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Solution state conformation and degradation of cyclopeptides containing an NGR motif

ANNA K. FÜZÉRY,[a] NIKOLETT MIHALA,[b] PÁL SZABÓ,[c] ANDRÁS PERCZEL,[a] RAFFAELLA GIAVAZZI[d] and HELGA SÜLI-VARGHA[b]*

[a] Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary
[b] Research Group for Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Budapest, Hungary
[c] Department of Mass Spectrometry, Chemical Research Center of the Hungarian Academy of Sciences, Budapest, Hungary
[d] Department of Oncology, Mario Negri Institute for Pharmacological Research, Bergamo, Italy

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Abstract: In contrast to the RGD-peptides, head to tail cyclization of LNGRV and LNGRv caused only a marginal change in their integrin receptor affinity as shown by the limited effect and selectivity on the adhesion of endothelial cells to ECM components. Structure determination of the two cyclopeptides by NMR and MD, semiempirical and *ab initio* methods revealed that both are very flexible and take on multiple stable conformers in solution. This structural diversity, along with the presence of the Asn-Gly peptide bond, enhances succinimide ring formation leading to the hydrolysis of Asn. It has been demonstrated that c(LNGRV) suffers deamidation with time both in solution and during storage. As the isoaspartyl-peptide may co-elute with the asparginyl-peptide in the course of HPLC analysis, MS measurement is necessary to check the purity of peptides containing the NGR sequence. Our stability investigations raise the question whether the NGR motif or its hydrolysis product is effective in *in vivo* experiments.

Keywords: NGR; cyclopeptide; integrin receptor; adhesion; NMR-conformation; MD and *ab initio* calculation; deamidation

INTRODUCTION

Integrins are $\alpha\beta$ heterodimeric transmembrane glycoproteins, which mediate cell–cell and cell–extracellular matrix (ECM) interactions. Presently about 30 members of the integrin receptor family are known and they are classified on the basis of their β chain. The ligands of these receptors, such as fibronectin, laminin, collagen and vitronectin, are components of the ECM and their main binding region contains an RGD tripeptide fragment as was first found for fibronectin in 1984 [1]. Structure–activity relationship (SAR) investigations soon revealed that rather than the character of the amino acids preceeding and following the RGD sequence, the secondary structure of the peptide containing the RGD sequence, determines receptor selectivity. For example, the distance between the two pharmacophores — the β-carboxyl group in the aspartic acid and the guanidino side chain in the arginine — determines receptor selectivity in the β_3 subgroup of integrins [2].

Along with SAR investigations of peptides containing the RGD sequence, a search for other integrin receptor ligands has also been launched. For this purpose phage-display peptide libraries [3] can be applied for both *in vitro* and *in vivo* testing. Using this method further linear and cyclic peptides containing RGD and non-RGD sequences have been found. From the non-RGD cell adhesion sequences the NGR motif was frequently identified in linear peptides — NGRAHA [4] — and in several cyclopeptides it was flanked by cysteine residues ($CysX_nCys$) [5]. The NGR sequence also occurs in fibronectin, and since it is found in its cell-binding region, it may contribute to this ligand's specific recognition by the classical $\alpha_5\beta_1$ integrin receptor. This receptor plays a crucial role in cell migration and consequently in tumour invasion and metastasis formation [6,7]. Although the linear NGR peptide has affinity for the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ receptors three orders of magnitude (IC_{50} 10^{-5}) lower than the linear RGD peptide, the cyclic CVLNGRMEC possesses a markedly increased affinity (IC_{50} 10^{-7}) toward $\alpha_5\beta_1$. This seems to indicate that by decreasing the flexibility of the peptide with a disulfide bond, its $\alpha_5\beta_1$ receptor selectivity increases.

Therefore it was hypothesized that by further constraining the structure of the ligand containing the NGR sequence greater receptor selectivity may be achieved similar to that observed for RGD peptides. The synthesis and secondary structural investigations of c(LNGRV) and c(LNGRv) were performed, the latter having a D-valine incorporated to promote the formation of a β-turn conformation. As a reference system, the linear sequence Ac-LNGRV-OH and the widely studied RGD-peptides, Ac-VRGDf-NH_2 and c(VRGDf),

Abbreviations: A lower case letter in a sequence defined by the one letter code indicates a D-residue. Head to tail cyclized sequences are indicated, e.g. c(LNGRv).

* Correspondence to: Helga Süli-Vargha, H-1518 Budapest 112, PO Box 32, Hungary; e-mail: suline@para.chem.elte.hu

as well as a cyclopeptide containing the retro RGD sequence c(FDGRV) [2] were prepared. Since there was a possibility of Asn → Asp hydrolysis, i.e. the transformation of NGR to DGR (the latter sequence can be regarded as retro RGD), the synthesis of c(LDGRV) was also completed.

MATERIALS AND METHODS

Chemicals and Equipment

Fmoc-amino acids were obtained from Novabiochem and Boc-amino acids were purchased from Reanal (Budapest, Hungary). Analytical reverse phase HPLC was performed on a Knauer model using a YMC-Pak ODS C18, 120 Å, 5 μm, (4.6 mm × 150 mm) column at a flow rate of 1 ml/min. Preparative reverse phase HPLC was performed on a Pharmazia instrument using a Waters Delta Pak C18, 300 Å, 15 μm, (19 × 300 mm) column at a flow rate of 20 ml/min. Detection in both cases was at 220 nm. HPLC eluents (if not indicated) were A: 0.1% TFA/water and B: 0.07% TFA/acetonitrile. The MS measurements were run on an Applied Biosystems/MDS Sciex API 2000 triple quadrupole instrument using a TurboIonspray source.

Peptide Synthesis

The linear Ac-LNGRV-OH **I** was prepared by Fmoc-technique on 2-chlorotritylchloride resin and Ac-VRGDf-NH_2 **V** by Boc-technique on benzhydrylamine resin, using the HBTU/HOBt/DIEA coupling method. The protected linear precursor peptides for cyclization were also synthesized with the latter strategy and were cleaved from the resin with a dichloromethane–methanol–acetic acid (8 : 1 : 1) mixture. Cyclization of H-Arg(Pbf)-Val-Leu-Asn(Trt)-Gly-OH, H-Arg(Pbf)-*D*-Val-Leu-Asn(Trt)-Gly-OH, H-Arg(Pbf)-Val-Phe-Asp(OBu^t)-Gly-OH, H-Asp(OBu^t)-*D*-Phe-Val-Arg(Pbf)-Gly-OH and H-Arg(Pbf)-Val-Leu-Asp(OBu^t)-Gly-OH hydrochloride salts was carried out in DMF using 3 eq. BOP, 3 eq. HOBt and 6 eq. DIEA. The side chain protecting groups were removed by TFA/H_2O/triisopropylsilane (95 : 2.5 : 2.5). The crude cyclopeptides c(LNGRV) **II**, c(LNGRv) **III**, c(FDGRV) **IV** c(VRGDf) **VI** and c(LDGRV) **VII** were purified on a C18 semipreparative HPLC column. All peptides were identified by MS and their homogeneity was assessed by analytical HPLC.

Adhesion Assay

Non-tissue-culture 96-well plates were coated with 625 ng/cm^2 fibronectin or vitronectin or 1% gelatin for 16 h at 4 °C. After washes, non-specific sites were blocked with 1% BSA in phosphate buffer solution (30 min at 37 °C). Human umbilical vein endothelial cells (HUVEC) were added (2.5×10^4/well), in the presence of serial dilutions of the peptides, and incubated at 37 °C for 90 min. After washing to remove non-adherent cells, the attached cells were fixed and stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The stain was eluted with a 1 : 1 solution of ethanol:0.1 M sodium citrate and absorbance at 540 nm was measured with a Multiscan MC. Data are presented as the percentage of control adhesion (cells exposed to vehicle).

NMR Measurements

The NMR solution structures of c(LNGRV) and c(LNGRv) in water (H_2O : D_2O/95 : 5) were determined by the analysis of ^{1}H-^{1}H-TOCSY (45-65ms spin-lock time) and ^{1}H-^{1}H-NOESY (200–400 ms mixing time) spectra. Structure calculations and refinement were carried out using XPLOR (online) Version 3.851 [8,9]. $^3J_{HN\alpha}$ coupling constants (involving the backbone amide and alpha hydrogens) were determined from the TOCSY spectra and their corresponding ϕ dihedral angles were calculated according to:

$$^3J_{HN\alpha} = 6.4\cos^2(f - 60) - 1.4\cos(f - 60) + 1.9$$

for L-residues[10]

$$^3J_{HN\alpha} = 6.4\cos^2(f + 60) - 1.4\cos(f + 60) + 1.9$$

for D-residues

These data were not used in the XPLOR calculations, rather they were used later to validate the molecular dynamics, semiempirical and *ab initio* structures.

MD, Semiempirical and *ab initio* Calculations

For both c(LNGRV) and c(LNGRv) 15 of the XPLOR structures were randomly chosen as starting geometries for simulated annealing runs using GROMACS 3.0.5 [11,12]. Each structure was energy minimized using a conjugate gradient algorithm, then subjected to 1 ns dynamics at 1000K with a 0.1 ps coupling to a temperature bath, followed by a 1 ns slow cooling from 1000K to 0K with a 0.1 ps coupling to the temperature bath. The resulting structures were subjected to geometry optimization using semiempirical (AM1) and *ab initio* (RHF/3-21G) methods. Single point energy calculations were carried out on the converged structures using the B3LYP density functional method in combination with the 6–31+G(d) basis set. All semiempirical and *ab initio* calculations were carried out using the Gaussian 98 [13] package.

Decomposition Experiments

1 mM solutions of peptides **II** and **III**, respectively, in 0.1 M pH 7.4 ammonium acetate buffer solution were left to stand at room temperature. At intervals samples were taken and injected onto an analytical HPLC column (conditions see above).

MS Measurements

LCMS measurements were run on an Applied Biosystems/MDS Sciex API-2000 tandem mass spectrometer equipped with a TurboIonspray source. The eluents were delivered by two Perkin Elmer 200 micro HPLC pumps. The samples were separated on a Vydac 218TP52 reversed-phase column (2.1 mm × 250 mm, 5 μm particle size). The mobile phase composition started off as 99% A (0.1% TFA in water) for 2 min, followed by a linear gradient of B (0.07% TFA in acetonitrile) to 40% in 30 min, held at 40% B for 1 min and then gradually changed back to the original composition over 5 min. The flow rate of the eluents was 400 μl/min. The source conditions were: ionization potential: 5000 V, curtain gas: 40 psi, nebulizer gas: 50 psi, aux gas: 60 psi, source temperature:

350 °C, declustering potential: 35 V, focusing potential: 280 V. The mass spectrometer was scanned in the range 500–600 with a step size of 0.1 amu and a scan time of 1 s and 10 μl of sample was injected using the built-in injector of the mass spectrometer. Analyst 1.4 software was used for instrument control and data processing.

RESULTS AND DISCUSSION

Synthesis of the Peptides

All linear peptides were synthesized manually on solid phase, Ac-LNGRV-OH **I** was prepared by Fmoc-technique on 2-chlorotritylchloride resin and Ac-VRGDf-NH_2 **V** by Boc-technique on benzhydrylamine resin. Protected linear peptides for cyclization were also synthesized by Fmoc-technique on 2-chlorotritylchloride resin. Cyclization of H-Arg(Pbf)-Val-Leu-Asn(Trt)-Gly-OH, H-Arg(Pbf)-D-Val-Leu-Asn(Trt)-Gly-OH, H-Arg(Pbf)-Val-Phe-Asp(OBu^t)-Gly-OH, H-Asp(OBu^t)-D-Phe-Val-Arg(Pbf)-Gly-OH and H-Arg(Pbf)-Val-Leu-Asp(OBu^t)-Gly-OH was carried out in solution, followed by the removal of the protecting groups. This resulted in cyclopeptides c(LNGRV) **II**, c(LNGRv) **III**, c(FDGRV) **IV** c(VRGDf) **VI** and c(LDGRV) **VII**, respectively.

Cell Adhesion Properties of the Peptides

The inhibition of the adhesion of human umbilical vascular endothelial cells (HUVEC) to ECM components by the peptides **I**–**VI** was used as a biological assay of their activity (Table 1). As expected [14], the peptides containing the RGD sequence (**V** and **VI**) prevented cell adhesion to vitronectin, with the cyclic peptide being more active (causing up to 93% inhibition of cell adhesion). The retro RGD sequence in cyclopeptide **IV** had a low ability to prevent HUVEC adhesion to vitronectin. As reported [4] the tested concentrations of the linear NGR-containing peptide **I** caused only a marginal reduction of cell adhesion to fibronectin and vitronectin. Cyclization of the linear peptides containing the NGR sequence (**II, III**) caused no significant change in the inhibiton of the adhesion of HUVEC to fibronectin, while **III** showed a marginal increase in the HUVEC adhesion inhibition to vitronectin. Neither receptor selectivity seemed to change significantly, only analogue **II** showed a slightly increased affinity toward fibronectin versus vitronectin.

Structure Determination of c(LNGRv)

A set of 25 nOes were obtained for c(LNGRv) from the measured NOESY spectrum (Table 2). Of these, ~84% were intraresidual and the remaining 16% sequential; no long range nOes were observed. Using these as constraints XPLOR calculations yielded a set

Table 1 Inhibition of HUVEC Adhesion to ECM Components by the Examined Peptides[a]

Peptide		IC_{50} (μM)	Maximal adhesion inhibition (%)		
			Fibronectin	Vitronectin	Gelatin
Ac-LNGRV-OH	**I**	—	13	8	nt
c(LNGRV)	**II**	—	20.5	6	22
c(LNGRv)	**III**	—	18.5	20.5	12
c(FDGRV)	**IV**	—	nt	16	Nt
Ac-VRGDf-NH_2	**V**	—	nt	27	Nt
c(VRGDf)	**VI**	25	nt	93	Nt

[a] Peptides were tested at concentrations ranging from 0.1 to 100 μM. Results are the mean value of data from at least two experiments.

Table 2 Unambiguously Assignable nOes for c(LNGRv)

Atom 1[a]	Atom 2[a]	Atom 1[a]	Atom 2[a]
Leu Hα	Leu Hβ1/Hβ2	Arg HN	Gly Hα1/Hα2
Leu Hα	Leu Hγ	Arg Hα	Arg Hβ1/Hβ2
Asn Hδ21/Hδ22	Asn Hα	Arg Hδ1/Hδ2	Arg Hβ1/Hβ2
Asn Hα	Asn Hβ1	Arg Hδ1/Hδ2	Arg Hγ1/Hγ2
Asn Hα	Asn Hβ2	Arg Hε	Arg Hβ1/Hβ2
Asn Hδ21/Hδ22	Asn Hβ1	Arg Hε	Arg Hγ1/Hγ2
Asn Hδ21/Hδ22	Asn Hβ2	Arg Hε	Arg Hδ1/Hδ2
Asn HN	Asn Hα	Arg HN	Arg Hα
Asn HN	Asn Hβ1/Hβ2	Arg HN	Arg Hβ1/Hβ2
Asn HN	Leu Hα	Val(D) Hβ	Leu HN
Asn HN	Leu Hβ1/Hβ2	Val(D) HN	Val(D) Hα
Gly Hα1	Gly Hα2	Val(D) HN	Val(D) Hβ
Arg Hβ1/Hβ2	Val(D) HN		

[a] Where two atoms are listed, the nOe peak could not be stereospecifically assigned.

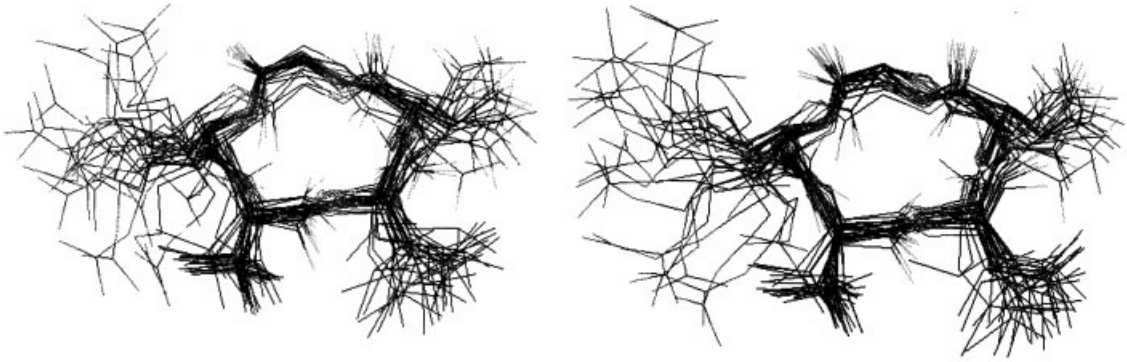

Figure 1 Superposition of 30 c(LNGRv) structures obtained from XPLOR runs. Carbon is shown in black, nitrogen and oxygen in dark and light grey, respectively. Left: without the use of nOes; right: with the use of the nOes listed in Table 2.

Table 3 Selected Geometry Optimized Conformers of c(LNGRv)

AA residue	Backbone	Conf.1	Conf.2	Conf.3	Conf.4	Conf.5	Conf.6
Leu	ϕ[a]	70.4	−83.2	−100.4	83.4	−61.2	−67.7
	ψ	−70.8	72.9	94.9	−128.1	133.0	98.7
Asn	ϕ	−86.0	75.3	−119.4	−80.2	60.3	52.6
	ψ	71.1	100.2	139.0	79.9	41.4	−122.2
Gly	ϕ	78.5	80.4	86.2	82.2	92.9	−68.9
	ψ	−87.1	−74.8	−66.4	152.7	147.8	−5.3
Arg	ϕ	−88.2	−87.9	−75.9	39.1	42.9	−126.6
	ψ	62.1	59.1	61.9	50.2	51.8	44.0
D–Val	ϕ	107.6	81.5	71.9	86.7	105.4	91.2
	ψ	88.4	−76.3	−97.5	103.0	−153.6	−61.2
ΔE[b]		0.00	5.12	5.62	5.67	5.71	6.05

For all structures listed, geometry optimizations were carried out at the RHF/3-21G level of theory while single point energies were calculated using the B3LYP/6–31+G(d) level of theory.
[a] All dihedral angles are given in degrees.
[b] Relative energies (in kcal/mol) are calculated relative to the global minimum structure.

of 30 structures shown in Figure 1 (right panel). Not surprisingly, there were very few violations of the nOe data even though the structures exhibit a wide range of conformations. This observation as well as the lack of long range nOes suggests that c(LNGRv) is intrinsically fairly flexible. The control experiment, carried out without the use of nOe constraints (Figure 1, left panel), yielded results very similar to the nOe-based calculations supporting the previous conclusion.

To gain a more detailed idea of the flexibility of c(LNGRv), a combination of MD, AM1 and *ab initio* calculations were used to explore its conformational space. Analysis of the geometry optimized structures (selected structures can be found in Table 3, and a complete list can be obtained from the authors upon request) shows that c(LNGRv) is able to take on many different conformations with very similar energies; all of these satisfy very well the nOes listed in Table 2 and the $^3J_{HN\alpha}$ coupling constant data (data not shown). This further supports the conclusion that c(LNGRv) exists as a conformationally fluctuating species in solution.

Structure Determination of c(LNGRV)

Two sets of resonances were present both in the TOCSY and in the NOESY spectra of this compound. Based on the solution stability results described earlier, these chemical shifts can be assigned to two species coexisting in solution, c(LNGRV) and its rearranged counterpart. A total of 44 nOes were obtained for c(LNGRV), all listed in Table 4. XPLOR and MD, semiempirical as well as *ab initio* calculations yielded results similar to those of c(LNGRv) (Figure 2, Table 5). Overall, the same conclusion can be drawn as before, c(LNGRV) is very flexible in solution and does not adopt a single structure. Rather, a time-averaged structure of multiple conformers is observed.

Decomposition of the NGR-peptides

Deamidation of asparagine — and also peptide bond cleavage — in peptides and proteins is known to happen spontaneously under mild conditions or even during storage [15]. In the first step of the deamidation

Table 4 Unambiguously Assignable nOes for c(LNGRV)

Atom 1[a]	Atom 2[a]	Atom 1[a]	Atom 2[a]
Asn HN	Asn Hα	Arg Hα	Arg Hβ1/Hβ2
Asn HN	Asn Hβ1	Arg Hα	Arg Hγ1/Hγ2
Asn HN	Asn Hβ2	Arg Hα	Arg Hδ1/Hδ2
Asn HN	Leu Hα	Arg Hα	Val HN
Asn HN	Leu Hβ1/Hβ2	Arg Hβ1/Hβ2	Arg Hδ1/Hδ2
Asn HN	Leu Hγ	Arg Hβ1/Hβ2	Arg Hε
Asn Hα	Asn Hβ1	Arg Hβ1/Hβ2	Val HN
Asn Hα	Asn Hβ2	Arg Hγ1/Hγ2	Arg Hδ1/Hδ2
Asn Hα	Asn Hδ21/Hδ22	Arg Hγ1/Hγ2	Arg Hε
Asn Hα	Gly HN	Arg Hδ1/Hδ2	Arg Hε
Asn Hδ21	Asn Hβ1	Val HN	Val Hα
Asn Hδ21	Asn Hβ2	Val HN	Val Hβ
Asn Hδ22	Asn Hβ1	Val Hα	Val Hβ
Asn Hδ22	Asn Hβ2	Val Hα	Val Hγ11/12/13/21/22/23
Gly HN	Gly Hα1	Val Hα	Leu HN
Gly HN	Gly Hα2	Val Hβ	Leu HN
Gly Hα1	Gly Hα2	Val Hβ	Val Hγ11/12/13/21/22/23
Arg HN	Arg Hα	Leu HN	Leu Hα
Arg HN	Arg Hβ1/Hβ2	Leu HN	Leu Hβ1/Hβ2
Arg HN	Arg Hγ1/Hγ2	Leu HN	Leu Hγ
Arg HN	Gly Hα1	Leu HN	Leu Hδ11/12/13/21/22/23
Arg HN	Gly Hα2	Leu Hβ1/Hβ2	Leu Hδ11/12/13/21/22/23

[a] Where two or more atoms are listed, the nOe peak could not be stereospecifically assigned.

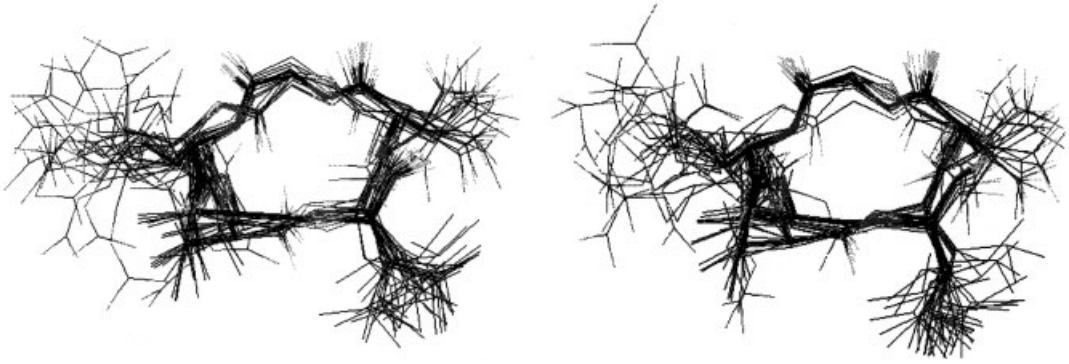

Figure 2 Superposition of 30 c(LNGRV) structures obtained from XPLOR runs. Carbon is shown in black, nitrogen and oxygen in dark and light grey, respectively. Left: without the use of nOes; right: with the use of the 44 nOes listed in Table 4.

process a succinimide ring is formed, via the release of ammonia, which then hydrolyses generating aspartyl and isoaspartyl peptides. The succinimide ring formation is enhanced when Asn is followed by Gly in the sequence, and if the peptide chain is flexible. Both of these premises are fulfilled in the case of **II** and **III**, since they contain an Asn-Gly peptide bond and, in spite of their cyclic structure, have a highly flexible backbone as well as side chains as shown by NMR.

As the results of the integrin receptor binding tests carried out at different times were not unambiguous, in spite of the fact that the samples had the same HPLC profile (Figure 3A), it was suspected that deamidation may occur in the solution of peptides containing the NGR motif. Therefore the stability of **II** and **III** at pH 7.4 in ammonium acetate buffer solution at room temperature was tested. At certain intervals samples of the solution were examined by HPLC. Some decomposition had already been observed after 24 h; in the case of both cyclopeptides a new peak had appeared. To identify the decomposition product, the solution of **II** was subjected to HPLC/MS analysis after 48 h. In the HPLC diagram (Figure 3B) the peak of c(LNGRV) **II** was at 19.86 min, while the decomposition product appeared at 21.36 min. MS measurements indicated that the latter, as expected, contained

Table 5 Selected Geometry Optimized Conformations of c(LNGRV)

AA residue	backbone	Conf.1	Conf.2	Conf.3	Conf.4	Conf.5	Conf.6
Leu	ϕ[a]	75.0	74.7	77.0	72.6	71.4	72.9
	ψ	−77.0	−52.5	−60.8	−60.2	−71.2	−59.4
Asn	ϕ	−88.4	−75.9	−106.3	−78.0	−87.6	−83.2
	ψ	64.5	52.6	144.1	84.3	63.8	82.5
Gly	ϕ	79.6	81.0	74.6	82.1	77.0	82.6
	ψ	−71.1	−60.7	−70.1	−50.4	−77.6	−49.4
Arg	ϕ	−80.5	−84.2	−137.9	−118.7	−81.1	−118.5
	ψ	55.1	−47.4	−53.4	−69.7	75.6	−72.1
Val	ϕ	82.4	−137.8	−86.3	−88.2	74.3	−87.8
	ψ	105.4	82.5	81.5	75.9	102.3	77.8
ΔE[b]		0.00	3.10	3.97	4.05	4.30	5.72

For all structures listed, geometry optimizations were carried out at the RHF/3-21G level of theory while single point energies were calculated using the B3LYP/6–31+G(d) level of theory.
[a] All dihedral angles are given in degrees.
[b] Relative energies (in kcal/mol) are calculated relative to the global minimum structure.

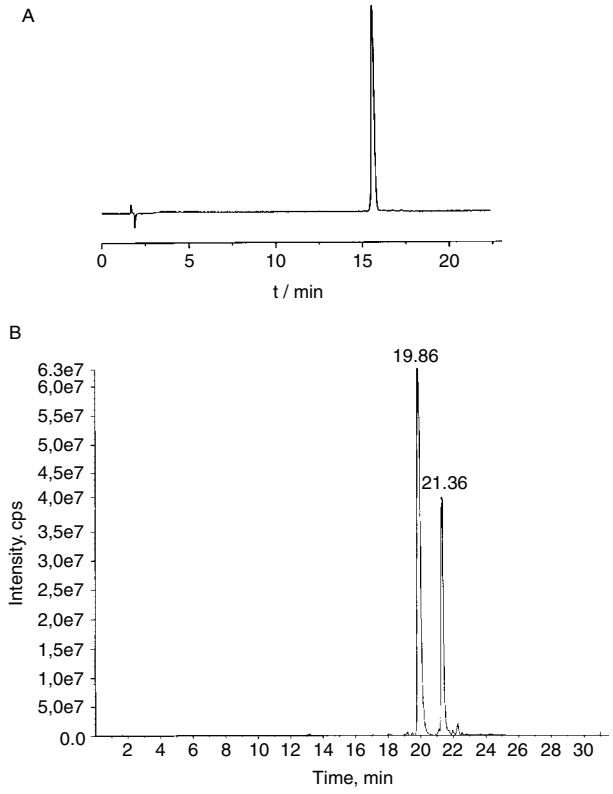

Figure 3 (A) HPLC diagram of c(LNGRV) after synthesis and purification (see Methods section). (B) HPLC chromatogram of c(LNGRV) in 0.1M NH_4OAc buffer solution at pH 7.4 after 48 h incubation; the second peak was identified with synthetic c(LDGRV).

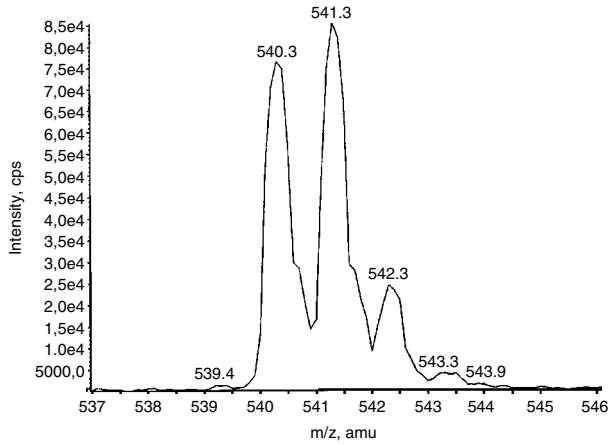

Figure 4 Electrospray mass spectrum of co-eluting compounds in HPLC peak at 19.86 min (for details see caption to Figure 3).

aspartic acid ($[M+H]^+ = 541.3$) and with the aid of the synthetic peptide **VII** it was identified as c(LDGRV). However, there still remained an unanswered question, namely, if there was a conversion of the NGR sequence to a DGR one, in which case a β-aspartyl-peptide should be formed at the same time as well. A more thorough study of the HPLC-MS results revealed the presence of another peak very close to the starting compound (Figure 4) with an $[M+H]^+ = 541.3$. This must be c(Leu-β-Asp-Gly-Arg-Val). Interestingly enough this iso-aspartyl peptide could also be detected in the original sample, which was stored in the refrigerator.

CONCLUSIONS

This study addressed the question whether the cyclization of linear peptides containing the NGR cell adhesion sequence leads to a less flexible conformation and at the same time to an increased $\alpha_5\beta_1$ integrin receptor selectivity. Our results indicate that contrary to the RGD-peptides the cyclization of LNGRV and LNGRv causes only a marginal change in their integrin

receptor affinity. This was shown by the limited effect and selectivity on the adhesion of endothelial cells to ECM components.

The structure determination of the two cyclopeptides by NMR, MD, semiempirical and *ab initio* methods revealed that in solution both are very flexible and take on multiple stable conformations of rather similar relative energy. This might be due to the lack of a stabilizing salt bridge, between the carboxyl group of Asp and the guanidino group of Arg, present in the RGD-cyclopeptides at neutral pH.

This flexibility and the presence of the Asn-Gly peptide bond enhance succinimide ring formation leading to the hydrolysis of Asn. Peptides containing Asp-Gly may also form succinimide rings, however, in the case of the RGD cyclopeptides this conversion is hindered by the salt bridge between the Arg and Asp side chains [16]. Here it has been demonstrated that c(LNGRV) both in solution and during storage suffers deamidation and as the isoaspartyl-peptide may co-elute with the asparginyl-peptide in the HPLC analysis, MS measurement is necessary to check the purity of peptides containing the NGR sequence.

The stability investigations raise the question of whether in *in vivo* experiments requiring a long lasting pharmacological distribution, the NGR motif or its hydrolysis product is effective? This question is of paramount interest, since the NGR motif is also regarded as a tumour homing peptide and, as such, a potential targeting device for inhibiting angiogenesis. For this reason its binding to different components of the tumour vessel is being widely investigated [17–19].

Acknowledgement

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