Radio-Analytical Determination of the Coating Efficiency of Cyclic RGD Peptides

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Coating of artificial surfaces with RGD (= arginine-glycine-aspartate) peptides to enhance cell adhesion is an ongoing issue. Thereby, the physiological adhesion process to the extra-cellular matrix (ECM) is mimicked by the peptide coating, leading to a strong cell-surface contact, followed by spreading and proliferation of the cells. For comparable cell adhesion studies, it is important to know the density of the RGD peptides on the surface. Here, we present an approach to determine the amount of bound cyclic RGD peptide by radio labeling with ^{125}I of a tyrosine-containing RGD peptide on different materials surfaces (poly(methyl methacrylate) (PMMA), titanium, and silicon). For all surfaces, the amount of bound peptides is in the range of pmol/cm^2.

Introduction. – Coating of artificial surfaces with cell-adhesive molecules provides a strong mechanical contact between cells and surface. Cell adhesion is mediated by integrins [1], a class of heterodimeric transmembrane receptors that bind selectively to different proteins of the extracellular matrix (ECM) [2]. Cellular binding sites, like the arginine-glycine-aspartate (RGD) sequence, have been reported to play a major role in mediating cell adhesion between extracellular matrix proteins and integrins [3]. These interactions allow inside out as well as outside in signal transduction. Many applications of coatings using cyclic RGD pentapeptides based on cyclo(-RGDfK-) have been reported by us and others [4]. The cyclic peptide binds with high activity and selectivity to $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins [5]. The αv integrins are known to adhere to vitronectin, a RGD-containing extracellular matrix protein. Only the $\alpha v\beta 3$ integrin is found in focal contacts and leads to spreading and migration of (endothelial) cells on vitronectin [6]. Surfaces coated with cyclic RGD peptides of the type cyclo(-RGDfK-) can be used in implantation medicine or for studying the behavior of cells attached to surfaces. One major problem of these coatings was that the amount of surface-bound RGD peptide could not be determined exactly. This problem is now solved by the radio-labeling approach presented here.

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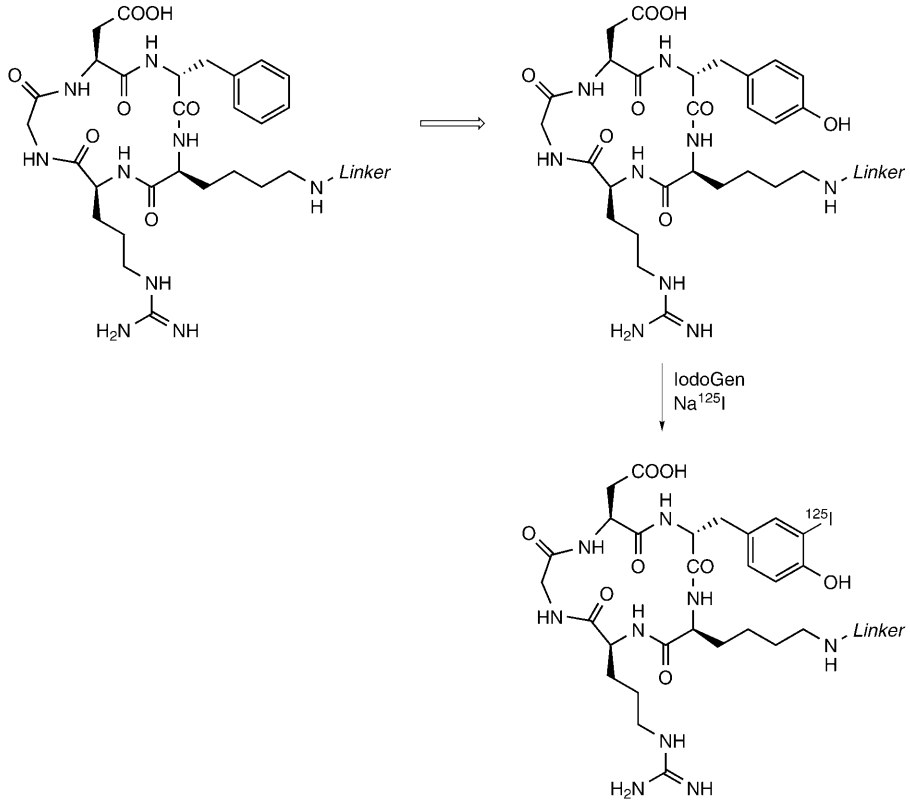
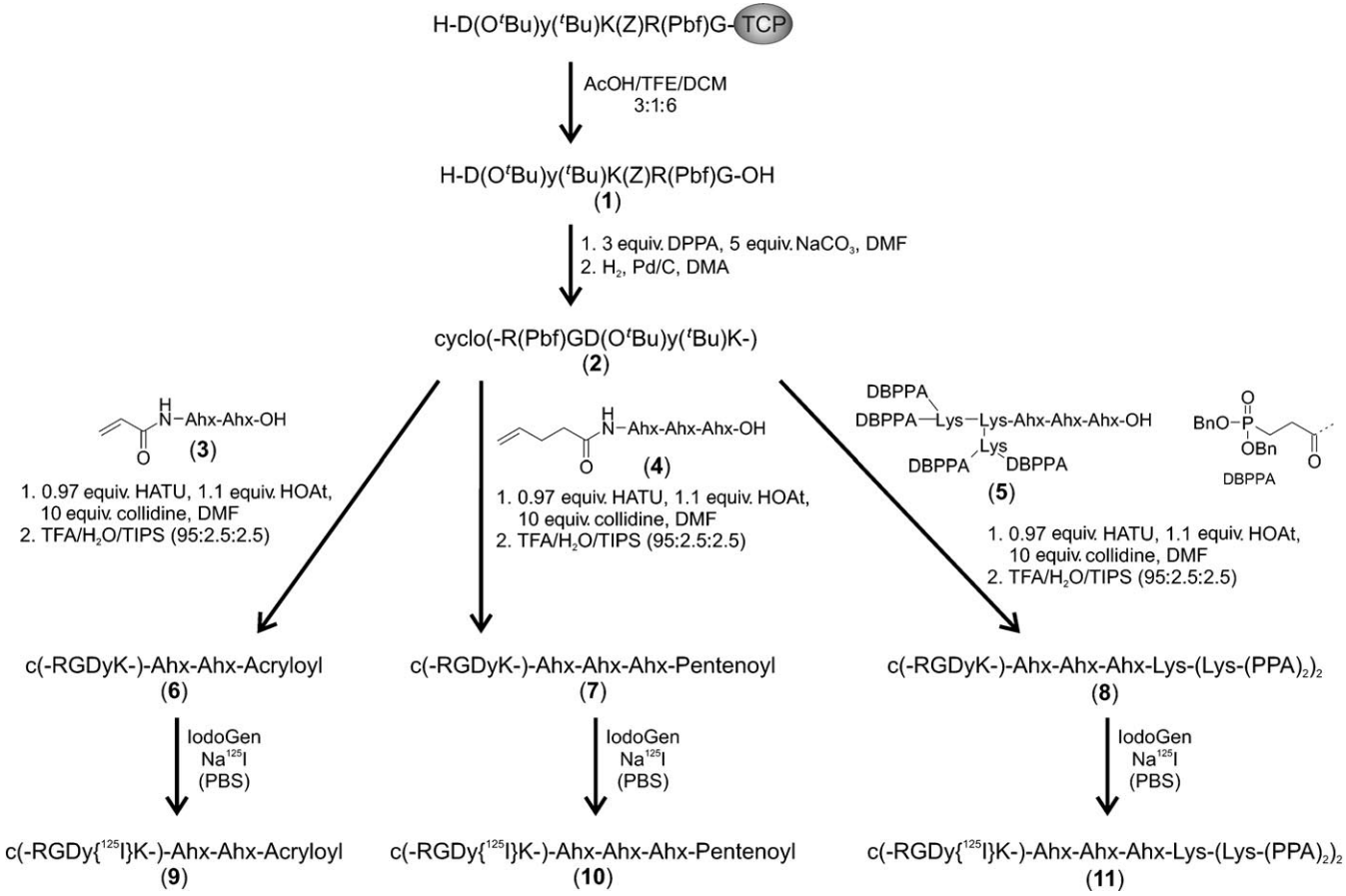
Results and Discussion. – To implement the radioactivity an approbate site for labeling had to be introduced into the peptide structure. As phenols can be smoothly labeled with ^{125}I ($t_{1/2}=59.4$ d, 81.3 TBq/mmol) by the *IodoGen®* (=1.3,4.6-tetrachloro-3α,6α-diphrenylglycoluril) method [7], we replaced the D-Phe in the cyclic pentapeptide with D-Tyr and used it for labeling (*Scheme 1*). The linear peptide H-D(O^{t}Bu)y(^{t}Bu)K(Z)R(Pbf)G-OH (**1**; Pbf=2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) was synthesized on trityl chloride polystyrol (TCP) resin [8] according to the Fmoc strategy [9]. After cyclization and deprotection of the lysine side chain, the peptide **2** was coupled with three different linker systems for three different surface types – an acryloyl anchor for poly(methyl methacrylate) (PMMA; **3**) [4a], a pentenoyl anchor for silicon (**4**) [4c], and a phosphonate anchor for titanium (**5**) [4e]. Thereafter, the peptides were deprotected with CF_3COOH (TFA)/H_2O/(i-Pr)$_3$SiH (TIPS) and purified by HPLC. The resulting compounds, **6**–**8**, were labeled with ^{125}I and purified by HPLC again (**9**–**11**; *Scheme 2*).

The corresponding ^{127}I-containing reference compounds, **9C**–**11C**, which are also used to characterize the radioactive compounds and to modify their specific activity,

Scheme 1. *Exchange of* D-*Phe through* D-*Tyr in the* $\alpha v\beta 3$*-Selective Cyclic RGD Peptide cyclo-(-RGDfK-) Enables the Radio-Labeling with* ^{125}I *of the Surface-Adhesive Peptide.*



Scheme 2. *Synthesis of the Radio-Labeled Compounds* **9**–**11**. For abbreviations of the reagents, see the *Exper. Part.*



were synthesized according to a reaction scheme, which needed some modifications because of the unprotected side chain of the Fmoc-D-3-iodotyrosine [10]. For the synthesis of the linear peptide H-K(ivDde)R(Pbf)GD(O^{t}Bu)y{I}-OH (**12**), Fmoc-D-3-iodotyrosine was attached to the TCP resin. Solid-state peptide synthesis (SPPS) was performed according to the standard coupling protocols. The cyclization of **12** was carried out using [(1*H*-benzotriazol-1-yl)-oxy]tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate (PyBOP) instead of diphenylphosphoryl azide (DPPA), because the use of DPPA led to quantitative formation of a diphenylphosphoric acid ester on the unprotected tyrosine OH group. Lysine side chain had to be protected with the 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene (ivDde) group [11] instead of the (benzyloxy)-carbonyl (Z) group, because its hydrogenolytical removal led also to a removal of the iodine from the tyrosine. The resulting peptide **13** was then coupled with the anchors **3**–**5**. Deprotection with TFA/H_2O/TIPS, followed by HPLC purification led to the compounds **9C**–**11C** (*Scheme 3*).

For the measurement of the amount of surface bound peptide, a reasonable specific activity was adjusted by mixing the radioactive compounds **9**–**11** with their corresponding non-radioactive analogs **9C**–**11C**. The different surface types poly(methyl methacrylate) (PMMA), silicon, and titanium were coated using these mixtures according to the protocols published previously [4a,c,e]. Briefly, **9**/**9C** was coated on PMMA discs (Ø 6 mm, 10 µl per disc) through UV irradiation, silicon was coated with **10**/**10C** in toluene at 80°, and **11**/**11C** was immobilized on titanium discs (Ø 1 cm, 300 µl per disc) from a phosphate-buffered saline (PBS) solution. After rinsing all probes thoroughly, the activity was detected in a γ-counter.

The percentage of bound peptide was calculated using the ratio of bound activity compared to the activity in the corresponding coating solution (same volume as used for one disc; *Table*). The percentage of bound immobilized activity is considered to be also the percentage of immobilized peptide; therefore, the amount of immobilized peptide can be calculated from the amount of peptide applied to each sample. The amount of bound peptide depends on the material and the concentration of the peptide in the coating solution used, but is in a pmol/cm^2 range for all surfaces examined (*Table*). For titanium, the highest amount (278 pmol/cm^2) could be reached.

Conclusions. – Replacment of D-Phe in the cyclic RGD pentapeptide with D-3-[^{125}I]-iodotyrosine resulted in a simple but efficient method to determine the amount of surface bound peptide. The only limitation of this approach is that the anchoring group used must be stable under the conditions of the radioiodination with *IodoGen*®.

Experimental Part

General. Amino acids and coupling reagents were purchased from *IRIS Biotech* (D-Marktredwitz), Trityl chloride polystyrol (TCP) resin from *Pepchem* (D-Tübingen), 3-(dibenzylphosphono)propionic acid (DBPPA) from *Novabiochem* (D-Schwalbach). All other chemicals were purchased from *Aldrich*, *Sigma*, or *Fluka*. NMR Spectra: *Bruker DMX-250*. ESI-MS measurements were performed on a *Finnigan LCQ* instrument.

Fmoc-D-3-iodo-tyrosine (*Fmoc*-D-*Tyr{I}-OH*). This compound was synthesized according to *Haubner et al*. [10]. M.p. 134–137°. ^{1}H-NMR (DMSO, 500 MHz): 12.50 (br. *s*, COOH); 10.12 (*s*, NH); 7.87

Scheme 3. *Synthesis of the Corresponding Compounds,* **9C**–**11C**, *with Nonradioactive* ^{127}I. For abbreviations of reagents, see the *Exper. Part.*

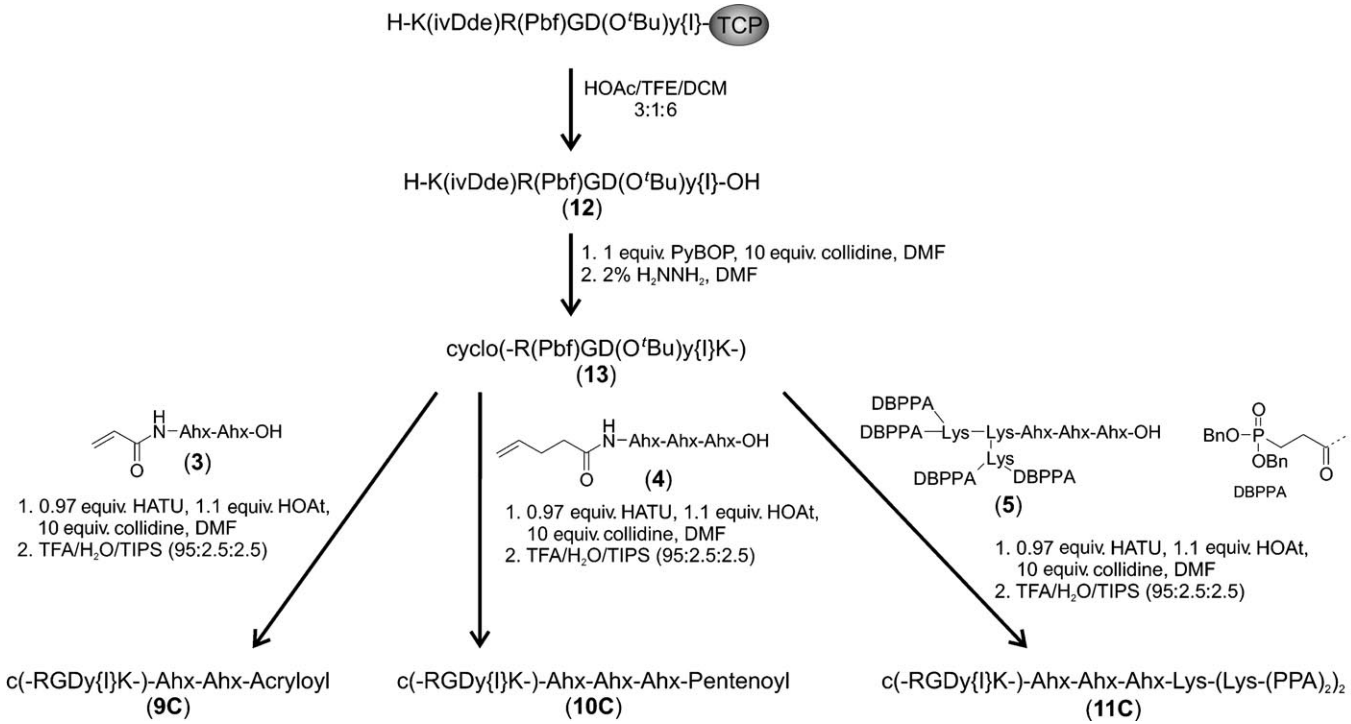


Table. *Amount of Surface-Bound RGD Peptide on Different Materials.* The mean value is the result of triplicate determinations.

Surface	Compound	Applied amount of peptide per probe [nmol]	Ratio cold/hot	Activity of coating solution [$\times 10^6$ Cpm]	Activity of coated sample [$\times 10^6$ Cpm]	Percentage of bound peptide [%]	Amount of bound peptide [pmol/cm^2]
PMMA	**9**	0.010	500 : 1	0.077	0.017	22.92	8.12 ± 1.23
discs Ø 6		0.10	625 : 1	0.77	0.14	17.95	63.6 ± 6.27
mm		1.0	500 : 1	9.77	0.35	3.58	127 ± 32.1
Silicon	**10**	1.0 mg/ml[a])	12800 : 1	307	–[b])	–[b])	138 ± 23.9
Titanium	**11**	0.035	6.3 : 1	0.0231	0.000442	1.92	0.47 ± 0.23
discs Ø 1		0.36	4.7 : 1	0.308	0.00298	0.97	2.47 ± 0.59
cm		3.6	4.7 : 1	3.13	0.0121	0.39	9.92 ± 0.89
		32	10.1 : 1	14.5	0.194	1.33	310 ± 85

[a]) Same solution (940 µl) was used for all samples. [b]) In the case of silicon samples with different areas were used; therefore, mean values can not be given.

(*d, J* = 7.5, 2 H, FmocCH); 7.69 (*d, J* = 8.5, 1 H, FmocCH); 7.65–7.59 (*m*, 3 H, PheCH); 7.39 (*m*, 2 H, FmocCH); 7.30 (*m*, 2 H, FmocCH); 4.20–4.15 (*m*, 3 H, $CHCH_2O$); 4.08 (*m*, H^{α}); 2.94 (*dd*, H^{β}); 2.72 (*dd*, H^{β}). ESI-MS: 308.0 ($[M+H]^+$).

H-D(O^t*Bu)y(*^{t}*Bu)K(Z)R(Pbf)G-OH* (**1**). The peptide was synthesized by solid-phase peptide synthesis (SPPS) on TCP resin [8] according to the Fmoc strategy [9]. The resin was loaded with Fmoc-Gly-OH. Subsequently, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Z)-OH, Fmoc-D-Tyr(^{t}Bu)-OH, and Fmoc-Asp(O^{t}Bu) (Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) were coupled with *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxy-1*H*-benzotriazole (HOBt) in twofold excess and $EtN(i\text{-}Pr)_2$ as base and 1-methylpyrrolidin-2-one (NMP) as solvent. The Fmoc deprotection between the couplings and at the end of the sequence was performed with 20% piperidine in NMP. The peptide was cleaved from the resin by treatment with AcOH/2,2,2-trifluoroethanol/CH_2Cl_2 3 : 1 : 6. ESI-MS: 1024.5 ($[M-2^t\text{Bu}+H]^+$), 1080.6 ($[M-{}^t\text{Bu}+H]^+$), 1136.5 ($[M+H]^+$), 1158.5 ($[M+Na]^+$).

Cyclo(-R(Pbf)GD(O^t*Bu)y(*^{t}*Bu)K-)* (**2**). Cyclization was performed *via in situ* activation with 3 equiv. diphenoxyphosphoryl azide (DPPA) and with 5 equiv. $NaHCO_3$ as solid base under high-dilution conditions (5 mM) in DMF for 16 h. Removal of the Z group was achieved by roughly stirring of a soln. in *N*,*N*-dimethylacetamide with Pd/C as catalyst under H_2 for 24 h. ESI-MS: 928.5 ($[M-{}^t\text{Bu}+2\ H]^+$), 984.4 ($[M+H]^+$), 1006.4 ($[M+Na]^+$).

Acryloyl-Ahx-Ahx-OH (**3**). This compound was synthesized according to *Pless et al.* [12]. ^{1}H-NMR (DMSO, 250 MHz): 8.03 (*m*, NH); 7.70 (*m*, NH); 6.16 (*dd*, *J* = 10, 17, 1 H, CH_2=CH); 6.03 (*dd*, *J* = 2.5, 17, 1 H, CH_2=CH); 5.53 (*dd*, *J* = 2.5, 10, 1 H, CH_2=CH); 3.15–2.95 (*m*, 2 NCH_2); 2.17 (*t*, *J* = 7, CH_2COOH); 2.02 (*t*, *J* = 7, CH_2CON); 1.55–1.15 (*m*, 2 $(CH_2)_3$). ESI-MS: 299.3 ($[M+H]^+$), 321.3 ($[M+Na]^+$).

Pent-4-enoyl-Ahx-Ahx-Ahx-OH (**4**). TCP Resin was loaded with Fmoc-Ahx-OH, followed by subsequent coupling of Fmoc-Ahx-OH, Fmoc-Ahx-OH, and pent-4-enoic acid. The linker was cleaved from the resin with 50% TFA in CH_2Cl_2. ^{1}H-NMR ((D$_6$)DMSO, 250 MHz): 7.70 (*m*, 3 NH); 5.76 (*m*, 1 H, CH_2=CH); 4.97 (*dd*, *J* = 19.2, 10.4, 2 H, CH_2=CH); 2.99 (*m*, 3 CH_2NH); 2.17 (*m*, =CH(CH_2)$_2$, CH_2COOH); 2.01 (*m*, 2 CH_2CO); 1.53–1.15 (*m*, 3 $(CH_2)_3$). ESI-MS: 440.2 ($[M+H]^+$); 462.1 ($[M+Na]^+$), 478.3 ($[M+K]^+$), 879.1 ($[2\ M+H]^+$).

(DBPPA$_2$*-Lys)*$_2$*-Lys-Ahx-Ahx-Ahx-OH* (**5**). TCP Resin was loaded with Fmoc-Ahx-OH, followed by subsequent coupling of Fmoc-Ahx-OH, Fmoc-Ahx-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Lys(Fmoc)-OH, and DBPPA as described above. After the first lysine, fourfold excess was used, for DBPPA eightfold excess. ESI-MS: 1004.8 ($[(M+2\ H)/2]^+$), 1015.7 ($[(M+H+Na)/2]^+$).

Coupling of the Linker to the Cyclic Peptide. The linker (1 equiv.) was activated with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazol (HOAt) and 10 equiv. of *syn*-collidine in DMF. After 2 h, 1 equiv. of cyclic peptide was added. After another 24 h, the peptide was precipitated in H_2O. Deprotection and purification by HPLC (*Amersham Pharmacia*, *YMC-RP*, 40 mm × 250 mm, *C18*; 8 ml/min; eluent *A* H_2O (0.1% CF_3COOH), eluent *B* MeCN (0.1% CF_3COOH), 220-nm UV detector) led to compounds **6**, **7**, and **8**.

Cyclo(-RGDyK-)-Ahx-Ahx-Acryloyl (**6**). ESI-MS: 451.1 $[(M+2\,H)/2]^+$; 900.6 $[M+H]^+$.

Cyclo(-RGDyK-)-Ahx-Ahx-Ahx-Pent-4-enoyl (**7**). ESI-MS: 521.8 ($[(M+2\,H)/2]^+$), 1041.8 ($[M+H]^+$), 1063.8 ($[M+Na]^+$), 1079.8 ($[M+K]^+$).

Cyclo(-RGDyK-)-Ahx-Ahx-Ahx-K-(K-PPA$_2$)$_2$ (**8**). ESI-MS: 942.9 ($[(M-2\,H)/2]^-$); 954.8 ($[(M-3\,H+Na)/2]^-$), 1885.9 ($[M-H]^-$), 1909.9 ($[M-2\,H+Na]^-$).

Labeling with ^{125}I. The peptide (300 μg) was dissolved in 150 μl of PBS in an *Eppendorf* cap coated with 150 μg of *IodoGen®*. 1.32 mCi ^{125}I as $Na^{125}I$ in 0.05M NaOH were added. After 30 min, the labeled compound was separated by HPLC.

H-K(ivDde)R(Pbf)GD(O^{t}Bu)y{I}-OH (**12**). The peptide was obtained also by SPPS on the TCP resin [8] according to the Fmoc strategy [9]. The resin was loaded with Fmoc-D-Tyr{I}-OH. Subsequently, Fmoc-Asp(O^{t}Bu), Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Lys(ivDde)-OH were coupled with TBTU, HOBt in twofold excess and EtN(i-Pr)$_2$ as base and NMP as solvent. The Fmoc deprotection between the couplings and at the end of the sequence was accomplished with 20% piperidine in NMP. The peptide was cleaved from the resin by treatment with AcOH/2,2,2-trifluoroethanol/CH_2Cl_2 3 : 1 : 6. ESI-MS: 888.4 ($[M-^t\!Bu+H]^+$), 944.3 ($[M+H]^+$), 984.3 ($[M+Na]^+$).

Cyclo(-R(Pbf)GD(O^{t}Bu)y(^{t}Bu)K-) (**13**). Cyclization was performed in DMF at a concentration of 5 mM with 1 equiv. of [(1*H*-benzotriazole-1-yl)oxy]tris(pyrrolidino-1-yl-phosphonium) hexafluorophosphate (PyBOP) and 10 equiv. *syn*-collidine as base. After 24 h, the solvent was removed under reduced pressure, and the peptide was precipitated in H_2O. The ivDde group was removed by treatment with 2% hydrazine in DMF. ESI-MS: 998.3 ($[M-^t\!Bu+2\,H]^+$), 1054.3 ($[M+H]^+$).

Cyclo(-RGDy{I}K-)-Ahx-Ahx-Acryloyl (**9C**). ESI-MS: 514.0 $[(M+2\,H)/2]^+$; 1026.5 $[M+H]^+$; 1048.5 $[M+Na]^+$.

Cyclo(-RGDy{I}K-)-Ahx-Ahx-Ahx-Pent-4-enoyl (**10C**). ESI-MS: 584.7 ($[(M+2\,H)/2]^+$), 1167.6 ($[M+H]^+$).

Cyclo(-RGDy{I}K-)-Ahx-Ahx-Ahx-K-(K-PPA$_2$)$_2$ (**11C**). ESI-MS: 1005.6 ($[(M-2\,H)/2]^-$).

Surface coating was performed as described in [4a,c,e].

The authors thank *Wolfgang Linke* for the help in radio-labeling. Financial support by the *Deutsche Forschungsgemeinschaft* (FOR-411) and the *Fonds der Chemischen Industrie* is gratefully acknowledged.

REFERENCES

[1] J. A. Eble, in 'Integrin-Ligand Interaction', Eds. J. A. Eble and K. Kühn, Springer-Verlag, Heidelberg, 1997, p. 1–40.

[2] K. Kühn, in 'Integrin-Ligand Interaction', Eds. J. A. Eble and K. Kühn, Springer-Verlag, Heidelberg, 1997, p. 41–83.

[3] M. D. Pierschbacher, E. J. Ruoslahti, *Nature* **1984**, *309*, 30; K. Okamoto, T. Matsuura, R. Hosokawa, Y. Akagawa, *J. Dent. Res.* **1998**, *77*, 481; T. Matsuura, R. Hosokawa, K. Okamoto, T. Kimoto, Y. Akagawa, *Biomaterials* **2000**, *21*, 1121; U. Hersel, C. Dahmen, H. Kessler, *Biomaterials* **2003**, *24*, 4385.

[4] a) M. Kantlehner, P. Schaffner, D. Finsinger, J. Meyer, A. Jonczyk, B. Diefenbach, B. Nies, G. Hölzemann, S. L. Goodman, H. Kessler, *ChemBioChem* **2000**, *1*, 107; b) H. Schliephake, D. Scharnweber, M. Dard, S. Rossler, A. Sewing, J. Meyer, D. Hoogestraat, *Clin. Oral Implants Res.* **2002**, *13*, 312; c) C. Dahmen, A. Janotta, D. Dimova-Malinovska, S. Marx, B. Jeschke, B. Nies, H. Kessler, M. Stutzmann, *Thin Solid Films* **2003**, *427*, 201; d) B. Elmengaard, J. E. Bechtold, K. Søballe, *Biomaterials* **2005**, *26*, 3521; e) J. Auernheimer, D. Zukowski, C. Dahmen, M. Kantlehner, A. Enderle, S.

L. Goodman, H. Kessler, *ChemBioChem* **2005**, *6*, 2034; f) J. Auernheimer, C. Dahmen, U. Hersel, A. Bausch, H. Kessler, *J. Am. Chem. Soc.* **2005**, *127*, 16107.

[5] M. Aumailley, M. Gurrath, G. Müller, J. Calvete, R. Timpl, H. Kessler, *FEBS Lett.* **1991**, *291*, 50; R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 7461.

[6] E. A. Wayner, R. A. Orlando, D. A. Cheresh, *J. Cell Biol.* **1991**, *113*, 919; D. I. Leavesley, G. D. Ferguson, E. A. Wayner, D. A. Cheresh, *J. Cell Biol.* **1992**, *117*, 1101.

[7] P. J. Fraker, J. C. Speck Jr., *Biochem. Biophys. Res. Commun.* **1978**, *80*, 849.

[8] K. Barlos, D. Gatos, J. Kallitsis, G. Papaphotiu, P. Sotiriu, Y. Wenqing, W. Schäfer, *Tetrahedron Lett.* **1989**, *30*, 3943.

[9] L. A. Carpino, G. Y. Han, *J. Org. Chem.* **1972**, *37*, 3404; C. D. Chang, J. Meienhofer, *Int. J. Pept. Protein Res.* **1978**, *11*, 246.

[10] R. Haubner, H. J. Wester, U. Reuning, R. Senekowitsch-Schmidtke, B. Diefenbach, H. Kessler, G. Stöcklin, M. Schwaiger, *J. Nucl. Med.* **1999**, *40*, 1061.

[11] S. R. Chhabra, B. Hothi, D. J. Evans, P. D. White, B. W. Bycroft, W. C. Chan, *Tetrahedron Lett.* **1998**, *39*, 1603.

[12] D. D. Pless, Y. C. Lee, S. Roseman, R. L. Schnaar, *J. Biol. Chem.* **1983**, *258*, 2348.

Received January 30, 2006