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Short Communication

Design, Synthesis, and Evaluation of Original Carriers for Targeting Vascular Endothelial Growth Factor Receptor Interactions

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Purpose. Angiogenesis is a key event in tumor growth and metastasis, chronic inflammatory disease, and cardiovascular disease. It is controlled by positive and negative regulators, which include vascular endothelial growth factor (VEGF) as the most active of these. VEGF/VEGF receptors are important targets not only for therapy but also for imaging. Based on the structural study of VEGF, we developed a novel cyclopeptide (cyclo-VEGI) that exhibits powerful antitumor properties. We herein report the design of novel molecules derived from cyclo-VEGI as potential targeting agents in cancer and other angiogenesis-related diseases.
Methods. We performed selective chemical modification of the most active VEGF-derived cyclopeptide (cyclo-VEGI). Original hydrophilic linkers were synthesized and coupled to cyclo-VEGI. These reactions provide nanocarriers for delivery. The inhibitory effect of the different compounds on VEGF binding was evaluated in competition assays with ^{125}I-VEGF. A fluorescent cyclo-VEGI peptide was synthezised to assess direct binding and internalization of cyclo-VEGI.
Results. Chemical modifications of cyclo-VEGI do not diminish the biological activity of cyclo-VEGI as measured in competition assays; in fact, it is even increased. Moreover there is a strong cellular accumulation of the fluorescent-labeled cyclo-VEGI. Conjugates synthesized in this study may be useful leads to design delivery systems for targeting approaches in cancer and other angiogenesis-related diseases.
Conclusion. The modified cyclo-VEGIs may have a wide range of applications and represent a useful tool to develop delivery/carrier systems for therapeutic targeting or imaging.

KEY WORDS: angiogenesis; chemotherapy; cyclopeptide modification; imaging; receptors targeting; solid-phase peptide synthesis; vascular endothelial growth factor.

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ABBREVIATIONS: Abs_{301}, absorbance at 301 nm; AcOH, acetic acid; All, allyl; Boc, tert-butoxycarbonyl; CHO, Chinese hamster ovary; cyclo-VEGI, cyclo-vascular endothelial growth inhibitor; Da, dalton; DMF, dimethylformamide; Flt-1, fms-like tyrosine kinase-1; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, hydroxybenzotriazol; KDR, kinase domain receptor; MALDI, matrix-assisted laser desorption/ionization; MsCl, mesyl chloride; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PhtNK, potassium phtalimidate; PVDF, poly(vinylidene difluoride); SPPS, solid-phase peptidic synthesis; TEG, triethyleneglycol; THF, tetrahydrofuran; Tis, triisopropylsilane; TsCl, tosyl chloride; Trt, trityl; VEGF, vascular endothelial growth factor.

INTRODUCTION

Angiogenesis is a process by which new capillaries sprout from preexisting blood vessels (1,2). It is involved both in physiological and pathological phenomena. Physiological angiogenesis occurs in the female reproductive system (3) such as during wound healing (4,5). In contrast, pathological neoangiogenesis occurs in a number of diseases such as cancer, mainly solid tumors and metastasis, or rheumatoid arthritis (6–8). Several growth factors have been proved to regulate these phenomena. Among these, vascular endothelial growth factor (VEGF) is a key regulator of both physiological and pathological angiogenesis (9–13). Inhibition of VEGF activity using various inhibitors results in suppression of tumor growth *in vivo* (14–17). VEGF receptors are also overexpressed in ischemic tissues (18). VEGF exists as different spliced forms, the most important being $VEGF_{165}$. Its signaling activity is mainly mediated by two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR). VEGF–KDR interaction has been demonstrated as the signaling pathway leading to tumoral neoangiogenesis (19,20). Domi-

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nant regions for VEGF binding to KDR have been investigated by structural studies and directed mutagenesis (21–23). They highlight that Arg82, Lys84, and His86 are the key residues for binding of VEGF to KDR; they are clustered within region 79–93 which forms a β-hairpin (21). Based on these structural studies, we designed a series of linear and cyclic peptides aimed to inhibit the VEGF–KDR binding. As described in previous reports (24–26) several cyclopeptides exhibited a good affinity for KDR receptor. All cyclopeptides which presented good affinities also inhibited endothelial cell proliferation and *in vivo* angiogenesis inhibition on CAM assays (differentiated chicken chorioallantoic membrane). One of most potent among these original derivatives is a 17-mer cyclopeptide we named cyclo-VEGI (for vascular endothelial growth inhibitor). It comprises VEGF residues 79–93, and among them basic residues Arg82, Lys84, and His 86 which have been characterized as the essential residues in VEGF– KDR binding and subsequent cell signaling. This novel active compound significantly decreases the growth of established intracranial human glioma in nude and syngenic mice and improves survival without side effects (24,26). Preliminary results also indicate a highly synergistic effect of cyclo-VEGI with alpha radioimmunotherapy.

Targeting molecules to neoangiogenic areas is of outstanding importance. Anticancer drugs may be delivered to neoangiogenic sites as well as radioisotopes for therapy or imaging. In addition to its role in tumors, imaging is important in cerebral infarct because penumbra areas overexpress VEGF receptors that may be specifically targeted. This would be of benefit since functional magnetic resonance imaging (MRI) is inadequate owing to blood flow alterations.

These data encouraged us to perform a series of chemical modifications of cyclo-VEGI to further improve these substrates for therapy or imaging. The design of scaffolds requires a specific strategy for synthesis to protect critical peptide side groups responsible for the biological activity of cyclo-VEGI. We herein report our results along with a preliminary *in vitro* evaluation of variously decorated cyclo-VEGI-derived peptides.

MATERIALS AND METHODS

General Information

Mass spectra were run using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) Reflex III Bruker apparatus for MALDI spectra and a Micromas VGAutospec-Q for high-resolution mass spectra (HRMS). For the nuclear magnetic resonance (NMR) spectra a Bruker Avance 300 was used. Chemicals shifts are reported in parts per million relative to tetramethylsilane as an internal standard (in NMR description, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad peak). Ultraviolet (UV) measurments were taken on a Genesys 5 Spectronic Instruments spectrophotometer using a quartz cell with 1-cm path length. For column chromatography 63–200 mesh silica gel 60 (VWR International) was used as the stationary phase. Infrared (IR) spectra were recorded on a Bruker Tensor 27 (Germany, Ettlingen) spectrometer equipped with an HTS-XT autosampler, a Globar (MIR) source (7 V), a KBr beam splitter, and a DTGS/B detector (18–36°C). The beam diameter at the sample location was 6 mm. In all experiments, a 2.0 cm^{-1} resolution was used and acquisitions were performed using 32 scans in transmittance.

The *N*-9-fluorenylmethoxycarbonyl (Fmoc)-protected natural amino acids and 2-chlorotrityl chloride resin were purchased from Advanced Chemtech. All trifunctional amino acids were suitably protected. The α-carboxyl group of glutamic acid was protected as allyl (All) group. The ε-amino group of lysine was protected as the (*tert*-butoxy)carbonyl (Boc) group. The histidine imidazole ring and the amide group of glutamine were protected with the triphenylmethyl (trityl, Trt) group (27,28) and the guanidino function of arginine as 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group (29,30). A 0.45 M solution of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in *N*-hydroxybenzotriazole (HOBt) was purchased from Applied Biosystems. Diisopropylethylamine (DIEA), magnesium sulfate, ninhydrine, phosphorus pentoxide, potassium hydroxide, potassium phthalimidate (PhtNK), sodium azide, sodium hydrogencarbonate ($NaHCO_3$), thioanisole, trifluoroacetic acid (TFA), triisopropylsilane (Tis), and triphenylphosphine (PPh_3) were purchased from Aldrich. Diethyl ether, *N*-methylmorpholine (NMM), piperidine, sodium hydroxide, sodium diethyldithiocarbamate, and triethylamine were purchased from Avocado. Dimethylformamide (DMF) and hydrazine hydrate were purchased from Acros. Acetic acid (AcOH), benzotriazol-1-yloxytris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP), hydrochloride acid, mesyl chloride (MsCl), tetrakis(triphenylphophine)palladium [$Pd(PPh_3)_4$], and tosyl chloride (TsCl) were purchased from Lancaster. Ammonia solution and phenol were purchased from Prolabo (France). Acetonitrile (CH_3CN), dichloromethane, ethanol, ethyl acetate, *n*-hexane, and methanol were purchased from J.T. Baker.

Binding Assays

$VEGF_{165}$ was labeled with ^{125}I-Na using Iodogen (Pierce, Rockford, IL, USA) as coupling agent according to the manufacturer's instructions. CHO-KDR cells or bovine aortic endothelial cells (BAE) were seeded at 2.5×10^5 density in gelatin-coated six-well plates and cultured in complete medium for 2 days. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated with 10 ng/ml of ^{125}I-VEGF and peptides at indicated concentrations in binding medium [Dulbecco's modified Eagle's medium (DMEM); 20 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4; 0.15% gelatin] on a shaker at 4°C. After 2 h, cells were washed three times with PBS and solubilized by the addition of 2% Triton, 10% glycerol, and 1 mg/ml of bovine serum albumin prior to γ-counting. Each condition was tested in duplicate and repeated at least two times. Data are expressed as percentage of total specific radioactivity.

Targeting of Coumarin-Labeled Cyclo-VEGI Peptides to Endothelial Cells *in Vitro*

BAEs were grown on Labtek chamber slides (Nunc, Naperville, USA) for 2 days. Préconfluent BAEs were incubated for different time intervals with coumarin-cyclo-

VEGI peptides or with uncoupled coumarin as a control. After specified time intervals, cells were washed three times with PBS and fixed with 1% glutaraldehyde. Cellular localization was analyzed with a fluorescence confocal microscope (Zeiss, LSM510 Meta). Experiments were repeated two times with identical results.

Anchoring of *N*-(9-Fluorenylmethoxycarbonyl)-Glutamic Acid Allyl Ester to the 2-Chlorotrityl Chloride Resin

This was accomplished by a general procedure recommended by Novabiochem. Fmoc-Glu-OAll (638.2 mg, 1.56 mmol) was dissolved in 10 ml of CH_2Cl_2 with DIEA (1.08 ml, 6.29 mmol). The solution was added to the 2-chlorotrityl chloride resin and was swollen for 2 h at room temperature. At the end of this time, the resin was washed with 3 × 10 ml of CH_2Cl_2–MeOH–DIEA (17:2:1), followed by 3 × 10 ml of CH_2Cl_2, 2 × 10 ml of DMF, and 2 × 10 ml of CH_2Cl_2. Then the resin was dried *in vacuo* over KOH. The substitution level was determined spectrophotometrically by Fmoc cleavage (31). Fmoc-Glu(resin)-OAll (5.05 mg) was introduced into a test tube and a solution of 20% piperidine in DMF was added (0.5 ml). 20% piperidine in DMF (0.5 mL) was also added to an empty test tube to serve as a blank. Over the next 15 min, the test tube with the resin was swirled two or three times to make sure all the resin had come in contact with the piperidine solution. DMF was added to both tubes to reach a 50-ml volume. The blank was used to zero the UV spectrophotometer at 301 nm. The absorbance of the solution is 0.346. The substitution level was calculated from the formula: $Abs_{301} \times Vol\ (ml)/[7800 \times m\ (g)]$ and was determined to be 0.44 mmol/g.

Peptide Synthesis

Cyclo-VEGI (*D*-FPQIMRIKPHQGQHIGE) was synthesized by Fmoc/t-Bu batch solid-phase synthesis on an Applied Biosystems 433A automated peptide synthesizer. Preloaded Fmoc-Glu(2-ClTrt resin)-OAll was used for the linear chain assembly. Subsequent Fmoc amino acids were coupled using a fourfold excess of amino acids activated as HOBt ester by means of a 0.45 M HBTU–HOBt solution. Removal of the allyl protecting group was performed before N-terminal Fmoc deprotection. The peptidyl resin was weighed (1.59 g, 0.27 mmol) and dried at 40°C under high vacuum. The reaction vessel was flushed with a stream of argon. $Pd(PPh_3)_4$ (965.83 mg, 0.83 mmol) was dissolved in a solution of $CHCl_3$–AcOH–NMM (37:2:1) (15 ml/g of resin) by bubbling a stream of argon through the solution. Thereafter this solution was added to the peptidyl resin under argon and was swelled manually every 15 min for 2 h at room temperature. Then the peptidyl resin was filtered and washed consecutively with 50 ml of 0.5% DIEA–NMP [N-methyl pyrrolidone] (% v/v), 50 ml of 0.5% sodium diethyldithiocarbamate–NMP (% w/w), 50 ml of CH_2Cl_2, 50 ml of 1 M HOBt in NMP, 50 ml of NMP, and 50 ml of CH_2Cl_2. The resin was dried under high vacuum over KOH overnight. Fmoc removal was achieved with a solution of 20% piperidine in NMP. The peptidyl resin was newly washed with 40 ml of 1 M HOBt in NMP, 40 ml of NMP, and 40 ml of CH_2Cl_2, and was dried overnight under high vacuum. Peptidyl resin was weighed (1.11 g, 0.20 mmol) and mixed with a solution of PyBOP (312.18 mg, 0.60 mmol), HOBt (81.07 mg, 0.60 mmol), and DIEA (207.63 μl, 1.2 mmol) in 20 ml of NMP. The mixture was swelled at room temperature until Kaiser's test was negative. The peptidyl resin was washed with 50 ml of each NMP, CH_2Cl_2, and MeOH and was dried under high vacuum. Final cleavage of cyclo-VEGI from the resin without loss of any side-chain protecting group was performed with a solution of 1% TFA in CH_2Cl_2 (10 ml/g of resin). Cyclopeptidyl resin was weighed (1.02 g), mixed with dilute TFA, and shaken for 2 min. Then the solution was filtered and the filtrate was collected in a flask containing a solution of 10% pyridine in MeOH (2 ml/10 ml of 1% TFA). It was repeated 10 times and cyclopeptidyl resin was washed with 3 × 30 ml of CH_2Cl_2, 3 × 30 ml of MeOH, 2 × 30 ml of CH_2Cl_2 and 3 × 30 ml of MeOH. Then filtrate was evaporated under reduced pressure to 5% of the volume. Thereafter, 40 ml of cold water were added to the residue to aid precipitation of the product which was subsequently isolated by filtration through a sintered glass funnel. Product was washed three times with fresh water and dried in a dessicator under vacuum over KOH, and later over P_2O_5. The product was analyzed by reverse-phase HPLC under the following conditions: eluant A, 0.05% TFA in water; eluant B, 0.05% TFA in CH_3CN–H_2O (70/30); gradient, 50–100% over 30 min; flow rate, 1 ml/min; detector 214 nm. Then 580 mg (65%) of protected cyclo-VEGI were obtained as a white solid without further purification. A portion of the product was treated with 0.75 g of phenol in a TIS–thioanisole–H_2O–TFA solution (1:2:2:40) for 3 h at room temperature. The product was precipitated from cold diethyl ether and filtered. Then MALDI mass spectrometry analysis gave the expected molecular mass of deprotected cyclo-VEGI (theoretical value: 1998.36 Da; experimental value: 1998.04 Da).

1-Allyloxy-8-Hydroxy-3,6-Dioxaoctane (**2**)

Sodium hydride (3 g, 0.08 mol) was added portionwise to a solution of triethyleneglycol (20 ml, 0.15 mol) in dry THF (50 ml). The solution was stirred at room temperature for 15 min. Then allyl bromide (4.3 ml, 0.05 mol) was added and stirring was maintained at room temperature for 1 h. Then solvent was evaporated under reduced pressure and the residue was dissolved in dichloromethane. The solution was filtrated and washed with brine (40 ml). The aqueous phase was extracted with dichloromethane (2 × 100 ml). The organic phase was dried over $MgSO_4$ and solvent were evaporated under reduced pressure. The resulting yellowish oil was purified by chromatography (*n*-hexane–ethyl acetate 70:30 and then 50:50). Thereafter evaporation of the solvent allowed to obtain 6.93 g (73%) of compound **2**.

^{1}H NMR ($CDCl_3$) δ ppm: 3.53–3.76 (m, 12H); 3.99 (d, 2H, $CH_2CH{=}CH_2$, $^3J_{H\text{-}H}$=5.61 Hz); 5.94–5.84 (m, 1H, $CH{=}CH_2$); 5.285.14 (m, 2H, $CH{=}CH_2$).

^{13}C NMR ($CDCl_3$) δ ppm: 61.6 (CH_2OH); 70.2–70.5 (CH_2O); 72.2 ($CH_2CH{=}CH_2$); 72.5 (CH_2CH_2OH); 117.1 ($CH{=}CH_2$); 134.6 ($CH{=}CH_2$).

1-[(*p*-Toluenesulphonyl)oxy]-9-Allyl-3,6,9-Trioxaoctane (3)

A solution of compound 2 (2.14 g, 11.3 mmol) in distilled triethylamine (30 ml) was cooled to 0°C. Then tosyl chloride was added (3.22 g, 16.9 mmol) to the medium and was stirred at room temperature for 24 h. Then the reaction mixture was poured into ice and ethyl acetate was added (100 ml). The aqueous phase was extracted with ethyl acetate (2 × 100 ml). The combined organic layers were washed with 2% AcOH and H_2O, and then dried over $MgSO_4$. The solvent was removed under vacuum to give a yellowish oil that was purified by chromatography (*n*-hexane/ethyl acetate, 75:25, 50:50, and 100% ethyl acetate). Then 3.1 g of compound 3 (80%) were obtained as a colorless oil.

^{1}H NMR ($CDCl_3$) δ ppm: 2.44 (s, 3H, $-CH_3$); 3.66–3.51 (m, 10H, $-CH_2-CH_2-O-$); 4.01 (d, 2H, $CH_2CH{=}CH_2$, $^3J_{H\text{-}H}$ = 5.61 Hz); 4.14 (t, 2H, $-CH_2-OTs$, $^3J_{H\text{-}H}$ = 4.7 Hz); 5.14–5.28 (m, 2H, $CH{=}CH_2$); 5.84–5.94 (m, 1H, $CH{=}CH_2$); 7.33 (d, 2H, –CHAr–, $^3J_{H\text{-}H}$ = 8.1 Hz); 7.77 (d, 2H, –CHAr–, $^3J_{H\text{-}H}$ = 8.1 Hz)

^{13}C NMR ($CDCl_3$) δ ppm: 21.2 ($-CH_3$); 68.2–68.9 ($-CH_2-CH_2-O-$); 70.1–70.2 ($CH_2CH{=}CH_2$, CH_2CH_2-OTs); 71.7 (CH_2–OTs); 116.6 ($CH{=}CH_2$); 127.5–129.5 ($-CH_{Ar}-$); 132.5 ($-C_{Ar}-SO_2-$); 134.3 ($CH{=}CH_2$); 144.5 ($-C_{Ar}-CH_3$).

1-Phthalimido-9-Allyl-3,6,9-Trioxaoctane (4)

To a solution of compound 3 (3.1 g, 9 mmol) in distilled DMF (100 ml) was added potassium phthalimidate (1.83 g, 9.9 mmol). The solution was heated at 120°C for 12 h and then the solution was cooled to room temperature. The solvent was evaporated under reduced pressure and the resulting residue was dissolved in CH_2Cl_2 (100 ml). H_20 (100 ml) was added. The aqueous phase was extracted three times with CH_2Cl_2 (3 × 100 mL). The combined organic layers were washed with H_2O (3 × 100 ml) and then dried over $MgSO_4$. Filtration and evaporation of the solvent made it possible to obtain 2.73 g of compound 4 (94%) without further purification.

^{1}H NMR ($CDCl_3$) δ ppm: 3.34–3.47 (m, 10H, $-CH_2-CH_2-O-$); 3.52 (t, 2H, CH_2–NPht, $^3J_{H\text{-}H}$ = 5.76 Hz); 3.67 (t, 2H, CH_2CH_2–NPht, $^3J_{H\text{-}H}$ = 5.85 Hz); 3.77 (d, 2H, $CH_2CH{=}CH_2$, $^3J_{H\text{-}H}$ = 5.61 Hz); 4.91–5.09 (m, 1H, $CH{=}CH_2$); 5.57–5.76– (m, 2H, $CH{=}CH_2$); 7.48–7.55 (m, 2H, CHAr); 7.57–7.63 (m, 2H, CHAr).

^{13}C NMR ($CDCl_3$) δ ppm: 37.1 (CH_2–NPht); 67.8 (CH_2CH_2–NPht); 69.3–72.0 ($-CH_2-CH_2-O-$); 116.9 ($CH{=}CH_2$); 123.1 (CHAr); 132.0 (CAr); 133.8 (CHAr); 134.6 ($CH{=}CH_2$); 168.1 (C=O).

IR (cm^{-1}): 2906; 2867; 1774; 1713; 1615; 1107; 1006; 928.

1-Amino-9-Allyl-3,6,9-Trioxaoctane (5)

To a solution of compound 4 (2.73 g, 8.55 mmol) in absolute ethanol (100 ml) was added hydrazine hydrate (1.66 ml, 34.23 mmol). The solution was heated at 80°C for 24 h. The solvent was evaporated under reduced pressure and the resulting residue was dissolved in CH_2Cl_2 (50 ml). After filtration the solvent was evaporated and then 1.53 g (95%) of compound 5 were obtained as a colorless oil.

^{1}H NMR ($CDCl_3$) δ ppm: 2.82 (t, 2H, CH_2NH_2, $^3J_{H\text{-}H}$ = 5.3 Hz); 3.43 (t, 2H $CH_2CH_2NH_2$, $^3J_{H\text{-}H}$ = 5.3 Hz); 3.54–3.68 (m, 10H, $-CH_2-CH_2-O-$); 4.01 (d, 2H, $CH_2CH{=}CH_2$, $^3J_{H\text{-}H}$ = 5.61 Hz); 5.14–5.28 (m, 2H, $CH{=}CH_2$); 5.84–5.94 (m, 1H, $CH{=}CH_2$).

^{13}C NMR ($CDCl_3$) δ ppm: 40.2 (CH_2NH_2); 68.5 ($CH_2CH_2NH_2$), 68.7–71.2 (CH_2-CH_2-O- and $CH_2CH{=}CH_2$); 116.2 ($CH{=}CH_2$); 133.9 ($CH{=}CH_2$).

IR (cm^{-1}): 2908; 2869; 1674; 1116; 997; 929.

1-[(*p*-Toluenesulphonyl)oxy]-11-Hydroxy-3,6,9-Trioxaundecane (7)

To a solution of tetraethyleneglycol (165 ml; 950 mmol) in CH_2Cl_2 (425 ml) under nitrogen atmosphere was added triethylamine (20 ml, 142.5 mmol). The solution was cooled at 0°C and then tosyl chloride was added (18.1 g; 95 mmol). Then the solution was heated at room temperature and was stirred for 20 h. Thereafter the solvent was evaporated under reduced pressure and the resulting residue was dissolved in dichloromethane (200 ml). This organic phase was washed with H_2O (3 × 300 ml) and the combined aqueous layers were extracted with dichloromethane (3 × 200 ml). The combined organic layers were washed with 2% AcOH and H_2O, and then dried over $MgSO_4$. After filtration the solvent was evaporated under reduced pressure to yield a colorless oil which was purified by silica gel chromatography (*n*-hexane/ethyl acetate, 70:30 to 50:50) to give 28.18 g (85%) of compound 7.

^{1}H NMR ($CDCl_3$) δ ppm: 2.43 (s, 3H, $-CH_3$); 2.68 (s, 1H, –OH); 3.58–3.68 (m, 14H, $-CH_2-CH_2-O-$); 4.14 (t, 2H, $-CH_2-OTs$, $^3J_{H\text{-}H}$ = 4.7 Hz); 7.33 (d, 2H, –CHAr–, $^3J_{H\text{-}H}$ = 8.1 Hz); 7.77 (d, 2H, –CHAr–, $^3J_{H\text{-}H}$ = 8.1 Hz)

^{13}C NMR ($CDCl_3$) δ ppm: 23.0 ($-CH_3$); 64.9 ($-CH_2-OH$); 68.7–71.9 ($-CH_2-CH_2-O-$); 73.8; 128.2–132.3 ($-CH_{Ar}-$); 134.1 ($-C_{Ar}-SO_2-$); 146.2 ($-C_{Ar}-CH_3$).

IR (cm^{-1}): 3422; 2915; 2876; 1189.

1-Azido-11-Hydroxy-3,6,9-Trioxaundecane (8)

To a solution of compound 7 (14 g, 40.18 mmol) in absolute ethanol (200 ml) was added sodium azide (5.22 g, 80.36 mmol). The solution was stirred at 85°C for 7 h. Thereafter the solution was cooled to room temperature. To the resulting residue were added CH_2Cl_2 (200 ml) and H_20 (200 ml). The organic phase was washed with H_2O (3 × 200 ml) and the combined aqueous layers were extracted with CH_2Cl_2 (3 × 200 ml). The solvent was dried over $MgSO_4$, filtrated, and evaporated under vacuum. The residue was then purified by silica gel chromatography (*n*-hexane/ethyl acetate, 50:50 to 25:75) to give 6.71 g (76%) of compound 8 as a brown oil.

^{1}H NMR ($CDCl_3$) δ ppm: 2.89 (s, 1H, –OH); 3.31 (br s, 2H, $-CH_2-N_3$); 3.52 (br s, 2H, $-CH_2-CH_2-N_3$); 3.59 (m, 12H, $-CH_2-CH_2-O-$).

^{13}C NMR ($CDCl_3$) δ ppm: 50.6 ($-CH_2-N_3$); 61.7 ($-CH_2$ –OH); 70.0–70.6 ($-CH_2-CH_2-O-$ and $-CH_2-CH_2-N_3$); 72.4 ($-CH_2-CH_2-OH$).

IR (cm^{-1}): 3429; 2914; 2875; 2113; 1125.

1-Azido-11-[(Methanesulfonyl)oxy]-3,6,9-Trioxaundecane (9)

To a solution of compound **8** (17.39 g; 79.32 mmol) in CH_2Cl_2 (500 ml) was added triethylamine (30 ml, 214.16 mmol). The solution was cooled to 0°C and then mesyl chloride (12.3 ml; 158.65 mmol) was added. The reaction mixture was heated at room temperature, stirred for 24 h, and then a solution of saturated $NaHCO_3$ (200 ml) was added. The organic layer was washed with H_2O (3 × 100 ml), dried over $MgSO_4$, and filtrated. The solvent is evaporated under reduced pressure to give a dark oil that was purified by silica gel chromatography (*n*-hexane–ethyl acetate, 10:90). Then 21.2 g (88%) of compound **9** were obtained as a brown oil.

^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.96 (s, 3H, $-CH_3$); 3.28 (br s, 2H, $-CH_2-N_3$); 3.51–3.54 (m, 10H, $-CH_2-CH_2-O-$); 3.65 (br s, 2H, $-CH_2-CH_2-OMs$); 4.25 (br s, 2H, $-CH_2-OMs$).

^{13}NMR ($CDCl_3$, 75 MHz) δ: 37.5 ($-CH_3$); 50.0 ($-CH_2-N_3$); 68.3–69.9 ($-CH_2-CH_2-O-$ and $-CH_2-CH_2-N_3$).

IR (cm^{-1}): 2912; 2873; 2110; 1678; 1134.

1-Azido-10-Phthalimido-3,6,9-Trioxaundecane (10)

To a solution of compound **9** (7.88 g, 26.5 mmol) in dry DMF (150 ml) was added potassium phthalimidate (5.4 g, 29.15 mmol). The solution was heated at 120°C for 12 h, and then the solvent was evaporated under reduced pressure. CH_2Cl_2 (100 ml) and H_20 (100 ml) were added to the resulting residue. The aqueous layer was extracted with CH_2Cl_2 (3 × 100 ml) and the combined organic layers were washed with H_20 (3 × 100 ml). The organic phase was dried over $MgSO_4$, filtrated and then the solvent was evaporated under reduced pressure. The residue was purified by silica gel chromatography (*n*-hexane/ethyl acetate, 80:20) to give 6.47 g of compound **10** (70%) as a brown oil.

^{1}H NMR ($CDCl_3$) δ ppm: 3.12 (t, 2H, CH_2NPht, $^3J_{H\text{-}H}$ = 5.25 Hz); 3.34–3.47 (m, 10H, $-CH_2-CH_2-O-$); 3.55 (t, 2H, CH_2-N_3, $^3J_{H\text{-}H}$ = 5.76 Hz); 3.69 (t, 2H, CH_2CH_2-NPht, $^3J_{H\text{-}H}$ = 5.85 Hz); 7.48–7.55 (m, 2H, CHAr); 7.57–7.63 (m, 2H, CHAr).

^{13}C NMR ($CDCl_3$) δ ppm: 36.7 (CH_2NPht), 50.1 (CH_2N_3), 67.3 (CH_2CH_2NPht and $CH_2CH_2N_3$), 69.5–70.1 (CH_2O), 123.5 (CHAr), 131.5 (CAr), 134.8 (CHAr), 167.6 (C=O).

1-Azido-12-[but-3-enyl]-3,6,9,12-Tetraoxaundecane (11)

To a solution of but-3-en-1-ol (1.1 ml; 13.28 mmol) in dry THF (10 ml), cooled at 0°C, was added sodium hydride (400 mg; 13.26 mmol) under an inert atmosphere. A solution of compound **9** (1.58 g, 5.31 mmol) in dry THF (5 ml) was added. The reaction mixture was heated at room temperature and was stirred overnight. Then a solution of 32% of aqueous ammonia was added until the reaction mixture was clear. Thereafter the solvent was evaporated under reduced pressure. The resulting residue was dissolved in dichloromethane (50 ml) and washed with H_2O (3 × 50 ml). The aqueous layers were extracted with dichloromethane (3 × 50 ml). After drying over $MgSO_4$ the solvent was filtrated and evaporated under vacuum. The residue was then purified by silica gel chromatography (*n*-hexane–ethyl acetate, 40:60) to give 1.07 g (74%) of compound **11**.

^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.26 (br s, 2H, $-CH_2-CH=CH_2$); 3.31 (br s, 2H, $-CH_2-N_3$); 3.45 (br s, 2H, $-CH_2-CH_2-N_3$); 3.54–3.59 (m, 14H, $-CH_2-CH_2-O-$ et $-CH_2-CH_2-CH=CH_2$); 4.93–5.03 (m, 2H, $-CH=CH_2$); 5.70–5.76 (m, 1H, $-CH=CH_2$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ: 33.9 ($-CH_2-CH=CH_2$); 50.5 ($-CH_2-N_3$); 69.8–70.4 ($-CH_2-CH_2-O-$ and $-CH_2-CH_2-CH=CH_2$); 116.1 ($-CH=CH_2$); 136.8 ($-CH=CH_2$).

IR (cm^{-1}): 2911; 2872; 2110; 1125.

1-Amino-10-Azido- 3,6,9-Trioxaundecane (12)

To a solution of compound **10** (3.51 g, 10.09 mmol) in absolute ethanol (100 ml) was added hydrazine hydrate (1.95 ml, 40.36 mmol) under an inert atmosphere. The reaction mixture was heated at 80°C for 24 h. The solvent was evaporated under reduced pressure and the resulting residue was diluted with water (50 ml) and concentrated HCl (8 ml) and heated at reflux for 1 h. The resulting suspension was cooled to 0°C and the solids were filtered. The aqueous filtrate was neutralize with 1 M NaOH and then concentrated under reduced pressure. The residue was diluted with dichloromethane (50 ml) washed with 4 M NaOH and dried over $MgSO_4$. Thereafter the solvent was evaporated to give 2.03 g of compound **12** (92%) as a yellowish oil.

^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.71 (t, 2H, CH_2NH_2); 3.25 (t, 2H, CH_2N_3); 3.37 (t, 2H, $CH_2CH_2NH_2$); 3.48–3.72 (m, 10H, CH_2CH_2O and $CH_2CH_2N_3$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ: 41.2 (CH_2NH_2), 50.3 (CH_2N_3), 69.9–70.3 ($-CH_2-CH_2-O-$ and $CH_2CH_2N_3$), 72.8 ($CH_2CH_2NH_2$).

IR (cm^{-1}): 3360; 2909; 2870; 1774; 2110; 1124.

HRMS (FAB^+): calculated for $C_8H_{19}N_4O_3$ $(MH)^+$ 219.1457, found 219.1462.

1-Amino-12-[but-3-enyl]-3,6,9,12-Tetraoxaundecane (13)

To a solution of compound **11** (1.02 g; 3.73 mmol) in dry THF (50 ml) was added triphenylphosphine (1.27 g; 4.7 mmol) under inert atmosphere. Stirring was maintained at room temperature overnight. The solvent was evaporated until 10% of the initial content and a solution of 1:1 ether–petroleum ether was added to the solution which cooled to 0°C. The solution was filtered to remove precipitated triphenylphosphine oxide. The filtrate was then evaporated. Purification of the crude product was achieved by silica gel chromatography (CH_2Cl_2–MeOH, 50:50). Thereafter the solvent was evaporated to give 1.07 g (78%) of compound **13**.

^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.23 (br s, 2H, $-CH_2-CH=CH_2$); 2.75 (br s, 2H, $-CH_2-NH_2$); 3.39 (br s, 2H, $-CH_2-CH_2-NH_2$); 3.51–3.54 (m, 14H, $-CH_2-CH_2-O-$ and $-CH_2-CH_2-CH=CH_2$); 4.89–4.99 (m, 2H, $-CH=CH_2$); 5.66–5.72 (m, 1H, $-CH=CH_2$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ: 33.9 ($-CH_2-CH=CH_2$); 41.5 ($-CH_2-NH_2$); 70.1–70.4 ($-CH_2-CH_2-O$ and $-CH_2-CH_2-CH=CH_2$); 73.2 ($-CH_2-CH_2-NH_2$); 116.2 ($-CH=CH_2$); 136.7 ($-CH=CH_2$).

IR (cm^{-1}): 2907; 2868; 1117.

HRMS (FAB^+): calculated for $C_{12}H_{26}NO_4$ $(MH)^+$ 248.1861 found 248.1869.

Compound **14**

To a solution of cyclo-VEGI (220.4 mg; 61.8 μmol) in NMP (0.1 ml/μmol) were added PyBOP (98.3 mg; 189 μmol) and HOBt (25.5 mg; 189 μmol). After complete dissolution, DIEA (65.4 μl; 378 μmol) and the reaction mixture was stirred over 5 min at room temperature. Then compound **12** (21.2 mg; 81.9 μmol) was added and the mixture was stirred over 24 h at room temperature. The solution was concentrated and the product was precipitated with cold water, filtered, and dissolved in a solution of $CH_3CN–H_2O$ (70/30) with 0.1% TFA (eluant B) and few drops of THF, and then loaded onto a preparative Hibar Purosphere C_{18} column. The elution was achieved using the following conditions: eluant A, 0.1% TFA in water; eluant B, 0.1% TFA in CH_3CN/H_2O (70:30); gradient, 0–100% B over 35 min; flow rate, 4 ml/min; detector, 214 nm. Then 220.9 mg (95%) of compound **14** were obtained. The purified product was analyzed by HPLC with a flow rate of 1 ml/min and using the same other conditions as described earlier. The MALDI mass spectrometry analysis of the purified product was unsuccessful, certainly because of the presence of the protecting groups which render the ionization of compound **14** difficult. A portion of the product was treated with 0.75 g of phenol in a TIS–thioanisole–H_2O–TFA solution (1:2:2:40) for 3 h at room temperature. The product was precipitated from cold diethyl ether and filtered. Then MALDI mass spectrometry analysis gave the expected result (theoretical value: 2198.60 Da; experimental value: 2198.28 Da).

Compound **15**

To a solution of cyclo-VEGI (733.5 mg; 0.2 mmol) in NMP (0.1 ml/μmol) were added PyBOP (325.1 mg; 0.62 mmol) and HOBt (84.4 mg; 0.62 mmol). After complete dissolution, DIEA (166.8 μl; 1.23 mmol) and the reaction mixture was stirred over 5 min at room temperature. Then compound **5** (50 mg; 0.26 mmol) was added and the mixture was stirred over 24 h at room temperature. The solution was concentrated and the product was precipitated with cold water, filtered, and dissolved in a solution of CH_3CN/H_2O (70:30) with 0.1% TFA (eluant B) and few drops of THF, and then loaded onto a preparative Hibar Purosphere C_{18} column. The elution was achieved using the following conditions: eluant A, 0.1% TFA in water; eluant B, 0.1% TFA in $CH_3CN–H_2O$ (70:30); gradient, 50–100% B over 40 min; flow rate, 4 ml/min; detector, 214 nm. Then 186 mg (94%) of compound **15** were obtained. The purified product was analyzed by HPLC with a flow rate of 1 ml/min and using the same other conditions as described earlier. The MALDI mass spectrometry analysis of the purified product was unsuccessful certainly because of the presence of the protecting groups which render the ionization of compound **15** difficult. A portion of the product was treated with 0.75 g of phenol in a TIS–thioanisole–H_2O–TFA solution (1:2:2:40) for 3 h at room temperature. The product was precipitated from cold diethyl ether and filtered. Then MALDI mass spectrometry analysis gave the expected result (theoretical value: 2169.59 Da; experimental value: 2169.32 Da).

Compound **16**

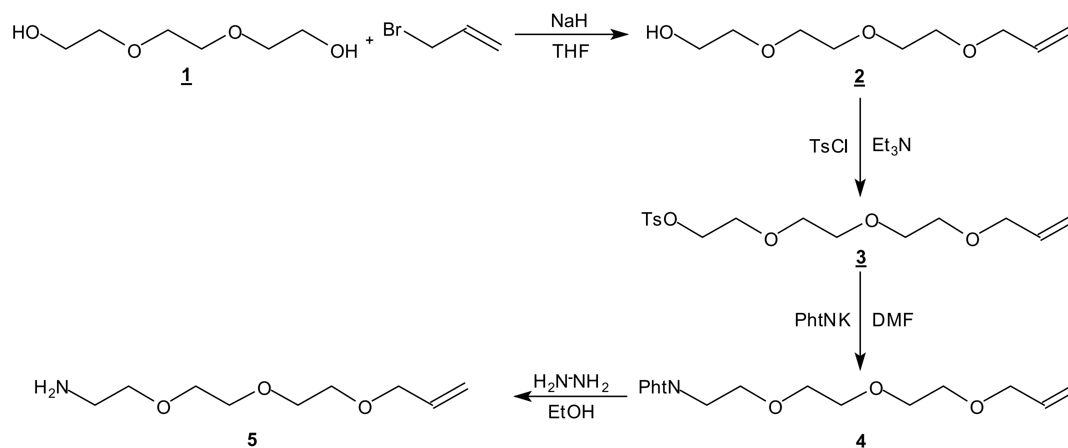
To a solution of cyclo-VEGI (300 mg; 84.2 μmol) in NMP (0.1 ml/μmol) were added PyBOP (133.6 mg; 256.7 μmol) and HOBt (34.7 mg; 256.7 μmol). After complete dissolution, DIEA (90 μl; 520.1 μmol) was added and the reaction mixture was stirred over 5 min at room temperature. Then compound **13** (27.4 mg; 110.9 μmol) was added and the mixture was stirred over 24 h at room temperature. The solution was concentrated and the product precipitated with cold water, filtered, and dissolved in a solution of $CH_3CN–H_2O$ (70:30) with 0.1% TFA (eluant B) and few drops of THF. Then it was loaded onto a preparative Hibar Purosphere C_{18} column. The elution was achieved under the following conditions: eluant A, 0.1% TFA in water; eluant B, 0.1% TFA in CH_3CN/H_2O; gradient 0–100% B over 25 min; flow rate, 4 ml/min; detector, 214 nm. The purified product was analyzed by HPLC with a flow rate of 1 ml/min and using the same other conditions as described earlier. The MALDI mass spectrometry analysis of the purified product was unsuccessful, certainly because of the presence of the protecting groups which render the ionization of compound **16** difficult. A portion of the product was treated with 0.75 g of phenol in a TIS–thioanisole–H_2O–TFA solution (1:2:2:40) for 3 h at room temperature. The product was precipitated from cold diethyl ether and filtered. Then MALDI mass spectrometry analysis revealed that the product was a mixture of cyclo-VEGI and compound **16** (theoretical values: 2227.67 Da and 1998.36; experimental values: 2227.35 Da and 1998.04 Da) which were unseparable under the protected form.

Compound **17**

Compound **14** (240.7 mg; 63 μmol), triphenylphosphine (18.2 mg; 69 μmol), and water (1.7 ml; 94 μmol) were mixed with 5 ml of THF. After the solution was stirred for 48 h, it was concentrated and the product was precipitated with cold water and filtered. The product was dissolved in a solution of $CH_3CN–H_2O$ (70:30) with 0.05% TFA (eluant B) and a few drops of THF, and then loaded onto a preparative Hibar Purosphere column C_{18}. The elution was achieved using the following conditions: eluant A, 0.05% TFA in water; eluant B, 0.05% TFA in $CH_3CN–H_2O$ (70/30); gradient, 0–100% over 35 min; flow rate, 4 ml/min; detector, 214 nm. Then 127.1 mg (54%) of compound **14** were obtained. The purified product was analyzed by HPLC with a flow rate of 1 ml/min and under the same conditions as described earlier. The MALDI mass spectrometry analysis of the purified product was unsuccessful certainly owing to the presence of the protecting groups which render the ionization of compound **17** difficult. A portion of the product was treated with 0.75 g of phenol in a TIS–thioanisole–H_2O–TFA solution (1:2:2:40) for 3 h at room temperature. The product was precipitated from cold diethyl ether and filtered. Then MALDI mass spectrometry analysis gave the expected result (theoretical value: 2172.60 Da; experimental value: 2172.85 Da).

Compound **18**

Fully experimental procedure and biological properties will be developed in a future paper. Analytical HPLC on



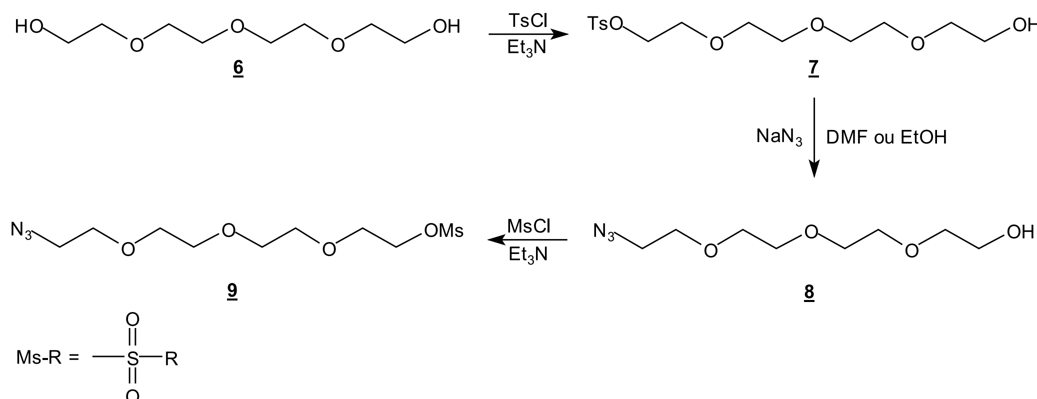
Scheme 1. Synthesis of a heterobifunctional linker with a terminal olefin.

Hibar purosphere column C_{18} show one compound with a retention time of 11.2 min. Elution was achieved using the following conditions: eluant A, 0.05% TFA in water; eluant B, 0.05% TFA in CH_3CN–H_2O (70:30); gradient: 10% at 0 min, 20% at 5 min, 100% at 7 min and over a period of 30 min; flow rate, 1 ml/min; detector, 410 nm. Then ES mass spectrometry analysis gave m/e (m+3) 806.8 Da.

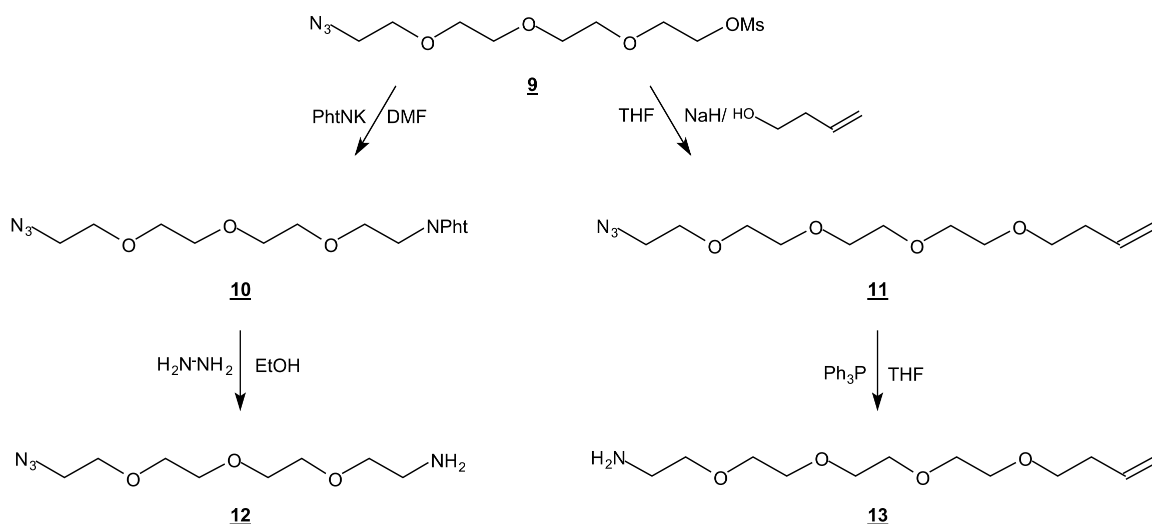
RESULTS AND DISCUSSION

Chemical modifications of cyclo-VEGI must not affect residues Arg82, Lys84, and His86 because they play a central role in the cyclo-VEGI–KDR interaction. The side chain group of Glu93, which is the only to be acidic, was selected to introduce new molecular decoration into cyclo-VEGI. Moreover, Glu93 is at the opposite site of key residues Arg82, Lys84, and His86 in cyclo-VEGI. Our first goal was to propose a synthetic route allowing to obtain a protected cyclopeptide with one deprotected side chain group, by solid-phase peptidic synthesis (SPPS), to introduce selectively molecular decoration. To our knowledge this strategic route had never been described previously. For this purpose, we performed the total synthesis of cyclo-VEGI on the extremely acid-labile 2-chlorotrityl chloride resin. The linear precursor peptide of cyclo-VEGI was assembled by standard Fmoc chemistry on a Fmoc-Glu(resin)-OAll carrier. Indeed, side-chain anchoring of Glu to this resin will allow its protection during SPPS and its selective deprotection during the final peptidic-resin cleavage performed under mild acidic conditions. We prepared this noncommercially available preloaded resin by formation of an ester bond between the γ-COOH group of Fmoc-Glu-OAll and the 2-chlorotrityl chloride resin, without any coupling reagent in the presence of diisopropylethylamine. Substitution level was determined by UV quantification at 301 nm (31) and suitable protecting groups were used for all trifunctional amino acids.

After SPPS completion, removal of allyl (All) protection was carried out according to the method of Kates and coworkers (32) utilizing $Pd(PPh_3)_4$–$CHCl_3$–AcOH–NMM. This reaction was performed under neutral conditions allowing the selective deprotection of the α-COOH group with the linear peptide still anchored to the resin, and thereafter on-resin cyclization. The cyclic peptide was then cleaved from the solid support using repeated mild acidic conditions to prevent any side-chain deprotection by extended exposure of the protected cyclopeptide to residual TFA. The final product was purified by reverse-phase HPLC. It was impossible to obtain any analytical result by MALDI mass spectrometry. It was hypothesized that the presence of the protecting groups and the cyclic structure prevent ionization of the protected cyclopeptide, and thus MALDI mass spectrometry analysis. Nevertheless, a portion of protected cyclo-VEGI was taken



Scheme 2. Synthesis of a common synthon for terminal amine/olefin linkers.

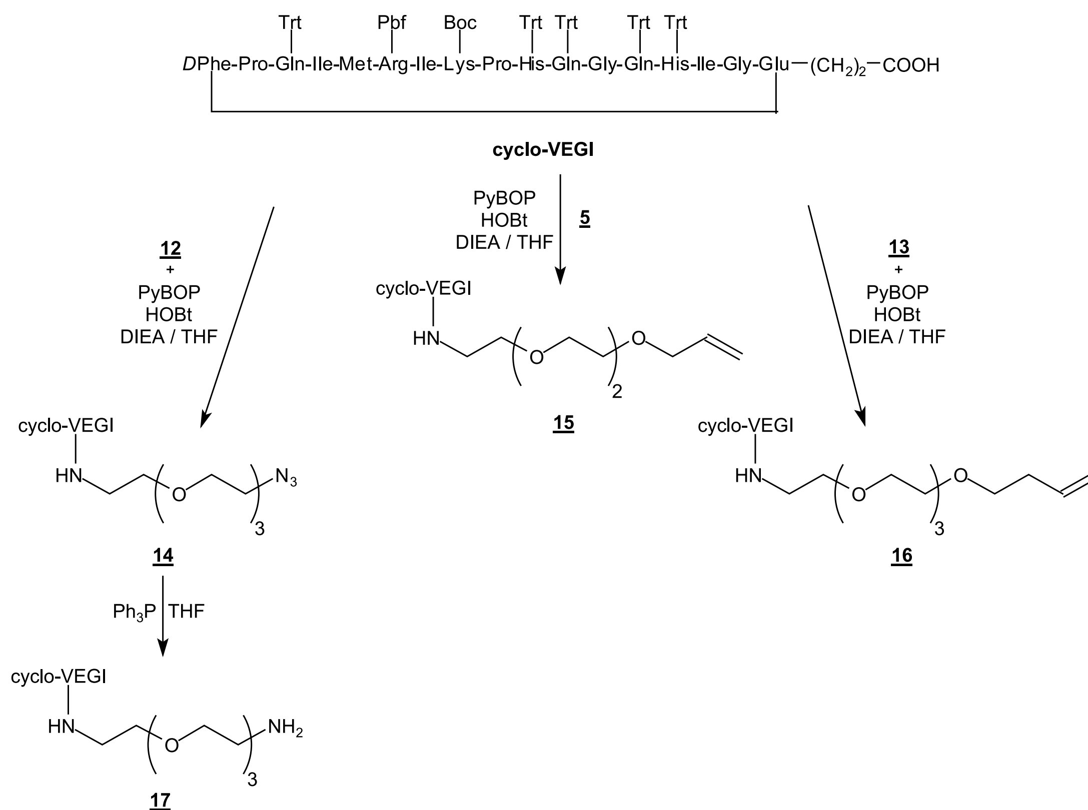


Scheme 3. Synthesis of terminal amine/olefin linker from compound 9.

and deprotected by a mixture of TFA in the presence of suitable scavengers. The free cyclopeptide was purified by reverse-phase HPLC and showed the expected molecular mass (theoretical value: 1998.36 Da, experimental value: 1998.04 Da). Based on the initial loading, overall yield of the supported-solid synthesis of protected cyclo-VEGI was calculated at 65%.

Our second goal was to determine possible molecular decorations of cyclo-VEGI for its use in angiogenesis imaging or in nanocarrier preparation. We have reported elsewhere the immobilization of linear peptides through their terminal amino function, onto poly(acrylic acid) radiation-grafted polyvinylidene fluoride (PVDF) films (33). The introduction of a linker molecule with an amino function would enable the grafting of cyclo-VEGI onto polymeric nanocarriers and therefore allow the design of drug delivery/carrier systems. Moreover, chemical modifications of cyclo-VEGI should be possible via linker molecules by amide bond formation with the γ-COOH group of Glu93. These linker molecules should also act as spacer arms to minimize steric hindrance between cyclo-VEGI and the solid support.

We next designed and synthesized three linkers. Oligoethylene glycol derivatives are ideal for this purpose because



Scheme 4. Functionalization of cylo-VEGI.

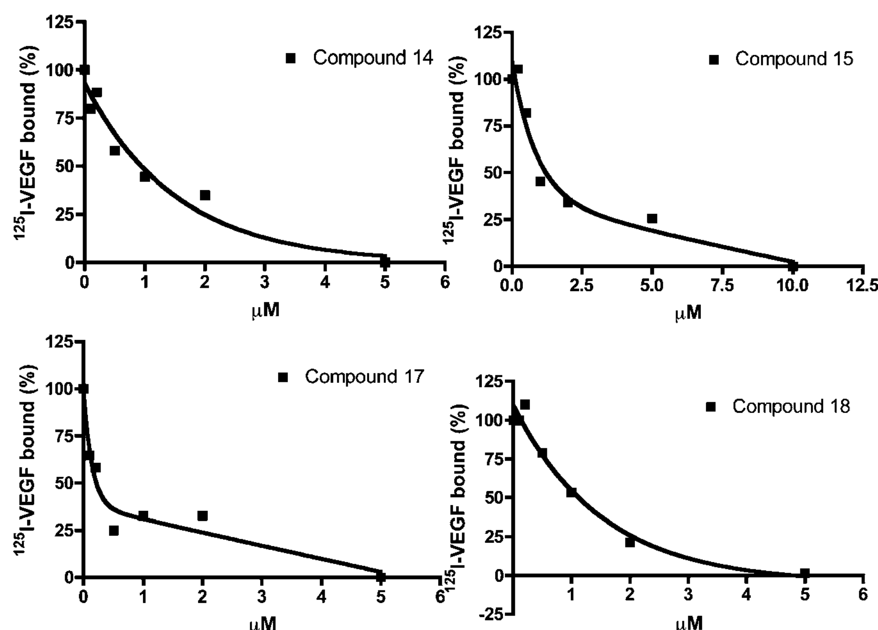


Fig. 1. Inhibition of ^{125}I-VEGF–KDR binding to Chinese hamster ovary (CHO) cells expressing KDR by modified cVEGI peptides. Cells were incubated in duplicates with increasing concentration of peptides in the presence of 10 ng/ml of ^{125}I-VEGF. Binding was analyzed after 2 h of incubation at 4°C as indicated in materials and methods. Results are expressed as percentages of specific binding in comparison to control.

they are water soluble and available in a variety of lengths. We first synthesized a heterobifunctional linker that possesses a terminal amino group for its anchoring to cyclo-VEGI, and a terminal allyl group allowing further modifications (i.e, radiolabeling). This linker was prepared from commercially available triethylene glycol **1** (TEG) (Scheme 1).

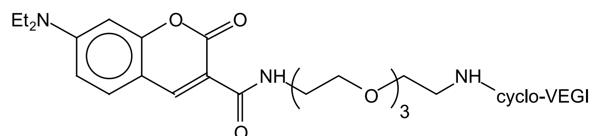
The critical step is the selective mono-allylation of TEG. It was successfully performed by using an excess of TEG with sodium azide and allyl bromide in dry THF to give compound **2** in good yield. Tosylation of compound **2** gave compound **3** which was subjected to a Gabriel's amination to give the original linker molecule **5** with an overall yied of 65%. Thereafter we synthesized two further linkers from a common synthon taking advantage of the procedure described by Bertozzi and Bednarski (34) (Scheme 2) yielding a synthon allowing to obtain a linker with a terminal olefin or amino group under protected form (azido or phthalimido form).

The critical step is the selective monotosylation of tetraethylene glycol **6**. It was performed in triethylamine with tosyl chloride and a large excess of tetraethylene glycol to give compound **7** in good yield. Compound **7** was reacted with sodium azide in DMF or absolute ethanol to provide compound **8**. Mesylation of compound **8** yielded synthon **9** from which two linker molecules with a terminal olefin or a terminal amino group could be obtained (Scheme 3).

Compound **9** subjected to a Gabriel's amination yielded linker **12** with an overall yield of 49%. The azido group would give the terminal amino group by reduction after decoration of cyclo-VEGI with linker **12**. On the other side, when compound **9** was subjected to the nucleophilic substitution of its mesylate group by an alcohol with a terminal olefin it gave compound **11**. We chose to replace the allyl group by a but-1-enyl group to determine what kind of terminal olefin would be the most convenient for functionalizations such as radiolabeling as we might be afraid of a too high lability of the allyloxy group. This could have a nefast effect on the functionalization step. The reduction of the azido group of compound **11** was performed with triphenylphosphine in THF to give the original linker **13** which was obtained with an overall yield of 33%. Thereafter cyclo-VEGI was functionalized with linkers **5**, **12**, and **13** (Scheme 4) using a mixture of PyBOP and HOBt with DIEA in THF to give compounds **14**, **15**, and **16**.

Compound **17** was obtained from compound **14** by reduction of the azido group under mild conditions possible because of the presence of triphenylphosphine in THF. Compounds **14**–**16** were purified by reverse-phase HPLC. The presence of all the side-chain protecting groups in cyclo-VEGI made the ionization of compounds **14**–**16** impossible in MALDI mass spectrometry analysis. Therefore, in a fraction of each compound, all the protecting groups were completely removed to allow MALDI mass spectrometry analysis which confirmed the integrity of compounds **14**, **15**, and **17**. MALDI analysis of unprotected compound **16** also showed the presence of non-modified cyclo-VEGI. Purification by reverse-phase HPLC of protected or unprotected compound **16** and cyclo-VEGI was unsuccessful owing to similar retention times.

We then investigated the ability of compounds **15** and **17** to inhibit the binding of ^{125}I-VEGF to KDR in comparison to nonmodified cyclo-VEGI. This was achieved by investigating



Scheme 5. Structure of compound **18**.

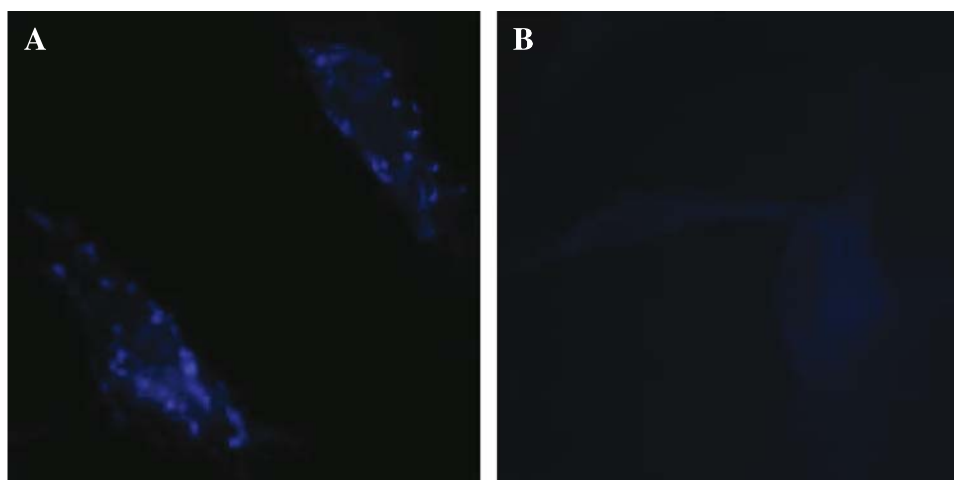


Fig. 2. (A, B) Targeting of endothelial cells by coumarin-labeled cyclo-VEGI in vitro. Endothelial cells were incubated with 1 μM of coumarin-labeled cyclo-VEGI (peptide **18**; A) or coumarin only (B) for 2 h. After 2 h, cells were washed three times and fixed with 1% glutaraldehyde. Fluorescence pictures were taken at 405 nm wavelength.

the capacity of these cyclopeptides to interfere with binding of ^{125}I-labeled VEGF$_{165}$ to Chinese hamster ovary (CHO) cells expressing KDR (Fig. 1).

As shown in Fig. 1, functionalization of cyclo-VEGI does not abrogate its ability to compete with ^{125}I-VEGF binding. Modified cyclo-VEGIs interfere even more strongly in ^{125}I-VEGF binding than nonmodified cyclo-VEGI. (IC$_{50}$ values of 1, 0.94, 0.16 μM for compound **14**, **15**, and **17**, respectively). This may be explained by an increase in basicity of the compounds through blocking the acidic function of the Glu residue by our linkers since basic residues play a key role in the VEGF–KDR interaction. Compound **17** showed even higher inhibitory activity when compared to the other modified peptides. This may be explained by the presence of another basic residue coming from the linker. We also tested the effect of coumarin-labeled cyclo-VEGI peptide on VEGF binding (compound **18**). Cyclo-VEGI was labeled with 7-diethylaminocoumarin-3-carboxylic acid as previously described (35,36) (compound **18**; Scheme 5). Inhibitory activity was similar to that of compounds **14** and **15** (IC$_{50}$ = 1 μM for **18**).

Furthermore, we investigated the ability of compound **18** to target endothelial cells *in vitro* (Fig. 2). Coumarin-labeled cyclo-VEGI (compound 1 μM) was incubated for 2 h with BAE cells (Fig. 2). Fluorescence labeling was seen in cells that were exposed to coumarin–cyclo-VEGI but not in cells exposed to coumarin only. There was a time-dependent accumulation of the fluorescent peptide. Fluorescence was already visible at 30 min and increased significantly up to 2 h. At 2 h, fluorescence was mainly intracellular and probably localized in the endosomal compartment.

CONCLUSION

As an innovative approach toward the preparation of original molecular nanocarriers, we herein described a new and convenient strategy for the decoration of cyclo-VEGI. These derivatives were obtained by an original synthetic route. Conjugation of cyclopeptide was achieved with three newly synthezised heterobifunctional linkers, two of them being original. Functionalized cyclo-VEGI conserved a strong inhibitory potency for VEGF-binding to KDR, compound **17** for instance being more efficient than non-modified cyclo-VEGI. Furthermore, functionalized cyclo-VEGI is targeted to endothelial cells *in vitro* where it is accumulated after binding in the endosomal compartment. An important point to be noticed at is that for all previously prepared cyclopeptides binding affinities were well correlated with *in vitro* cellular activity as well as *in vivo activity*. The full biological evaluation is in progress.

These compounds could be useful not only as inhibitors or targeting agents in tumors or other angiogenesis-related diseases but also for molecular imaging Chemical conjugation to polymers and nanoparticules as well as extensive biological evaluation of the different systems is in progress.

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