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Communication

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Tackling Lipophilicity of Peptide Drugs: Replacement of the Backbone *N*-Methyl Group of Cilengitide by *N*-Oligoethylene Glycol (*N*-OEG) Chains

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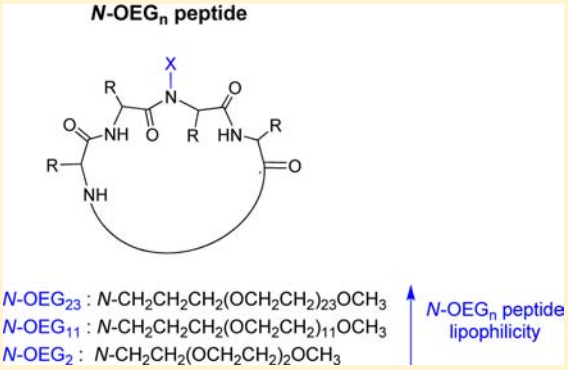
Supporting Information

ABSTRACT: Cilengitide is an RGD-peptide of sequence cyclo-[RGDf*N*MeV] that was was developed as a highly active and selective ligand for the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. We describe the synthesis of three analogues of this peptide in which the *N*-Me group has been replaced by *N*-oligoethylene glycol (*N*-OEG) chains of increasing size: namely *N*-OEG_2, *N*-OEG_{11}, and *N*-OEG_{23}, which are respectively composed of 2, 11, and 23 ethylene oxide monomer units. The different *N*-OEG cyclopeptides and the original peptide were compared with respect to lipophilicity and biological activity. The *N*-OEG_2 analogue was straightforward to synthesize in solid phase using an Fmoc-*N*-OEG_2 building block. The syntheses of the *N*-OEG_{11} and *N*-OEG_{23} cyclopeptides are hampered by the increased steric hindrance of the *N*-substituent, and could only be achieved by segment coupling, which takes place with epimerization and thus requires extensive product purification. All the *N*-OEG analogues were found to be more hydrophobic than the parent peptide, and their hydrophobicity was systematically enhanced upon increasing the length of the OEG chain. The *N*-OEG_2 cyclopeptide displayed the same capacity as Cilengitide to inhibit the integrin-mediated adhesion of HUVEC endothelial, DAOY gliobastoma, and HT-29 colon cancer cells to their ligands vitronectin and fibrinogen. The *N*-OEG_{11} and *N*-OEG_{23} analogues also inhibited cell adhesion to these immobilized ligands, but their IC_{50} values dropped by 1 order of magnitude with respect to the parent peptide. These results indicate that replacement of the backbone *N*-Me group of Cilengitide by a short *N*-OEG chain provides a more lipophilic analogue with a similar biological activity. Upon increasing the size of the *N*-OEG chain, liophilicity is enhanced, but synthetic yields drop and the longer polymer chains may impede targeted binding.



In peptide-based medicinal chemistry, backbone *N*-methylation is an established tool to improve pharmacological properties of peptide drugs. The introduction of backbone *N*-Me groups has allowed to optimize the activity and selectivity of numerous peptide ligands as a result of conformational modulation.[1] Furthermore, the introduction of *N*-Me residues into peptides increases their hydrophobicity, proteolytic resistance, and membrane permeability, which can enhance their bioavailability, thus amplifying their therapeutic potential.[1,2] Surprisingly, little research has been conducted on modifying the peptide backbone with other *N*-alkyl substituents. Besides the so-called peptoids (*N*-substituted glycine oligomers)[3] and *N*-backbone cyclic peptides introduced by Gilon,[4] only a few examples have been reported in which other *N*-alkylated peptides have been accessed, and such examples are limited to modification with small *N*-alkyl groups [i.e., *N*-ethyl,[5,6] *N*-allyl,[5,7,8] *N*-butyl,[9] and *N*-guanidylbutyl[10]]. In the field of *N*-backbone cyclic peptides, which are usually prepared using *N*-functionalized building blocks [e.g., *N*-aminoalkyl, *N*-carboxyalkyl, and *N*-sulfanyl],[11−15] the solution synthesis and purification of *N*-alkylated dipeptide building units have been described numerous times.[11,12,16−21]

Very recently, we showed that peptides bearing an *N*-triethylene glycol (*N*-TEG) chain are straighforward to access using standard solid-phase techniques.[22] With our methodology, we synthesized several *N*-TEG analogues of Sansalvamide A peptide, all of which were found to be slightly more lipophilic than their corresponding *N*-Me homologues. Since

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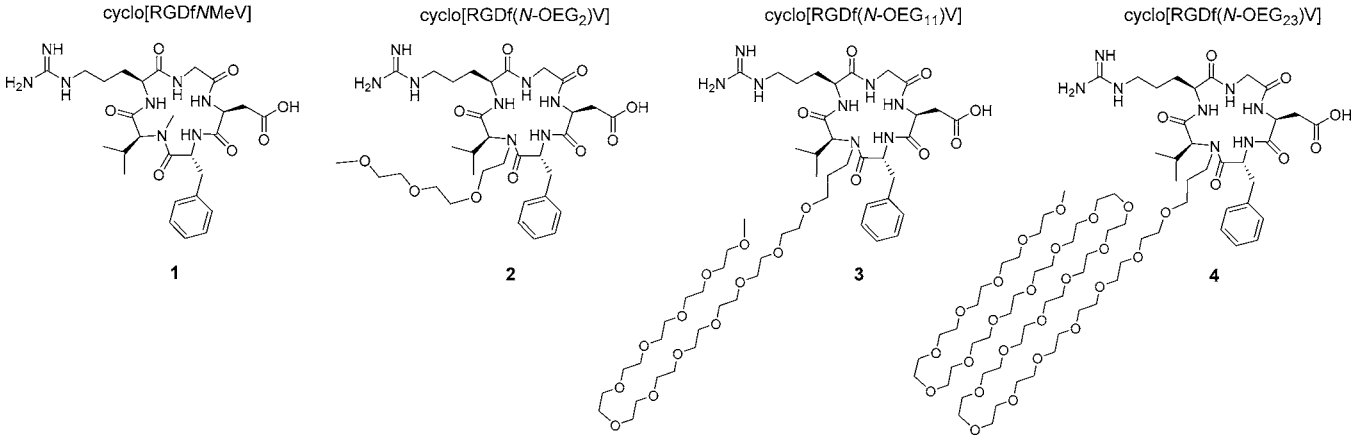


Figure 1. Structure of cyclo[RGDf*N*MeV] (**1**) and the *N*-OEG cyclopeptides (**2**–**4**) synthesized and tested.

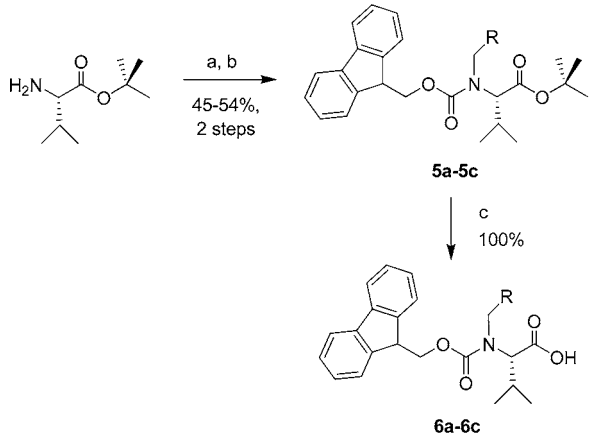
lipophilicity is a key pharmacological parameter,[23] we found it desirable to know if such an enhancement in lipophilicity would also be observed upon incorporation of an *N*-oligoethylene glycol (*N*-OEG) chain into other peptides. We also sought to evaluate the effect of such structural modification on biological activity in more detail. To investigate this, we chose Cilengitide (**1**) as model peptide. This peptide is an RGD-peptide of sequence cyclo[RGDf*N*MeV] that was developed as a highly active and selective ligand for the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors.[24]

In this work, we have investigated the effect of replacing the backbone *N*-Me group of Cilengitide by different *N*-OEG chains on its lipophilicity and biological activity. Herein we describe synthesis of three analogues of **1** in which the *N*-Me group of Val has been replaced by *N*-OEG chains of increasing length, namely, *N*-OEG_2, *N*-OEG_{11}, and *N*-OEG_{23}, which are respectively composed of 2, 11, and 23 repeating ethylene oxide monomer units (Figure 1).

In a first attempt to synthesize cyclopeptides **2**–**4**, we adopted the strategy that we had employed for the synthesis of the *N*-TEG Sansalvamide A peptide analogues. Such an approach involved the use of Fmoc-protected *N*-TEG building blocks in the solid-phase synthesis of the linear peptide precursors, which were cyclized in solution. To prepare the required Fmoc-*N*-OEG Val derivatives (**6a**–**6c**), valine *tert*-butyl ester was subjected to reductive alkylation with a suitable aldehyde.[25] In all cases, the reductive alkylation mixture consisted of unreacted starting material, *N*-monoalkylated product, and *N*,*N*-dialkylated product. The latter two were found to be inseparable, but after Fmoc-protection of the amino group, the desired products (**5a**–**5c**) could be isolated by flash chromatography. Remarkably, the increased length of the *N*-OEG chain did not prevent the acylation of the secondary amine with Fmoc-Cl and did not hamper the purification of **5a**–**5c** by flash chromatography. Finally, acidic cleavage of the *tert*-butyl ester yielded **6a**–**6c** in 45–54% overall yield (Scheme 1).

The solid-phase peptide synthesis (SPPS) of the linear pentapeptides **9a**–**9c** was performed on the 2-chlorotrityl chloride (CTC) resin by Fmoc-/^{t}Bu- chemistry (Scheme 2). The use of a low functionalization (0.2–0.3 mmol/g) was aimed to facilitate the sterically hindered coupling step.[26] In all the pentapeptides (**9a**–**9c**), the *N*-OEG Val residue was placed in the middle of the sequence to render Gly at the *C*-terminus, which minimizes steric hindrance during cyclization and rules

Scheme 1. Synthesis of the Fmoc-*N*-OEG Val Derivatives (6a–6c)[a]



a. *N*-OEG_2 : R- = $CH_3O(CH_2CH_2O)_2CH_2$-
b. *N*-OEG_{11} : R- = $CH_3O(CH_2CH_2O)_{11}CH_2CH_2$-
c. *N*-OEG_{23} : R- = $CH_3O(CH_2CH_2O)_{23}CH_2CH_2$-

[a]Reagents and conditions: a. R-CHO (1.1 equiv), $NaBH_3CN$ (1.34 equiv), MeOH/AcOH 99:1; b. Fmoc-Cl (1.2 equiv), DIEA (2.0 equiv), DCM; c. TFA/DCM 1:1.

out the possibility of epimerization in this step. The Fmoc-*N*-OEG Val derivatives (**6a**–**6c**) were coupled onto the peptidyl-resin in a 3-fold excess using DIPCDI/OxymaPure activation. With these conditions, the coupling of **6a** and **6b** took place efficiently and with no detectable epimerization, whereas the coupling of **6c** was found to be hampered by its longer *N*-OEG_{23} chain and required PyBOP/HOAt as a stronger activation method, although the coupling did not proceed to completion. After having assembled the three *N*-OEG tripeptidyl-resins, we investigated the coupling of Fmoc-D-Phe using bis(trichloromethyl)carbonate (BTC) as activating reagent.[27,28] It is important to note that this coupling is hampered not only by the *N*-substituent, but also by the *β*-branched side-chain of Val. With this activation method, resin-bound *N*-OEG_2 Val was completely acylated after two treatments (15 h) with the *in situ* generated acid chloride of Fmoc-D-Phe (as checked by HPLC analysis of a cleaved resin sample). The use of BTC also allowed for coupling Fmoc-D-Phe onto resin-bound *N*-OEG_{11} Val, but the conversion was low and did not improve after a third coupling cycle. However,

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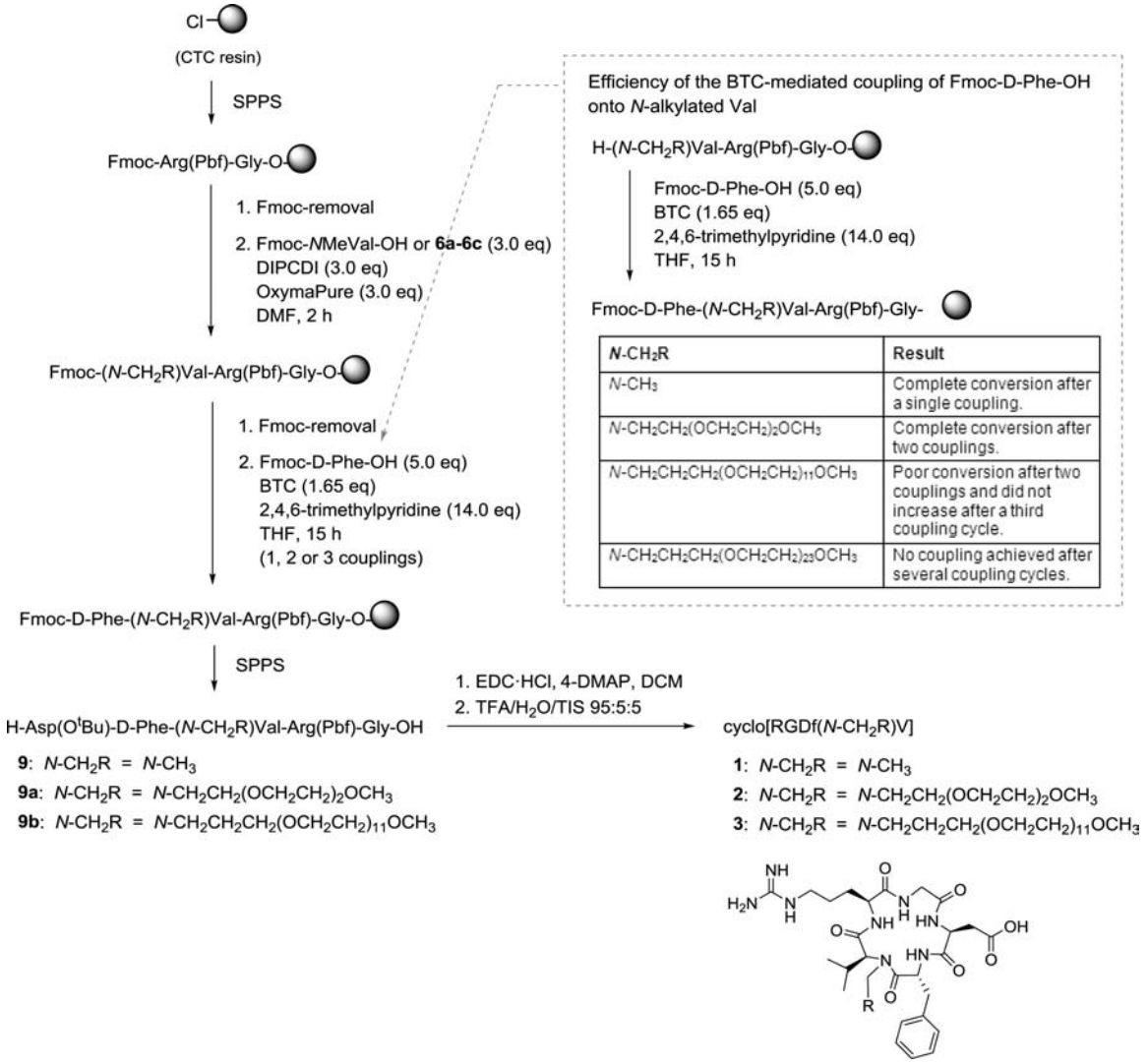
Scheme 2. Synthesis of Cyclo[RGDf*N*MeV] (1) and the *N*-OEG Cyclopeptides 2−3



N-CH_2R	Result
N-CH_3	Complete conversion after a single coupling.
N-$CH_2CH_2(OCH_2CH_2)_2OCH_3$	Complete conversion after two couplings.
N-$CH_2CH_2CH_2(OCH_2CH_2)_{11}OCH_3$	Poor conversion after two couplings and did not increase after a third coupling cycle.
N-$CH_2CH_2CH_2(OCH_2CH_2)_{23}OCH_3$	No coupling achieved after several coupling cycles.

for the *N*-OEG_{23} peptidyl-resin, no coupling could be achieved. We tested other activation methods, but all of them failed to form the desired amide bond. Thus, we discontinued the synthesis of pentapeptide **9c** by this approach. For the resin-bound *N*-OEG_2 and *N*-OEG_{11} peptides, further peptide chain elongation and cleavage afforded the desired pentapeptides (**9a** and **9b**), which were cyclized with EDC and catalytic amounts of 4-DMAP. Cyclization proceeded smoothly and, after removal of side-chain protecting groups with 95% TFA in the presence of H_2O and TIS, the *N*-OEG_2 and *N*-OEG_{11} cyclopeptides (**2** and **3**) were isolated by semipreparative RP-HPLC.

Thus, the synthetic strategy shown in Scheme 2 allows the efficient preparation of peptides bearing an *N*-OEG chain of 2 ethylene oxide units, and enables us to obtain peptides with an 11-monomer unit *N*-OEG chain, though in very low amount. However, the synthesis of our target *N*-OEG_{23} pentapeptide (**9c**) was not found feasible, since no coupling of Fmoc-D-Phe onto resin-bound *N*-OEG_{23} Val could be achieved. To avoid the difficult formation of the D-Phe-(*N*-OEG_{11})Val and D-Phe-(*N*-OEG_{23})Val amide bonds in solid phase, we sought to prepare suitable Fmoc-D-Phe-(*N*-OEG)Val dipeptides in solution and use them as solid-phase building blocks. To prepare the required dipeptides (**8b** and **8c**), valine *tert*-butyl ester was subjected to reductive alkylation with a suitable aldehyde, and the resulting *N*-OEG_{11} or *N*-OEG_{23} Val derivatives were reacted with the acid chloride of Fmoc-D-Phe. This procedure enabled us to obtain dipeptides **7b** and **7c** with no detectable epimerization, and acidic cleavage of the *tert*-butyl ester yielded the desired building blocks, **8b** and **8c** (Scheme 3).

In the SPPS of the *N*-OEG_{11} and *N*-OEG_{23} pentapeptides (**9b** and **9c**), dipeptides **8b** and **8c** were coupled onto the peptidyl-resin using PyBOP/HOAt activation (Scheme 4). Since these dipeptides are time-consuming to synthesize, only 1.0 equiv was coupled. After 3 h coupling, further PyBOP (1.0 equiv) and DIEA (1.0 equiv) were added and the reaction was allowed to proceed overnight. These couplings proceeded with acceptable efficiency. However, epimerization of *C*-activated dipeptides can occur through various mechanisms and is known to be a major disadvantage of segment peptide synthesis.[29] In our case, considerable epimerization took place at the dipeptidic *C*-terminal Val residue. The occurrence of epimerization was confirmed by the presence of two peaks with the mass of the desired product in the HPLC spectra of a cleaved peptidyl-resin sample. The high degree of epimerization observed was expectable, as the epimerization of *C*-activated

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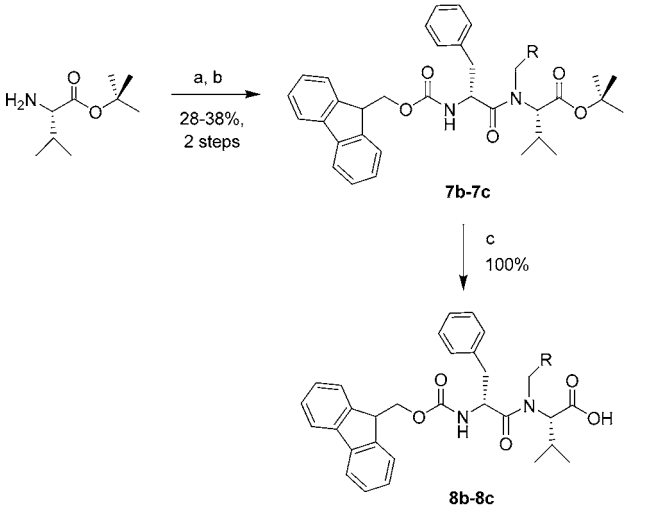
Scheme 3. Synthesis of the Fmoc-D-Phe-(*N*-OEG)Val Dipeptides (8b–8c)[a]



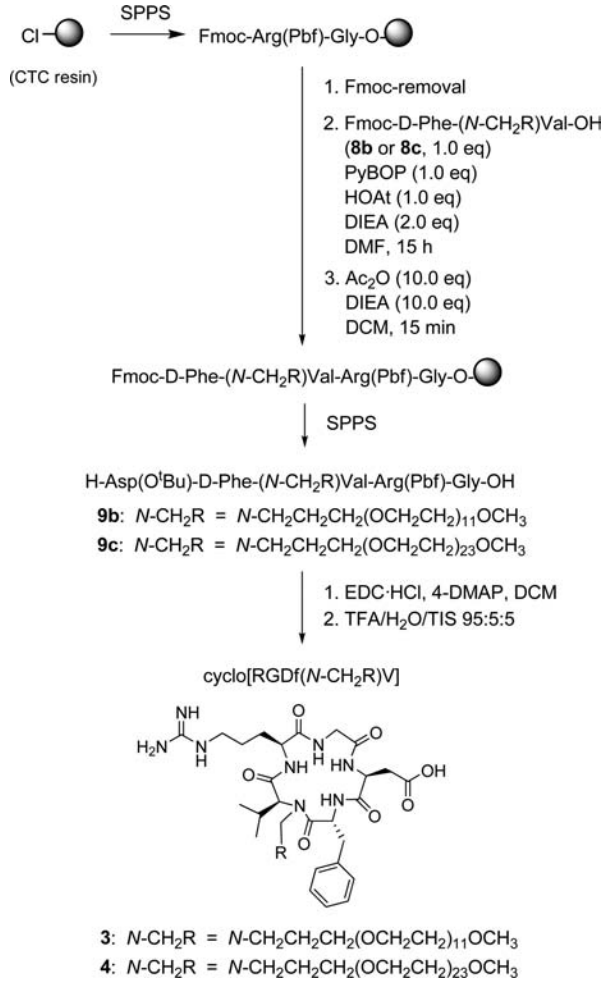
[a]Reagents and conditions: a. R-CHO (1.1 equiv), $NaBH_3CN$ (1.34 equiv), MeOH/AcOH 99:1; b. Fmoc-D-Phe-Cl (1.5 equiv), DIEA (2.5 equiv), DCM; c. TFA/DCM 1:1.

peptides is favored by itself (no carbamate as protecting group of the *C*-terminal residue) and, in this case, by the presence of D- and/or *N*-alkyl residues.[30,31] Further peptide elongation and cleavage from resin yielded pentapeptides **9b** and **9c** epimerized at Val. After cyclization and deprotection, the desired *N*-OEG_{11} and *N*-OEG_{23} cyclopeptides (**3** and **4**) were separated from their nondesired Val epimers by semipreparative RP-HPLC. For the two isolated *N*-OEG_{11} cyclopeptides, the stereoisomer having a lower retention time was found to coelute with stereochemically pure cyclo[RGDf(*N*-OEG_{11})V] (**3**), which had been previously prepared using Fmoc-*N*-OEG_{11} Val (**6b**) as building block. Analogously, the isolated *N*-OEG_{23} cyclopeptide with a lower RP-HPLC retention time was assumed to be the product with the desired stereochemistry, cyclo[RGDf-(*N*-OEG_{23})V] (**4**) (see Supporting Information).

To study the effect of the *N*-OEG chain on lipophilicity, we coinjected the *N*-OEG cyclopeptides (**2**–**4**) and cyclo-[RGDf*N*MeV] (**1**) onto a C18 column and compared their RP-HPLC retention times.[32] This is reported as a reliable method to estimate the relative hydrophobicities of a series of modified analogues.[33] The RP-HPLC behavior of a compound depends on its hydrophobic interactions with the nonpolar stationary phase: the more hydrophobic a compound is, the stronger its retention on the column. All the *N*-OEG analogues (**2**–**4**) were found to be more lipophilic than **1**, and their lipophilicity was systematically enhanced upon increasing the size of the *N*-OEG chain (Figure 2). These results can be explained by the amphiphilic nature of OEG.[34,35] Although OEG-modified peptides often show better aqueous solubility than their corresponding nonmodified peptides, this is due to the polymer chain preventing intermolecular aggregation, and not due to an increase in hydrophilicity.[36,37] Along these lines, the attachment of an OEG chain to peptides can improve their solubility in organic solvents.[38,39]

Our findings upon incorporation of *N*-OEG into Cilengitide, which has two ionizable side-chain functionalities, are

Scheme 4. Synthesis of the *N*-OEG Cyclopeptides 3–4 Using a Dipeptide Building Block



consistent with our previously reported findings for the *N*-OEG analogues of the totally aliphatic Sansalvamide A peptide: for both model peptides, replacement of a backbone *N*-Me group by an *N*-OEG chain provides a higher lipophilicity. Therefore, we can reasonably expect that *N*-Me-for-*N*-OEG substitution will also increase lipophilicity for other *N*-methylated peptide scaffolds. Such an enhancement in lipophilicity can be conveniently exploited for improving the absorption of peptide drug candidates that are too hydrophilic to cross biological membranes via transcellular passive diffusion,[40,41] which is the most common transport route for peptides. Indeed, the covalent modification of peptides by attaching lipophilic moieties has proven to be an effective approach to improve their intestinal permeability[42–46] and oral bioavailability.[47,48]

The parent peptide, cyclo[RGDf*N*MeV] (**1**), and its *N*-OEG analogues (**2**–**4**) showed no degradation when incubated in human serum at 37 °C over a period of 48 h (see Supporting Information). The high enzymatic stability of **1**–**4** was expected, since cyclic peptides show increased resistance to proteolytic cleavage, and the presence of D- and *N*-alkyl residues confers them further stability.[49]

The biological activity and selectivity of cyclo[RGDf*N*MeV] (**1**) and the *N*-OEG cyclopeptides (**2**–**4**) were evaluated in cell adhesion inhibition assays (Table 1). Adhesion studies were carried out with HUVEC endothelial, DAOY glioblastoma, and

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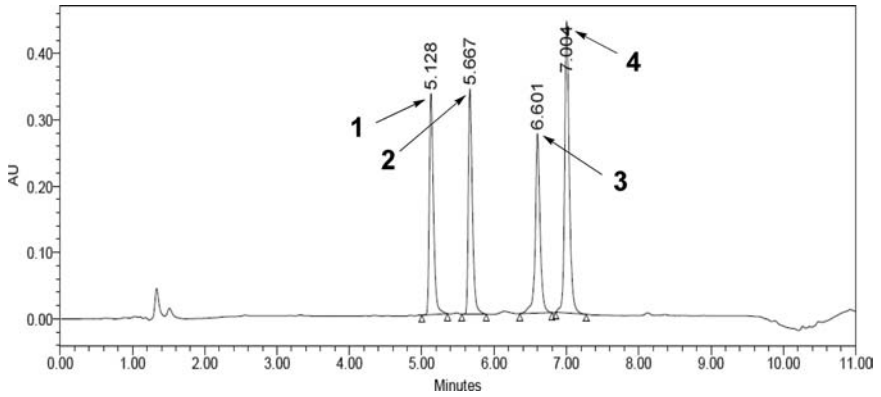

Figure 2. HPLC chromatogram obtained after coinjection cyclo[RGDf*N*MeV] (**1**) and the *N*-OEG cyclopeptides (**2**–**4**) on a C18 column, linear gradient from 10% to 50% ACN over 8 min.

Table 1. Adhesion Inhibition Assays of Cyclo[RGDf*N*MeV] (1) and the *N*-OEG Cyclopeptides (2–4)[a]

	vitronectin (VN)			fibrinogen (FB)	
compound	HUVEC on VN $\alpha_v\beta_3$ + $\alpha_v\beta_5$	DAOY on VN $\alpha_v\beta_3$ + $\alpha_v\beta_5$	HT-29 on VN $\alpha_v\beta_5$	HUVEC on FB $\alpha v\beta_3$	DAOY on FB $\alpha_v\beta_3$
cyclo[RGDf*N*MeV] (**1**)	0.37	2.69	3.11	0.076	0.44
cyclo[RGDf(*N*-OEG$_2$)V] (**2**)	0.42	3.62	2.17	0.036	0.14
cyclo[RGDf(*N*-OEG$_{11}$)V] (**3**)	12.42	171.5	n.d.	1.38	4.55
cyclo[RGDf(*N*-OEG$_{23}$)V] (**4**)	22.30	143.1	n.d.	0.28	2.31

[a]IC_{50} values are given in μM.

HT-29 colon cancer cells using vitronectin (VN) or fibrinogen (FB) as ligands. For HUVEC and DAOY cells, adhesion to their ligand VN is mediated by integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, whereas their adhesion to FB is only mediated by integrin $\alpha_v\beta_3$. In contrast, the adhesion of HT-29 cells to VN is only $\alpha_v\beta_5$-dependent. For all the cell/ligand systems, the compounds inhibited cell adhesion in a concentration-dependent manner, resulting in the expected sigmoid curves. The inhibitory activities of the *N*-OEG$_2$ cyclopeptide (**2**) were very similar to those of **1**, which indicates that replacement of its *N*-Me group by a short *N*-OEG$_2$ chain does not affect binding affinity. In contrast, the *N*-OEG$_{11}$ and *N*-OEG$_{23}$ analogues (**3** and **4**) inhibited cell adhesion with less potency, which is probably due to their longer OEG chains interfering with the RGD-receptor interaction. Indeed, the reduced biological activity of peptides upon attachment of a bulky polymer chain is an issue of major concern, especially in the case of small peptides.[50]

In summary, the analogue of Cilengitide bearing an *N*-OEG$_2$ chain instead of a backbone *N*-Me group (**2**) was straightforward to synthesize in solid phase. The acylation of resin-bound *N*-OEG$_2$ amines can be achieved by activating the following amino acid with BTC, and the protocol is compatible with the acid-labile CTC resin. However, for resin-bound *N*-OEG$_{11}$ and *N*-OEG$_{23}$ residues, this method does not work. The increased steric hindrance of their *N*-substituents impedes the acylation in solid phase (1 and 10 atom-chains for **1** and **2**, versus 38 and 74 atom-chains for **3** and **4**). We were able to obtain the *N*-OEG$_{11}$ and *N*-OEG$_{23}$ cyclopeptides (**3** and **4**) by using a dipeptidic building block, but considerable epimerization took place during dipeptide coupling, and extensive RP-HPLC purification was required to separate the desired cyclic products from their nondesired diastereoisomers.

All the *N*-OEG analogues (**2**–**4**) were found to be more hydrophobic than the parent peptide (**1**), and their hydrophobicity was systematically enhanced upon increasing the length of the OEG chain. The *N*-OEG$_2$ cyclopeptide (**2**) displayed the same capacity as Cilengitide (**1**) to inhibit integrin-mediated cell adhesion, but the inhibitory activities of the *N*-OEG$_{11}$ and *N*-OEG$_{23}$ cyclopeptides (**3** and **4**) were 1 order of magnitude lower. Taken together, the results show that, in the case of the cyclic pentapeptide Cilengitide, substitution of a backbone *N*-Me group by a short *N*-OEG chain provides a more lipophilic analogue with a similar biological activity. Upon increasing the length of the OEG chain, lipophilicity is enhanced, but the synthesis is not efficient and steric hindrance may impede targeted binding. Thus, replacement of a backbone *N*-Me group by a short *N*-OEG chain is a feasible way to enhance the lipophilicity of peptide drug candidates, which can result in a better membrane permeability via passive diffusion, and may not have a negative impact on biological activity. Considering that backbone *N*-Me groups are common structural motifs in many biologically active peptides, we want to communicate that modification at this position is a valuable alternative to introduce chemical diversity or alter pharmacologically important parameters when modification at any other position of the peptide is not wished or possible.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details of the syntheses, cellular assays, serum stability assays, characterization data, HPLC traces and HRMS data of the pure cyclopeptides (**1**–**4**), and copies of the NMR spectra of selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

4-DMAP, 4-dimethylaminopyridine; ACN, acetonitrile; BTC, bis(trichloromethyl)carbonate; CTC, 2-chlorotrityl chloride; DIEA, *N,N′*-diisopropylethylamine; DIPCDI, *N,N′*-diisopropylcarbodiimide; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; FB, fibrinogen; MeOH, methanol; OEG, oligoethylene glycol; OxymaPure, ethyl-2-cyano-2-(hydroxyimino)acetate; TEG, triethylene glycol; VN, vitronectin

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