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Cyclic Aza-peptide Integrin Ligand Synthesis and Biological Activity

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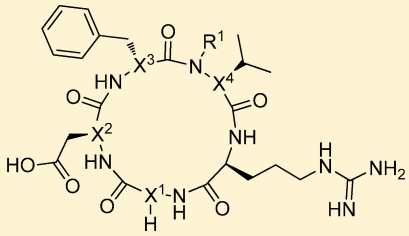
Supporting Information



cilengitide: $X^1 = X^2 = X^3 = X^4 = CH$, $R^1 = Me$
peptide 1: $X^1 = N$, $X^2 = X^3 = X^4 = CH$, $R^1 = H$
peptide 2: $X^2 = N$, $X^1 = X^3 = X^4 = CH$, $R^1 = H$
peptide 3: $X^3 = N$, $X^1 = X^2 = X^4 = CH$, $R^1 = H$
peptide 4: $X^4 = N$, $X^1 = X^2 = X^3 = CH$, $R^1 = H$
peptide 5: $X^1 = N$, $X^2 = X^3 = X^4 = CH$, $R^1 = Me$
peptide 6: $X^2 = N$, $X^1 = X^3 = X^4 = CH$, $R^1 = Me$
peptide 7: $X^3 = N$, $X^1 = X^2 = X^4 = CH$, $R^1 = Me$

ABSTRACT: Aza-peptides are obtained by replacement of the α-C-atom of one or more amino acids by a nitrogen atom in a peptide sequence. Introduction of aza-residues into peptide sequences may result in unique structural and pharmacological properties, such that aza-scanning may be used to probe structure−activity relationships. In this study, a general approach for the synthesis of cyclic aza-peptides was developed by modification of strategies for linear aza-peptide synthesis and applied in the preparation of cyclic aza-pentapeptides containing the RGD (Arg-Gly-Asp) sequence. Aza-amino acid scanning was performed on the cyclic RGD-peptide Cilengitide, *cyclo*[R-G-D-f-*N*(Me)V] **1**, and its parent peptide *cyclo*(R-G-D-f-V) **2**, potent antagonists of the αvβ3, αvβ5, and α5β1 integrin receptors, which play important roles in human tumor metastasis and tumor-induced angiogenesis. Although incorporation of the aza-residues resulted generally in a loss of binding affinity, cyclic aza-peptides containing aza-glycine retained nanomolar activity toward the αvβ3 receptor.

INTRODUCTION

Aza-peptides are mimics possessing an α-carbon to nitrogen atom replacement in the peptide sequence. Although a configurationally labile α-center is installed by this substitution, enhanced rigidity is also introduced into the aza-peptide backbone as a result of the planarity of the urea moiety and electronic repulsion between nitrogen atoms in the *N*,*N*′-diacyl hydrazine constituent.[1] Of particular note, aza-amino acids may induce β-turn conformations within peptides, as has been shown in a variety of theoretical,[1−5] spectroscopic,[3,5−7] and crystallographic[8−12] studies.

Several different strategies have been explored for the synthesis of aza-peptides featuring combinations of hydrazine and peptide chemistry. The aza-residue has been typically constructed from a hydrazine component and a carbonyl-donating reagent.[13,14] For example, hydrazides have been reacted, respectively, with resin-bound isocyanate and carbamoyl halides derived from activation of the amine terminus of a peptide chain.[15−17] Moreover, *N*′-alkyl carbazates, such as *N*-Fmoc,[18,19] *N*-Boc,[20−22] and *N*-Ddz-*N*′-alkyhydrazines[23] have been activated using phosgene equivalents and the resulting aza-amino acid building blocks coupled onto resin bound peptides, with enhanced yields using microwave irradiation.[24] In addition, a submonomer approach for the synthesis of aza-peptides by alkylation of a resin-bound aza-glycine residue has been used to circumvent the limitations of solution-phase hydrazine chemistry and expand the diversity of aza-residue side chains.[25−27]

Aza-amino acids have been introduced into biologically active peptides to study structure−activity relationships (SARs) as well as to generate analogues with superior pharmacological and pharmacokinetic properties. For example, incorporation of aza-residues has resulted in greater stability to enzymatic degradation, enhanced receptor affinity, and increased selectivity compared to the parent peptide.[13,14,28] Although several linear peptides have been scanned by systematic substitution of aza-residues for each amino acid in the sequence,[18,19,22,24,25,27] to the best of our knowledge, no cyclic peptide has similarly been systematically scanned. Moreover, only few aza-cyclopeptides have been reported in the literature.[29,30]

Searching for a general strategy for the preparation of cyclic aza-peptides, we have explored the synthesis of aza-derivatives of the cyclic RGD-pentapeptide Cilengitide, *cyclo*[R-G-D-f-(*N*Me)V] **1**, and its parent counterpart *cyclo*(R-G-D-f-V) **2** (Figure 1). Cilengitide was discovered by a mono-*N*-methylation scan of **2**, from which the *N*-(methyl)valine analogue exhibited subnanomolar activity and increased selectivity for the αvβ3 integrin.[31,32] Peptides **1** and **2** are

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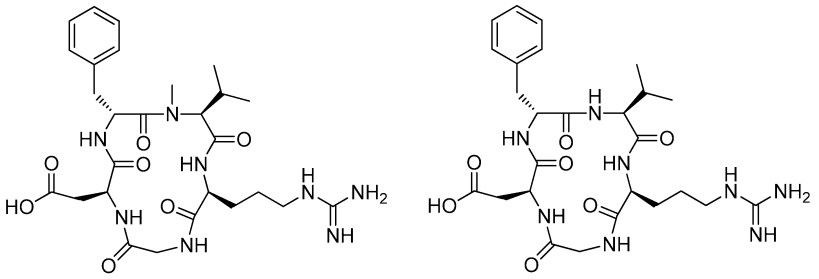
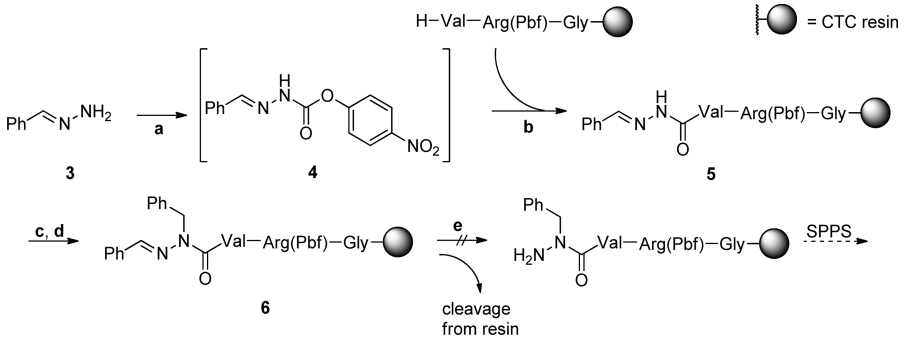


1 2

Figure 1. Cilengitide (*cyclo*[R-G-D-f-(*N*Me)V], (**1**)) and its parent peptide *cyclo*(R-G-D-f-V) (**2**).

Scheme 1. Attempted Submonomer Synthesis of an AzaF Peptide Ester[a]



[a]Reagents and conditions: (a) *p*-nitrophenyl chloroformate, DCM, 0 °C → rt, 2 h; (b) DIEA, DCM, rt, 16 h; (c) KO^{t}Bu, THF, 30 min; (d) benzyl bromide, THF, 12 h; (e) $NH_2OH{\cdot}HCl$, pyridine, 60 °C, sonication.

both potent inhibitors of the $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ integrins, which play important roles in human tumor metastasis and tumor-induced angiogenesis. Cilengitide, the first antiangiogenic small molecule targeting the $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ integrins, is currently in clinical phase II and III trials for the treatment of glioblastoma and other cancer types.[33,34] The role of these pro-angiogenic integrins in cancer, however, is not yet fully understood, and new molecules with improved selectivity profiles (i.e., targeting only one integrin subtype) would represent very promising tools to study the specific function of integrins in malignant angiogenesis and for selective tumor imaging applications.[35]

The receptor selectivity and activity of Cilengitide and related RGD-ligands has been shown to be dependent on backbone conformation and the relative populations of active conformers.[36,37] Aza-amino acids have already been successfully employed in the synthesis of linear RGD-ligands, which exhibited modifications in their receptor binding profiles.[38] In an earlier communication, replacement of aza-glycine for glycine in cyclic aza-analogues of Cilengitide and *cyclo*(R-G-D-f-V) provided analogues with nanomolar activity and varying selectivity for the $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, and $\alpha IIb\beta 3$ integrin receptors.[29] Considering these earlier reports, a more detailed aza-residue scan of cyclic peptides **1** and **2** was merited to further study influences of aza-substitution on conformation, activity, and selectivity.

Insight is now given for the design of cyclic aza-peptides in general. Moreover, seven aza-analogues of cyclic peptides **1** and **2** were prepared possessing aza-aspartic acid (azaD), aza-glycine (azaG), aza-phenylalanine (azaF), and aza-valine (azaV) residues. Aza-arginine (azaR) analogues were not included in the scope of this study. Two additional diastereomeric analogues were isolated, resulting from epimerization of the C-terminal residue during the cyclization step. In addition, biological activity is presented for the cyclic aza-peptides toward the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrin receptors.

RESULTS AND DISCUSSION

Peptide Design. The strategy for the synthesis of the cyclic aza-peptides was based on the established protocols for the preparation of their cyclic integrin ligand counterparts.[39] Side-chain-protected linear aza-peptides were assembled on an acid-labile solid support using the Fmoc/^{t}Bu-strategy for solid-phase peptide synthesis (SPPS) and cleaved for subsequent cyclization in solution and final removal of side chain protection to yield the cyclic aza-peptides.

Particular synthetic challenges for the preparation of cyclic,[35,40,41] *N*-methylated,[35,42] and aza-peptides[14] were taken into consideration. For example, glycine was usually positioned at the carboxylate terminus to minimize steric hindrance and racemization during cyclization.[32] *N*-Fmoc-*N*-methyl-L-valine was prepared by reduction of the corresponding *N*-Fmoc-oxazolidinone,[43] and its linkage directly to the resin was avoided to prevent premature cleavage due to diketopiperazine formation.[44] At the *N*-terminal position of the linear peptide, *N*-methyl and aza-amino acid residues were usually avoided, because their amines are generally less reactive than those of conventional amino acids and necessitate the use of demanding coupling conditions.

Aza-peptide Synthesis. In an initial attempt to circumvent the issues of solution-phase hydrazine chemistry, we introduced the aza-residue into the resin-bound linear peptide using a submonomer approach (Scheme 1), which has been successful for the synthesis of various aza-derivatives of biologically active linear peptides.[25,27] Benzylidene carbazate **4**, which was generated *in situ* by reaction of benzaldehyde hydrazone **3**

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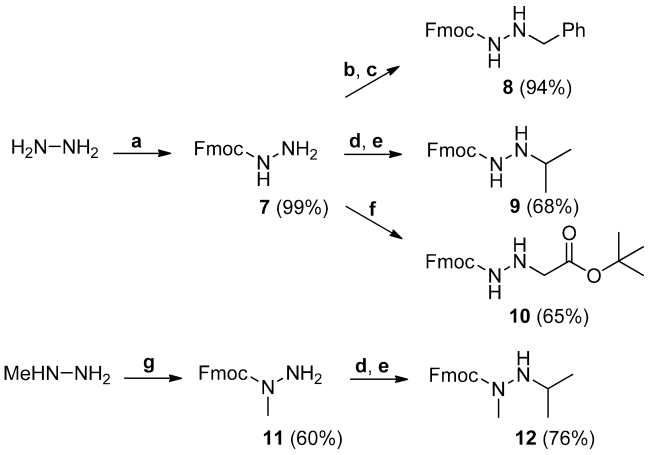
with *p*-nitrophenyl chloroformate, was linked to a resin-bound peptide to yield semicarbazone protected aza-glycine **5**. Alkylations with benzyl bromide, isopropyl iodine, and *tert*-butyl bromoacetate were used to introduce the side chains of the azaF (**6**), azaV, and azaD residues, respectively. Semicarbazone deprotection caused, however, concomitant cleavage of the aza-peptide from the acid-labile 2-chlorotrityl chloride (CTC) solid support (Scheme 1). Effective conditions for removing semicarbazones on rink-amide resin,[25] such as hydroxylamine hydrochloride in pyridine, caused aza-peptide cleavage from the CTC resin. In the face of several failed efforts to surmount this obstacle, the submonomer approach on CTC resin was abandoned.

N-Fmoc-aza-amino acid chlorides were next employed to introduce the aza-residue onto the resin-bound peptide. *N*′-Alkyl-fluoren-9-ylmethyl carbazate precursors were thus synthesized in solution and subsequently reacted with phosgene prior to coupling onto the solid-supported peptide sequence (Scheme 2).[18,38] The appropriate carbazate building-blocks for

Scheme 2. Synthesis of Aza-amino Acid Precursors *N*-Fluoren-9-ylmethyl Carbazates 8−10 and *N*-Fluoren-9-ylmethyl-*N*-methyl Carbazate 12[a]



[a]Reagents and conditions: (a) Fmoc-Cl, acetonitrile/water, 0 °C → rt, 14 h; (b) benzaldehyde, EtOH, at reflux, 2 h; (c) H_2 (100 psi), $Pd(OH)_2/C$, THF, rt, 14 h; (d) acetone, at reflux, 1 h; (e) $NaBH_3CN$, AcOH, THF, rt, 1 h; EtOH, at reflux, 3 h; (f) *tert*-butyl bromoacetate, K_2CO_3, DMF, rt, 20 h; (g) Fmoc-Cl, NEt_3, DCM, −78 °C to rt, 16 h.

azaG, azaF and azaV were obtained as described before.[18] Acylation of excess hydrazine with fluoren-9-ylmethyl chloroformate afforded the azaG precursor, fluoren-9-ylmethyl carbazate **7**, in quantitative yield. Condensation of **7** with benzaldehyde and acetone, respectively, and reduction of the resulting Fmoc-hydrazones yielded the azaF and azaV precursors, *N*′-benzyl- and *N*′-isopropyl-fluoren-9-ylmethyl carbazates **8** and **9**.[18] Although the azaD precursor, *N*′-*tert*-butyl-acetate-fluoren-9-ylmethyl carbazate **10**, was previously prepared by a multiple-step synthesis from an aldehyde precursor,[18] a more expedient route was explored based on the alkylation of **7** with *tert*-butyl bromoacetate.[23,45] Employing **7**, *tert*-butyl bromoacetate, and K_2CO_3 in DMF, a mixture of mono- and bisalkylated carbazates were obtained, from which the former **10** was isolated in 65% yield. In the synthesis of the *N*-methyl-aza-valine precursor, *N*′-isopropyl-*N*-fluoren-9-ylmethyl-*N*-methyl carbazate **12**, the differential reactivity of *N*-methylhydrazine was exploited during acylation with Fmoc-chloride in the presence of triethylamine to afford *N*-Fmoc-*N*-methylcarbazate **11**.[38] Condensation of **11** with acetone gave the corresponding hydrazone, which was reduced using sodium cyanoborohydride to afford *N*′-isopropyl-*N*-fluoren-9-ylmethyl-*N*-methyl carbazate **12**.

Aza-amino acid residues were coupled onto the resin-supported peptide after activation of the appropriate Fmoc-*N*′-alkyl carbazate with phosgene. Coupling reactions were performed overnight and repeated twice in the case of azaG[46] to ensure full conversion as monitored by HPLC−MS analysis.[18]

Although couplings of aza-residues on primary amines were generally efficient, LC−MS analysis revealed that acylation of *N*-methyl-L-valine with Fmoc-aza-phenylalanine acid chloride gave only ca. 15% conversion after two couplings. In order to enhance coupling yield, microwave irradiation was investigated, because it had previously been demonstrated to minimize reaction times and improve yields in SPPS.[47−49] Furthermore, microwave irradiation has recently been applied to enhance coupling of activated aza-amino acids.[24] A higher amount of base was employed to prevent potential cleavage from the acid-sensitive CTC-resin due to *in situ* generated HCl, without fear of racemization during coupling of the achiral Fmoc-aza-amino acid. Although coupling times could be reduced significantly, conversions remained modest. Under our best conditions, a 56% overall conversion was achieved using three consecutive coupling reactions of a 5-fold excess of activated Fmoc-aza-amino acid in DMF at 60 °C with microwave irradiation. Unreacted *N*-methyl-L-valinyl peptide was capped with acetic anhydride, and SPPS was continued.

Amino groups of aza-amino acid residues are typically less reactive than those of natural amino acids, requiring more vigorous coupling conditions and double couplings.[14] Coupling to aza-glycine was achieved using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt). Coupling to more bulky aza-residues required bis(trichloromethyl)carbonate (BTC) as a more reactive activating reagent.[18,19,24] More basic conditions were needed to prevent peptide cleavage from the acid-labile CTC resin during BTC couplings.[50,51] Furthermore, additional care was required during activation of the Fmoc-amino acid, and treatment of the BTC reaction with 2,4,6-collidine was performed slowly at 0 °C to avoid undesired side products with molecular ions corresponding to the product mass +26.

In general, the BTC protocol provided clean couplings and complete conversion in the acylation of the aza-amino-residue, except for *N*-methyl-aza-valine. The latter case gave low conversion with <8% of the Fmoc-phenylalanine coupled product, even after two treatments with extended reaction times. The combination of an aza-residue and *N*-methylation was expected to require more forcing coupling conditions, due to difficulties encountered with each moiety in individual coupling experiments. Attempts failed to effect coupling onto *N*-methyl-aza-valine using other reagents, such as PyBOP[52] and BOP-Cl,[53] after extended reaction times or microwave irradiation using excess reactant. Moreover, acetylation of the *N*-methyl-aza-valine residue with a mixture of acetic anhydride and DIEA in DMF resulted in only 15% conversion, confirming its poor reactivity. To circumvent this issue, the synthesis of a dipeptide building block in solution and subsequent coupling to the resin-bound peptide may be necessary; however, such an approach was beyond the scope of this work.

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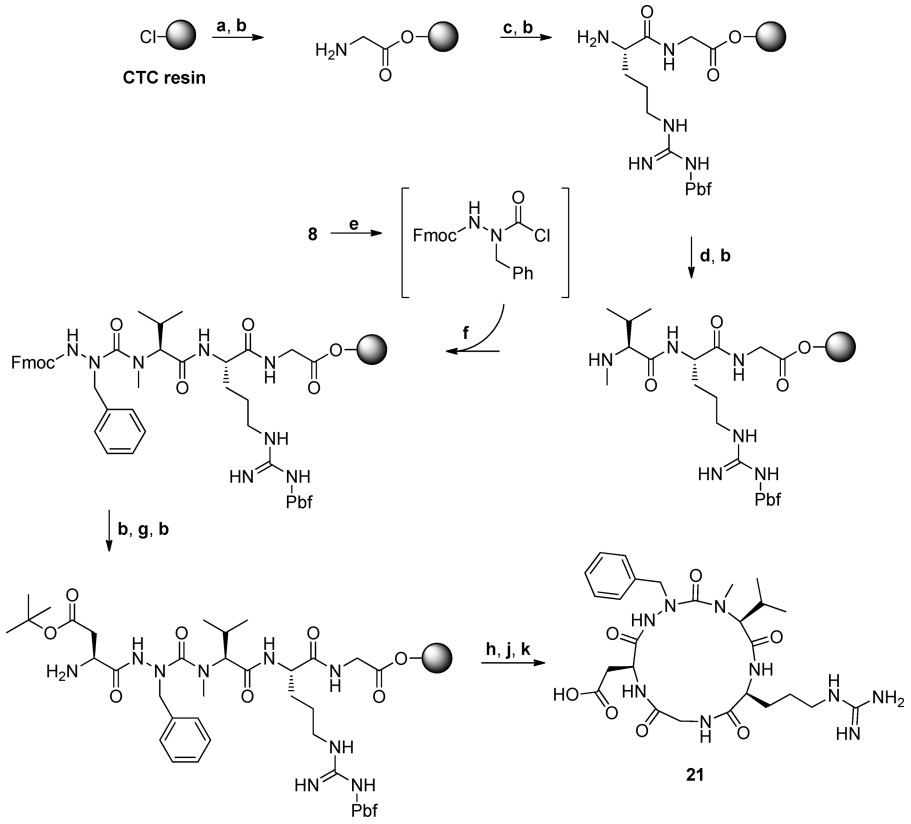
Scheme 3. Synthesis of Cyclic Aza-peptide 21[a]



[a]Reagents and conditions: (a) Fmoc-Gly, DIEA, DCM; (b) piperidine in DMF (20%, v/v); (c) Fmoc-Arg(Pbf), HBTU, HOBt, DIEA, DMF; (d) Fmoc-*N*Me-Val, HBTU, HOBt, DIEA, DMF; (e) phosgene in toluene (20 wt %), DCM, 0 °C; (f) DIEA, DMF, microwave irradiation; (g) Fmoc-Asp(O^{t}Bu), BTC, 2,4,6-collidine, 0 °C to rt; DIEA, THF, rt; (h) TFA in DCM (1%); (j) HATU, HOAt, 2,4,6-collidine, DMF; (k) TFA/TES/H_2O/DCM (60/2.5/2.5/35, v/v/v/v).

Table 1. Synthesized Aza-peptides

compd	structure	crude purity[a]		final peptide[d]		HRMS	
		linear peptide[b]	cyclic peptide[c]	yield[e]	purity[a]	calcd.	found
13	*cyclo*[R-azaG-D-f-V]	68%	56%	4.2%	>95%	576.2889	576.2890
14	*cyclo*[R-G-azaD-f-V]	78%	54%	4.7%	>96%	576.2889	576.2884
15	*cyclo*[R-G-azaD-f-v][f]	–	23%	3.9%	>96%	576.2889	576.2884
16	*cyclo*[R-G-D-azaF-V]	58%	61%	3.2%	>98%	576.2889	576.2898
17	*cyclo*[R-G-D-f-azaV]	65%	72%	4.2%	>99%	576.2889	576.2902
18	*cyclo*[R-azaG-D-f-*N*(Me)V]	72%	38%	2.7%	>99%	590.3045	590.3050
19	*cyclo*[R-G-azaD-f-*N*(Me)V]	73%	15%	4.4%	>99%[g]	590.3045	590.3059
20	*cyclo*[R-G-azaD-f-*N*(Me)v][f]	–	8%	3.0%	>97%	590.3045	590.3058
21	*cyclo*[R-G-D-azaF-*N*(Me)V]	25%	11%	1.1%	>88%	590.3045	590.3044

[a]Peptide purity ascertained by UV absorption at λ = 214 nm; see Experimental Section. [b]After cleavage from resin. [c]After cyclization and side chain deprotection. [d]After HPLC purification; see Experimental Section. [e]Yields are calculated on the basis of resin loading. [f]Product isolated as a diastereomeric product after cyclization. [g]After HPLC purification, the LC trace of the product exhibited inseparable overlapping peaks that were attributed to interconverting conformers.

Cyclization and Deprotection. Although incorporation of turn-inducing residues, such as *N*-methyl-, D-, and aza-amino acids into the linear sequence may be expected to facilitate cyclization due to backbone preorganization,[40,41] the cyclization step proved another challenging point of the synthesis. After cleavage of the linear, side-chain-protected peptides from the solid support, formation of the macrocycle was performed at high dilution via *in situ* activation using diphenyl phosphorazide (DPPA) and $NaHCO_3$.[54] Some of the peptides required extended reaction times, up to 5 days, as well as employment of more powerful activation conditions such as HATU, HOAt, and 2,4,6-collidine, to ensure full conversion. Linear peptides possessing valine (i.e, **14** and **15**) as well as *N*-methyl-valine (i.e., **19** and **20**), respectively, at the carboxylate terminus, epimerized during the cyclization step, providing diastereomeric mixtures, despite the use of the relatively mild DPPA conditions (Table 3). Moreover, conditions for the removal of side chain protection, such as reaction time and TFA concentration, needed to be optimized to avoid ring opening by hydrolysis. As previously observed employing multiple *N*-methylations of Cilengitide,[35] the incorporation of aza-residues may enhance backbone rigidity favoring conformers, which may inhibit head-to-tail cyclization, due in part to added ring strain.

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Table 2. Integrin Binding Affinity

compd	Sequence	IC_{50} αvβ3 [nM]	IC_{50} α5β1 [nM]	α5β1/ αvβ3
1	*cyclo*[R-G-D-f-*N*(Me)V]	1.7 ± 0.3	28 ± 1	17
13	*cyclo*[R-azaG-D-f-V]	5.7 ± 0.4	255 ± 9	45
14	*cyclo*[R-G-azaD-f-V]	> 1000	n.d.[a]	n.d.
15	*cyclo*[R-G-azaD-f-v]	> 1000	n.d.	n.d.
16	*cyclo*[R-G-D-azaF-V]	> 1000	n.d.	n.d.
17	*cyclo*[R-G-D-f-azaV]	53 ± 7	n.d.	n.d.
18	*cyclo*[R-azaG-D-f-*N*(Me)V]	18 ± 2	180 ± 15	10
19	*cyclo*[R-G-azaD-f-*N*(Me)V]	> 10000	n.d.	n.d.
20	*cyclo*[R-G-azaD-f-*N*(Me)v]	> 10000	n.d.	n.d.
21	*cyclo*[R-G-D-azaF-*N*(Me)V]	> 2000	n.d.	n.d.

[a]n.d. = not determined.

The overall process for synthesizing a cyclic aza-peptide is summarized for the preparation of analogue **21** (Scheme 3). Yields, purities and HRMS characterization of all aza-peptides are summarized in Table 1.

Biology. The affinity of the cyclic aza-peptide analogues for the $\alpha v\beta 3$ integrin receptor was evaluated using a competitive solid-phase binding assay (see Experimental section for details). From the series, only the nonmethylated azaG derivative (peptide **13**) retained the antagonistic activity for $\alpha v\beta 3$ in the low nanomolar range (Table 2). Peptide **18**, which contains the azaG moiety and *N*-methyl-Val, also showed $\alpha v\beta 3$-binding activity, albeit 1 order of magnitude lower than that of the control peptide Cilengitide.[29]

Aza-residue substitution at other positions was not tolerated and caused reduced or total loss of affinity for the $\alpha v\beta 3$ integrin receptor. Aza-amino acids may induce conformational changes in the peptide backbone,[14] which distort the bioactive $\alpha v\beta 3$-binding conformation[32,55] causing loss of activity. In this regard, variations in the conformation of cyclic RGD peptides have resulted in dramatic effects in both antagonistic affinity and selectivity profiles for different integrin subtypes.[35]

Selectivity profiles of selected cyclic aza-peptides were also evaluated at the integrin $\alpha 5\beta 1$ receptor. Interest in this integrin has increased due to its crucial role in angiogenesis.[34,56] Peptides **13** and **18** showed a decreased binding-activity for this integrin receptor (Table 2); however, selectivity ratios with respect to $\alpha 5\beta 1$ and $\alpha v\beta 3$ were similar to those found for Cilengitide.[29] Although additional structural studies are needed, these observations may suggest a similar binding geometry for Cilengitide and peptides **13** and **18** on interaction with the integrin receptors.

CONCLUSION

A strategy for the synthesis of cyclic aza-peptides has been developed and employed in the aza-scan of cyclic RGD-peptides. Linear aza-peptides were assembled on solid support using aza-amino acid chloride building blocks, cleaved without the removal of side chain protection and cyclized in solution. The acid sensitive chlorotrityl solid support necessitated the employment of mild reaction conditions for the introduction and modification of the aza-residue. Examining the cyclic pentapeptides Cilengitide (**1**) and *cyclo*(R-G-D-f-V) (**2**), seven cyclic aza-peptides and two diastereomeric aza-analogues were prepared and evaluated for biological activity. Decreased affinities for the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrin receptors were generally observed and were consistent with previous findings demonstrating the importance of the backbone geometry for the biological activity of **1**. Only the aza-glycine analogues retained nanomolar activity, and relative to the other aza-analogues, *cyclo*[R-azaG-D-f-V] (**13**) exhibited better receptor selectivity, albeit with decreased affinity compared to Cilengitide. A more detailed structural analysis is necessary to elucidate the reason for the limited tolerance of **1** and **2** to the incorporation of aza-residues. The reported strategy for the synthesis of cyclic aza-peptides has surmounted considerable synthetic challenges and offers thus significant potential for application in the study of other biologically active cyclic peptides.

EXPERIMENTAL SECTION

Hazards. Phosgene solution and triphosgene (BTC) are both highly toxic and may cause death by inhalation. These substances should be handled in a well-ventilated hood with extreme caution.

Chemistry. Fmoc-*N*-methyl valine,[43] Fmoc-carbazates **7**, **8**, and **9**,[18] and *N*-Fmoc-*N*-methyl carbazate **11**[38] were synthesized as reported.

N′-1-(tert-Butyloxycarbonylmethylamino)-N-fluoren-9-ylmethyl Carbazate ***10***. To a well-stirred solution of fluoren-9-ylmethyl carbazate **7** (2.29 g, 9.0 mmol, 1.0 equiv) and K_2CO_3 (1.05 g, 9.9 mmol, 1.1 equiv) in dry DMF (36 mL, 0.25 M) was added *tert*-butyl bromoacetate (1.33 mL, 9.0 mmol, 1.0 equiv) dropwise at 0 °C. The mixture was stirred at 0 °C for 5 min, allowed to warm to room temperature, and stirred for another 20 h when dialkylation became significant, as observed by TLC (R_f (monoalkylation) = 0.25, R_f (dialkylation) = 0.58; hexane/EtOAc 1:1). The reaction mixture was diluted with 250 mL of water and extracted with EtOAc (3 × 200 mL). The combined organic layers were washed with water (250 mL) and brine (250 mL), dried over $MgSO_4$, and filtered. The volatiles were removed under reduced pressure, and the residue was purified by flash chromatography[57] (hexane/EtOAc 1:1) on silica gel to yield **10** as a white foam (2.12 g, 6.0 mmol, 65%): R_f = 0.25 (hexane/EtOAc 1:1); mp 84−85 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.75(s, 9H), 3.80 (brs, 2H), 4.42 (m, 2H), 4.69 (d, J = 6.8 Hz, 2H), 7.55 (t, J = 7.4 Hz, 2H), 7.64 (t, J = 7.4 Hz, 2H), 7.83 (d, J = 7.3 Hz, 2H), 8.00 (d, J = 7.5 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.1 (3C), 47.1, 53.4, 67.1, 82.0, 120.0 (2C), 125.0 (2C), 127.0 (2C), 127.7 (2C), 141.3 (2C), 143.7 (2C), 156.8, 170.7. MS (ESI): m/z = 369.2 $[M + H]^+$.

N′-Isopropyl-N-fluoren-9-ylmethyl-N-methyl Carbazate ***12***. A suspension of *N*-Fmoc-*N*-methyl carbazate **11**[38] (2.28 g, 8.5 mmol, 1 equiv) in acetone (25 mL, 340 mmol, 40 equiv) was heated at reflux until complete formation of the hydrazone was observed by TLC [(1:1 hexane/EtOAc), R_f (hydrazone) = 0.28]. The mixture was concentrated under reduced pressure. The hydrazone was dissolved in dry THF (43 mL, 0.2 M), treated with AcOH (0.54 mL, 9.4 mmol, 1.1 equiv) and $NaBH_3CN$ (0.80 g, 12.8 mmol, 1.5 equiv), stirred at room temperature for 2 h, when completion of the reaction was observed by TLC [(1:1 hexane/EtOAc), R_f (hydrazone) = 0.28], and concentrated under reduced pressure to yield a pale yellow oil that was dissolved in EtOAc (100 mL), washed with 1 M aqueous $KHSO_4$ (2 × 100 mL) and brine (100 mL), and dried over $MgSO_4$. The volatiles were removed under reduced pressure, and the residue was dissolved in EtOH (50 mL, 0.2 M) and heated at reflux for 3 h to hydrolyze the aminoborane adduct. The mixture was concentrated *in vacuo* to yield a yellow oil that was isolated by flash chromatography on silica gel (hexane/EtOAc 7:3) to obtain compound **12** as a colorless, viscous oil

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Table 3. Synthesis of Cyclic Aza-peptides

compd	Sequence[a]	Introduction of aza-residue[b]	Cycl. method[c] (reaction time)	Depr. cond.[d]
13	*cyclo*[R-azaG-D-f-V]	A	A (12 h)	A
14	*cyclo*[R-G-azaD-f-V]	A	A (24 h)	A
15	*cyclo*[R-G-azaD-f-v][e]	A	A (24 h)	A
16	*cyclo*[R-G-D-azaF-V]	A	B (12 h)	A
17	*cyclo*[R-G-D-f-azaV]	A	A (12 h)	A
18	*cyclo*[R-azaG-D-f-*N*(Me)V]	A	B (3 d)	B
19	*cyclo*[R-G-azaD-f-*N*(Me)V]	A	A (5 d)	B
20	*cyclo*[R-G-azaD-f-*N*(Me)v][e]	A	A (5 d)	B
21	*cyclo*[R-G-D-azaF-*N*(Me)V]	B	B (12 h)	A

[a]The amino acid loaded to the resin is highlighted in gray. [b]Conditions A and B for the introduction of the aza-residue were as described in the text. [c]The cyclization methods A and B were as described in the text. [d]Deprotection conditions A and B were as described in the text. [e]Product was isolated as a side product after cylization.

(2.20 g, 6.6 mmol, 76%). R_f = 0.63 (hexane/EtOAc 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.02 (m, 6H), 3.09 (brs, 3H), 3.26 (brs, 1H), 4.25 (t, J = 4.9 Hz, 1H), 4.46 (brs, 1H), 4.65 (brs, 1H), 7.32 (td, J = 7.4 Hz, J = 1.3 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.59 (d, J = 7.5 Hz, 2H), 7.77 (d, J = 7.5 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.8 (2C), 39.2, 47.4, 50.1, 67.6, 120.0 (2C), 124.9 (2C), 127.1 (2C), 127.8 (2C), 141.5(2C), 144.0 (2C), 157.4. MS (ESI): m/z = 311.0 $[M + H]^+$, 333.0 $[M + Na]^+$. HRMS (ESI): calcd m/z for $C_{19}H_{22}N_2O_2$ $[M + H]^+$ = 311.17540, found 311.17682.

General Procedures for Peptide Synthesis. Peptides were prepared by Fmoc SPPS methods in an automated shaker using 2-chlorotrityl chloride resin[58] (manufacturer's reported loading 0.8 mmol/g). The resin was swollen in dry DCM, treated with the first amino acid (1.2 equiv) and DIEA (3.5 equiv), and shaken for 1 h. The loaded resin was washed with DCM (3×) and DMF (3×). Fmoc deprotection was achieved by treatment with a 20% solution of piperidine in DMF (1 × 10 min, 1 × 15 min). Couplings of Fmoc amino acids (3 equiv) were performed in DMF using HBTU (3 equiv) and HOBt (3 equiv) as coupling reagent and DIEA (3 equiv) as base (usually for 2 h). Coupling to *N*-methyl valine was achieved by treatment with Fmoc amino acid (3 equiv), DIEA (6 equiv), HATU (3 equiv), and HOAt (3 equiv) (usually for 1 h). The resin was washed after each coupling and deprotection step with alternatively DMF (3×), DCM (3×), and DMF (3×). Complete coupling reactions were confirmed by LC−MS monitoring. In case of unreacted starting material, coupling reactions were repeated, and remaining free amino groups were capped by treatment with a solution of acetic anhydride and DIEA in DMF (10/20/70, v/v/v) (1 × 10 min and 1 × 15 min) if necessary. Finally, the resin was washed with DMF (3×), DCM (3×), and DMF (3×).

Introduction of Aza-residues. Fmoc-aza-amino acid chlorides were prepared and coupled to the solid-supported peptides as previously described.[18] Briefly, a solution of phosgene in toluene (20 wt %, 2 equiv) was added dropwise to a stirred 0.1 M solution of *N*′-alkyl Fmoc-carbazate **7**−**10** or *N*′-isopropyl-*N*-Fmoc-*N*-methyl carbazate **12** (1 equiv) in dry DCM under argon at 0 °C. The mixture was stirred at 0 °C for 5 min and then allowed to warm up to room temperature. After TLC indicated complete consumption of starting material (usually after 30 min), the reaction mixture was concentrated under reduced pressure to yield the Fmoc-aza-amino acid chloride. *Coupling method A*: The Fmoc-aza-amino acid chloride (3 equiv relative to resin loading) was dissolved in dry DCM (0.15 M), treated with DIEA (6 equiv), stirred for 1−2 min, and transferred to the resin swollen in dry DCM. The resin mixture was shaken overnight at room temperature. To ensure full conversion, the resin was washed with dry DCM (3×) and treated under the same coupling conditions. Finally, the resin was washed with DCM (3×) and DMF (3×). *Coupling method B*: The resin was transferred into a glass microwave reaction vessel equipped with a magnetic stir bar and swollen in dry DMF. After the addition of DIEA (15 equiv) followed by a solution of Fmoc-aza-amino acid chloride (5 equiv) in dry DMF (0.3 M), the vessel was sealed with a septum cap and irradiated in the microwave at constant-temperature mode at 60 °C for 30 min. Resin was washed with dry DMF, and coupling was repeated twice. Finally, the resin was washed with DMF (3×), DCM (3×), and DMF (3×). Unreacted starting material was capped by treatment with a solution of acetic anhydride and DIEA in DMF (10/20/70, v/v/v, 1 × 10 min and 1 × 15 min).

BTC Coupling to the Aza-residue. To a well-stirred solution of Fmoc-amino acid (3 equiv) and BTC (1.15 equiv) in dry THF, 2,4,6-collidine (10 equiv) was slowly added dropwise at 0 °C. The resulting suspension was allowed to warm to room temperature and stirred for another 5 min. The resin was swollen in dry THF, treated with DIEA (8 equiv) followed immediately by the suspension containing the activated Fmoc-amino acid, shaken for 3−4 h, and washed with DMF (3×) and DCM (3×).

Peptide Cleavage and Cyclization. After coupling of the last amino acid and Fmoc-deprotection as described above, the side-chain-protected linear peptides were cleaved off solid support using a solution of 1% TFA in DCM (10 × 2 min). Two different methods were used for the backbone cyclization of the linear peptides. *Method A*: The linear, side-chain-protected peptide was diluted with DMF to 10^{-3} M. After addition of DPPA (3 equiv) and $NaHCO_3$ (5 equiv), the mixture was stirred until LC−MS monitoring indicated complete consumption of the starting material. The solution was concentrated under reduced pressure, and the cyclic peptide was precipitated by dropwise addition into a 1:1 mixture of water/brine (v/v). The peptide was spun down in a centrifuge, washed twice with water, and dried under vacuum. *Method B*: The linear, side-chain-protected peptide was diluted with DMF to 10^{-3} M. After addition of HATU (1.5 equiv), HOAt (1.5 equiv), and collidine (1.5 equiv) the mixture was stirred until LC−MS monitoring indicated complete consumption of starting material. The solution was concentrated under reduced pressure, taken up in saturated aqueous $NaHCO_3$ solution, and extracted with EtOAc (3×). The combined organic layers were washed with brine (1×), dried over Na_2SO_4, and concentrated under reduced pressure.

Side Chain Deprotection. *Condition A:* Cyclic aza-peptides were treated with a mixture of TFA/H_2O/TES/DCM (60/2.5/2.5/35, v/v/v/v) at room temperature until complete removal of side chain protection was indicated by ESI-MS monitoring. *Condition B*: In case the monitoring indicated ring-opening hydrolysis, side chains protection was removed using a mixture of TFA/H_2O/TES/DCM (40/2.5/2.5/55, v/v/v/v) and stirred at room temperature until ESI-MS monitoring indicated that ring-opening hydrolysis became the dominant reaction over removal of side chain protection.

The resulting crude peptide was precipitated into chilled diethyl ether, spun down in a centrifuge, and washed with chilled diethyl ether twice. After final centrifugation the peptide was dissolved in an acetonitrile/water (1:1, v/v) solution and lyophilized. HPLC purification yielded a white powder (Table 3).

Analysis and Purification of Aza-peptides. Analytical LC−MS analyses were performed on a Gemini 5μ C18 110A reverse-phase column (Phenomenex Inc., Torrance, CA, USA, 150 mm × 4.6 mm, 5 μm) with a flow rate of 0.5 mL/min using linear gradients of methanol (0.1% formic acid) in water (0.1% formic acid) or acetonitrile (0.1% formic acid) in water (0.1% formic acid). Purification of the aza-peptides was performed on a semipreparative column (250 mm × 21.2 mm, 5 μm, C18 Gemini column) using specified linear gradients of

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methanol (0.1% formic acid) in water (0.1% formic acid) with a flow rate of 10.6 mL/min.

Competitive Solid-Phase Integrin Binding Assay. The *in vitro* inhibition of integrin−extracellular matrix (ECM) protein binding was measured in a solid-phase binding assay using soluble integrins and coated ECM proteins, following previously described methods.[59] Briefly, for the $\alpha v\beta 3$-binding activity, flat-bottom 96-well plates were coated at 4 °C overnight with vitronectin (1.0 μg/mL). After a blocking step, soluble integrin $\alpha v\beta 3$ (1.0 μg/mL) was next incubated with a serial dilution of integrin inhibitors (10 to 0.0032 μM) for 1 h at room temperature. Then, primary (CD51/CD61, 2.0 μg/mL) and secondary antibodies (anti-mouse-horseradish peroxidase (HRP) conjugate, 1.0 μg/mL) were applied each for 1 h at room temperature, respectively. The detection of HRP was performed using a HRP substrate (3.3.5.5′-tetramethylethylenediamine, TMB), adding 2 M H_2SO_4 to stop the reaction. The absorbance of each well was recorded at 450 nm with a POLARstar Galaxy plate reader. Every concentration was analyzed in duplicate, and the resulting data points were fitted to a sigmoidal curve using OriginPro 7.5G software. The turning point describes the IC_{50} value. Alternatively, for $\alpha 5\beta 1$, the plates were coated with fibronectin (0.50 μg/mL), and the inhibitors were incubated with soluble $\alpha 5\beta 1$ (1.0 μg/mL). After treatment with primary (CD49e, 1.0 μg/mL) and secondary (anti-mouse-HRP, 2.0 μg/mL) antibodies, the binding was visualized as described above. In all assays, each plate contained Cilengitide[32,34] as internal control.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Characterization data. 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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