt) and

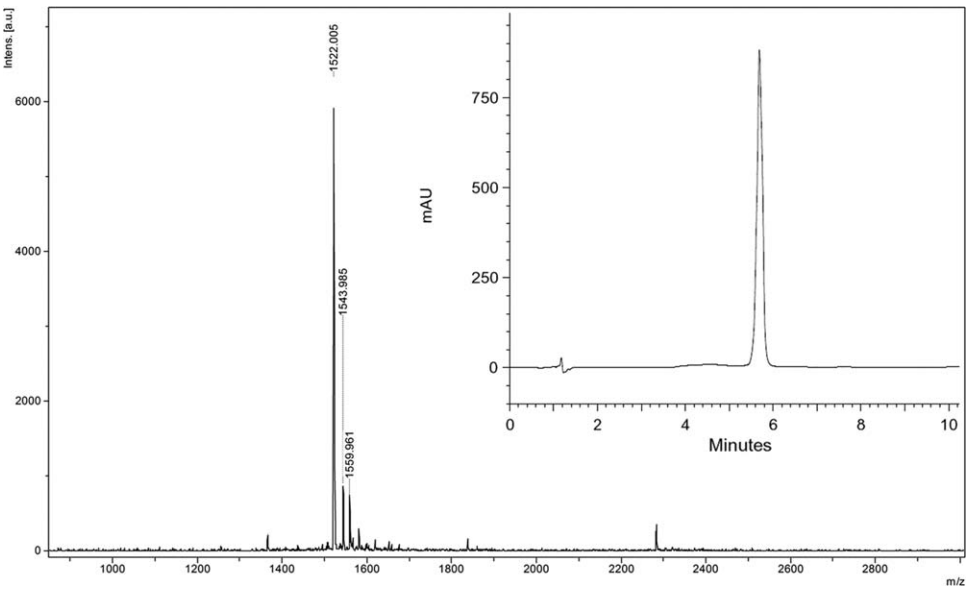


FIGURE 4 MALDI spectrum and HPLC profile of pure CBO-P23M dimer.

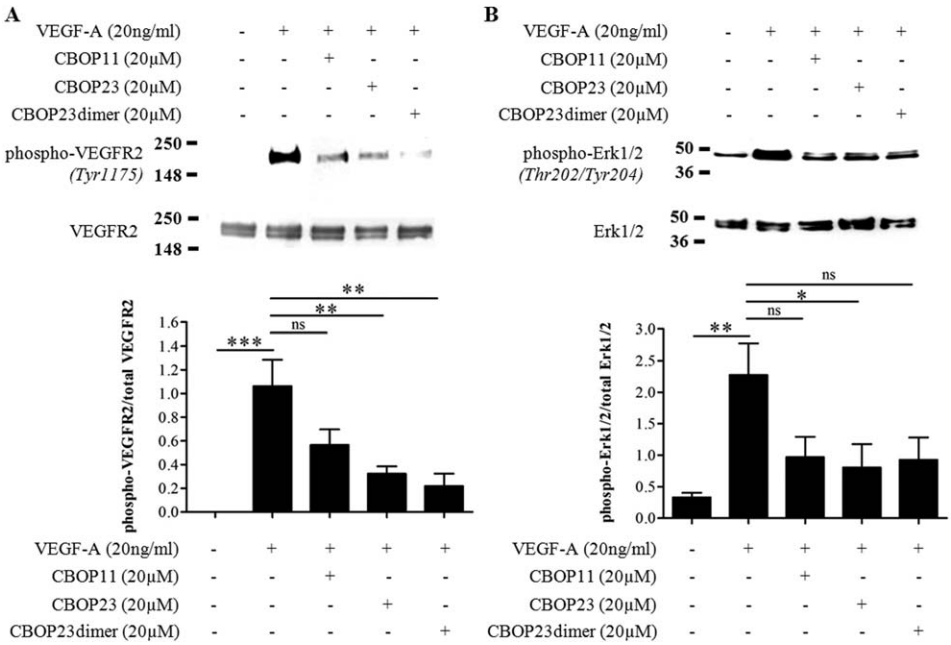


FIGURE 5 Inhibition of VEGF-A induced VEGFR-2 (A) and Erk1/2 (B) activation.

N,*N*′-diisopropylcarbodiimide (DIC) were obtained from Iris Biotech GMBH. All other chemicals were commercially available by Sigma-Aldrich and Acros.

Solid-Phase Peptide Synthesis General Experimental Procedures.

The syntheses were performed manually at 0.25 – 0.5 mmol scale using Fmoc/*t*Bu chemistry on 2-chlorotrityl chloride resin (1.6 mmol/g).

Resin Swelling. Swelling was performed by treating the resin for 20 min in dichloromethane (DCM).

First Amino Acid Coupling. After swelling of the resin, Fmoc-Pro-OH or Fmoc-Glu-OAllyl (2.3 eq) in DCM at 0.25*M* concentration and DIEA (6.9 eq) were added. The reaction mixture was left under mechanic stirring for 3 h at room temperature. The resin was

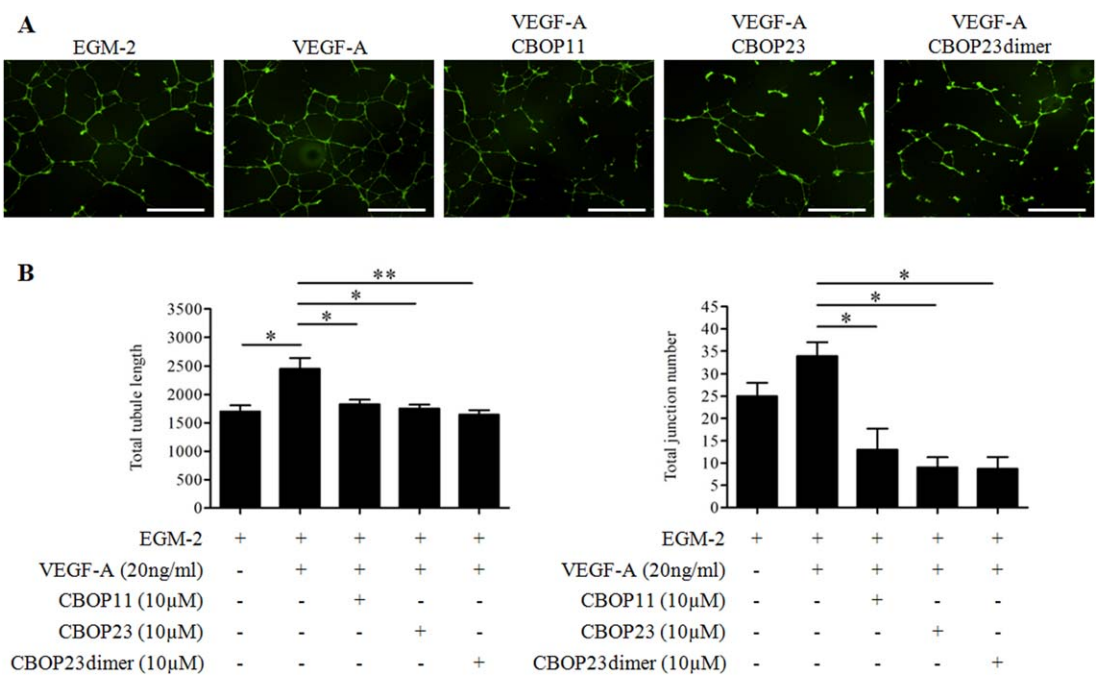


FIGURE 6 Inhibition of capillary-like network.

left under vacuum overnight. Then the resin loading was determined from the Fmoc-release monitored by UV absorption at 301 nm.

Fmoc-Deprotection. Fmoc-deprotection steps were performed by treating the resin 2 × 20 min in Piperidine (Pip)/DMF (80/20, v/v) solution.

Washing. Washing steps after coupling and deprotection steps were performed by treating the resin in dimethylformamide (DMF) (3 × 1 min), methanol (MeOH) (1 × 1 min), and DCM (3 × 1 min), successively.

Amino Acid Coupling. The resin was immersed in a DMF solution containing: Fmoc-protected amino acids (5 eq), HBTU (5 eq), and DIEA (10 eq) and left under mechanic stirring for 2 h.

Peptide Cyclization

On Resin. After peptide elongation, the Allyl-protection was removed from the peptide C-terminal carboxylate by treating the resin with $Pd(PPh_3)_4$ (0.35 eq) and $PhSiH_3$ (20 eq) in DCM, 3 h. The peptide was then cyclized on solid support by using DIC (5 eq) and HOBt (5 eq) in DMF overnight. Then the peptide was cleaved by immersing the resin in a trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (90/5/5, v/v/v) solution for 3 h. The cleavage cocktail was filtered from the resin and concentrated. The peptide was precipitated with ice-cold diethyl ether, centrifuged and decanted. Precipitation, centrifugation, and decantation operations were repeated twice. The resulting white solid was solubilized with H_2O/CH_3CN solution (50/50, v/v) and freeze-dried.

In Solution. After swelling of the resin, side-chain protected peptide was removed from the solid support by reacting the resin with a TFA/DCM (1/99, v/v) solution for 1 min, five times. Then the resin was washed with DCM (2 × 1 min). The cleavage and washing solutions were collected and evaporated. The product was solubilized with H_2O/CH_3CN solution (50/50, v/v) and freeze-dried.

The peptide was solubilized in DCM at 0.24 m*M* concentration and added dropwise (over 2 h) to a DCM solution containing HOBt (5 eq), DIC (5 eq), and DIEA (10 eq). This reaction mixture was left under magnetic stirring overnight and then evaporated.

Side-chains deprotection was performed by treating the peptide with a TFA/H_2O/TIS (90/5/5, v/v/v) solution for 3 h under magnetic stirring. Then the cleavage cocktail was concentrated and the peptide was precipitated with ice-cold diethyl ether, centrifuged and decanted. Precipitation, centrifugation, and decantation operations were repeated twice. The resulting white solid was solubilized with H_2O/CH_3CN solution (50/50, v/v) and freeze-dried.

For analysis, peptides were dissolved in a H_2O/CH_3CN/TFA solution (50:50:0.1, v/v/v) at 1 mg/mL concentration and analyzed by HPLC and Maldi.

RP-HPLC Analysis and Purification of Peptides

The HPLC characterization of products was performed by analytic reverse-phase HPLC using a VWR Hitachi instrument equipped with an L-2450 auto sampler, two L-2130 pumps, a Satisfaction RP18-AE column (5 μm, 250 × 4.6 mm) and a L-2450 diode array detector, at a flow rate of 0.8 mL/min. The compounds were purified by preparative reverse-phase HPLC using a VWR LaPrep system consisted of a P202 injector, two P110 pumps, a Satisfaction RP18-AB C18 column (5 μm, 250 × 20 mm) and a P314 UV detector, at a flow rate of 10 mL/min. The following eluents were used in a gradient mode: (A) 0.1% TFA in H_2O/CH_3CN (95/5) and (B) 0.1% TFA in CH_3CN/H_2O (95/5). Water was of Milli-Q quality and was obtained after filtration of distilled water through a Milli-Q® cartridge system. CH_3CN and TFA were of HPLC use quality. Degassing of solvents was performed using argon bubbling. Gradient used for the analytic and preparative RP-HPLC was respectively from 0% B to 100% B in 15 min and from 0% to 50% B in 50 min; UV detection at 214 nm. CH_3CN was evaporated and the aqueous solution was freeze-dried to give purified peptides as white solids.

Peptide Mass Analysis

Peptide identity was confirmed by mass spectrometry analyses performed on an Ultraflex III TOF/TOF system (Bruker Daltonics, Bremen, Germany), equipped with 200 Hz smartbeam laser (355 nm) and operating in reflectron positive ion mode. Mass spectra were acquired over the *m*/*z* range 300 to 5000 by accumulating data from 1000 laser shots for each spectrum. The instrumental conditions employed to analyze molecular species were the following: ion source 1: 25.08 kV; ion source 2: 21.98 kV, lens: 11.03 kV, pulsed ion extraction: 30 ns, reflector: 26.39 kV, reflector 2: 13.79 kV. Matrix suppression was activated by deflection mode: suppression up to 450 Da. Mass calibration was performed for each sample in range of 400 to 2000 Da with a peptide calibration mixture (8206195, Peptide Calibration Standard, Bruker Daltonics). The instrument was controlled using Bruker's flexControl 3.4 software and mass spectra were analyzed in Bruker's FlexAnalysis 3.4 software.

Cell Culture

Human umbilical vein endothelial cells (HUVECs, Lonza) were cultured in EGM-2 composed by endothelial cell basal medium-2 (EBM-2) and EGM-2 SingleQuot Kit Suppl. & Growth Factors (FBS, hydrocortisone, hFGF, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-1000 and heparin). HUVECs were used at early passages (1 to 8). Cells were kept at 37°C in a 5% CO_2 humidified incubator.

Western Blotting

HUVECs were plated in 12-well plates at a density of 5×10^4 cells/well in EGM-2 medium. After 3 days, cells were switched 16 h in basal medium EBM-2 before exposition to VEGF and peptides at the indicated concentrations and for the specified durations. Cells were collected by scraping and then lysed with Laemmli reducing buffer. Equivalent amounts of protein for each sample were analyzed by SDS-PAGE, transferred onto nitrocellulose membrane and probed with the following primary antibodies: anti-VEGFR2, anti-phospho-VEGFR Tyr1175, anti-Erk1/2 and anti-phospho-Erk1/2 Thr202/Tyr204 (Cell Signaling). Primary antibodies were detected using HRP-conjugated goat anti-mouse antibody (Dako) and Luminata HRP Substrate (Millipore). Densitometric analyses were performed in duplicate by 2 independent observers using ImageJ software. Statistical significance was determined by ANOVA test and Dunnet post-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: no statistical difference. Experiments were done three times.

Tube Formation Assay

Forty-eight-well plates were coated with 10 mg/mL Matrigel® (BD Biosciences) and kept at 37°C for 4 h to allow gel formation. HUVECs (5×10^4 cells/well) were then plated onto the Matrigel® in EGM-2 supplemented by VEGF-A (20 ng/mL) and investigated peptides (10 μ*M*). Sixteen hours later, networks were stained with calcein-AM (25 μ*M*, 30 min at 37°C) and fixed in PBS-2% formaldehyde (1 h at room temperature). The three-dimensional cell organization was photographed using MVX10 macroscope (Olympus). Capillary-like structures were quantified by automatic counting using the AngioQuant software. Images are representative of three independents experiments. Statistical significance was determined by ANOVA test and Dunnet post-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: no statistical difference. Scale bar: 1 mm.

CONCLUSIONS

In summary, we designed the CBO-P23M as a novel cyclic peptide with potential anti-angiogenic activity. CBO-P23M is a cyclic hexamer peptide encompassing the 82 to 86 VEGF sequence with the key residues Arg82, Lys84, and His86 involved in the interaction with VEGFR-2. We demonstrated that CBO-P23M can be prepared using in solution cyclization, therefore overcoming the peptide cyclodimerization recorded during solid-phase cyclization

The anti-angiogenic activity of CBO-P23M was investigated together with that of its cyclic dimer (CBO-P23M dimer), obtained as the main side product during the synthesis of monomer. Our biological evaluations highlight an anti-angiogenic capacity of CBO-P23M and CBO-P23M dimer comparable to that of CBO-P11 peptide which was previously described by our group as promising anti-angiogenesis inhibitor. However, as CBO-P23M includes exclusively six amino acid residues, its synthesis appears much simpler than that of the 17-mer CBO-P11. Overall, these results make CBO-P23M an ideal candidate for the further development of new anti-angiogenic compounds.

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