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The synthesis of alternative diketopiperazines as potential RGD mimetics

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Abstract: Alternative RGD mimetics – with the exception of glycine – c(Arg-Asp) **1**, c(Arg-Glu) **2** and c[Arg-Asp(Phe-OH)] **3** were synthesized. The DKPs were prepared on solid phase with orthogonal protection allowing further derivatization in solution. During solution phase cyclization in NH_3/methanol, the side chain benzyl ester group of H-Arg(Tos)-Asp(OBzl)-OMe and H-Arg(Tos)-Glu(OBzl)-OMe suffer transesterification, while β-*t*-butyl or β-cyclohexyl esters are stable under the same conditions. In spite of the simple structure, all compounds bind selectively to the $\alpha_v\beta_3$ integrin receptor, **3** showing the highest affinity with an IC_{50} value of 0.74 μM value. On the other hand only **3** binds with measurable activity to the $\alpha_{IIb}\beta_3$ receptor (IC_{50} 159 μM). The binding affinities seem to be in accordance with the distances between the arginine guanidino and the aspartic acid carboxyl group in extended conformation determined by semiempirical geometry optimization. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: diketopiperazine; RGD mimetics; integrin receptor; transesterification

INTRODUCTION

Recently, increasing interest has been attracted by diketopiperazinesas, reflected by two reviews which have appeared in the last few years on this topic [1,2]. The reason for this growing curiosity is that the relative rigid diketopiperazine ring may ensure in many cases the appropriate spacial arrangement of pharmacophore groups for the interaction between biologically active peptides and their receptors or between substrates and enzymes. Diketopiperazines have the advantage of forming both hydrogen bonds and by substitution, aromatic side chain interactions as well. Thus diketopiperazines have gained importance in medicinal chemistry as peptidomimetics.

On the understanding that the integrin receptor selectivity of the RGD peptides is determined by the distance between the arginine δ-guanidino and the aspartic acid β-carboxyl groups [3], we decided on the synthesis of such RGD mimetics, where the distance between the functional groups is increasing. In the case of c(Arg-Asp) and c(Arg-Glu) the pharmacophores are located on a diketopiperazine template at different distances. For further elongation of the distance between the basic and acidic groups, phenylalanine was coupled to the β-carboxyl group of the aspartic acid. The novelty of these mimetics lies in the omission of glycine, which is considered to play a crucial role in the integrin receptor binding of the RGD sequence. The receptor binding of the models was tested on the two β_3 integrin receptors, namely on $\alpha_v\beta_3$ and on $\alpha_{IIb}\beta_3$.

MATERIALS AND METHODS

Chemicals and Equipment

Boc-Glu(OBzl)-OH, Boc-Asp(OBzl)-OH, Boc-Arg(Tos)-OH were purchased from Reanal (Budapest, Hungary), Boc-Glu(OcHex)-OH, Fmoc-Asp(OBu^t)-OH, Fmoc-Glu(OBu^t)-OH, Boc-Arg(Tos)-*O*-PAM from Novabiochem (Merck Kft, Budapest, Hungary). Merck Kieselgel precoated sheets (art. no. 5553) and solvent system, ethyl acetate/pyridine/acetic acid/water 60/20/6/11 were used for TLC. Analytical reverse phase hplc was performed on a Knauer model using Vydec C_{18}, 300 Å, 5μ, (4.6 mm × 250 mm) column at a flow rate of 1 ml/min. Preparative reverse phase hplc was performed on a Pharmacia instrument using Vydac C18, 300 Å, 15μ, (19 × 300 mm) at a flow rate of 12 ml/min. Detection was in both cases at 220 nm. Hplc eluents: A: 0.1% TFA/water, B: 0.07% TFA/acetonitrile. NMR spectra were recorded in DMSO-d_6 solution on a Bruker DRX 500 spectrometer at 500 (^{1}H)-, 125 (^{13}C) and 50 (^{15}N) MHz, with the deuterium signal of the solvent. Each assignment is based on 2D-HMQC and 2D-HMBC spectra obtained by using the standard Bruker pulse programs INV4GS and INV4GSLPLRND, respectively. MS spectra were recorded on a Bruker Esquire 3000 equipment. HRMS measurements were run on a VG ZAB SEQ HRMS high resolution mass spectrometer equipped with Cs ion SIMS ion source. The resolution was 10 000. Glycerol was used as matrix and the glycerol cluster peaks were used in the peak matching experiments.

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Semiempirical Geometry Optimization (AM1)

Molecular geometry optimizations were performed in the case of c(Arg-Asp), c(Arg-Glu) and c[Arg-Asp(Phe-OH)] at RAM1 level of theory using Gaussian03 [4]. The highest possible fixed distance values were used for each peptide.

General Procedure for Diketopiperazine Formation in Solution

Method A [5]: H-Arg(Tos)-Glu(OX)-OMe. HCl or H-Arg(Tos)-Asp (OX)-OMe.HCl (X: OBzl, OBu^t, OcHex) was dissolved in NH_3/methanol and the solution was allowed to stand for several hours at room temperature until the disappearance of the ninhydrin positive spot on TLC. The solution was concentrated *in vacuo*, the residue triturated with ice-cold water and the protected diketopiperazine filtered off as a white powder practically in quantitative yield.

Method B [6]: H-Arg(Tos)-Asp(OBzl)-OMe.HCl (0.2 mmol) was dissolved in 3 ml of 0.1 M acetic acid/2-butanol in the presence of an equivalent amount of NMM and the reaction mixture was heated at 120 °C for 3 h. According to TLC analysis, a multicomponent mixture was formed, containing the starting dipeptide as well.

Method C [7]: H-Arg(Tos)-Asp(OBzl)-OMe.HCOOH (0.2 mM) was boiled for 1 h in the mixture of 20 ml 2-butanol and 5 ml toluol, and the reaction mixture stood overnight at room temperature. After evaporation of the solvent *in vacuo* the oily residue was triturated with ether and c[Arg(Tos)-Asp(OBzl)] was filtered off as a white powder in 60% yield.

Synthesis of Diketopiperazines on Solid Phase

After removal of the Boc group from 500 mg Boc-Arg(Tos)-*O*-PAM (0.6mM/g), Fmoc-Asp(OBu^t)-OH and Fmoc-Glu(OBu^t)-OH (3 equivalent) respectively, were coupled to it with the aid of DIC/HOBt (3 equivalent). The Fmoc group was split with 0.1M TBAF/DMF containing 2% ethanol for 15 min. The resin was washed twice with DMF and twice with 0.1M acetic acid/DCM, and left for two days in 0.1M acetic acid/DCM solution. The solution was evaporated *in vacuo*, the residue triturated with ether and filtered off. The white powder was triturated with water and filtered off to give c[Arg(Tos)-Asp(OBu^t)] and c[Arg(Tos)-Glu(OBu^t)] respectively, in 35–40% yield. The protected diketopiperazine was treated with liquid HF for 1.5 h at 0 °C, then ether was added and the precipitate was filtered off and washed on the filter with ether. Purification was performed by preparative hplc.

Synthesis of c(Arg-Asp(Phe-OH))

c[Arg(Tos)-Asp(O^{t}Bu)] (100 mg) was kept for 1.5 h in the mixture of 1 ml of 4N HCl/dioxan and 0.3 ml of formic acid solution. The solution was concentrated *in vacuo*, the residue triturated with ether and filtered off to give c[Arg(Tos)-Asp] in quantitative yield (R_f 0.39). To the solution of H-Phe-OBzl.HCl (0.1 mM, 29 mg) in 1 ml DMF, DIEA (0.1 mM, 176 µl), c[Arg(Tos)-Asp] (0.1 mM, 42 mg) HOSu (0.1 mM, 115 mg) and DCC (0.1 mM, 206 mg) in 0.4 ml DMF were added at 0 °C. The reaction mixture was stirred at 0 °C for 3 h then stood at room temperature overnight. DCU was filtered off and the solvent was evaporated. The residue, c[Arg(Tos)-Asp(Phe-OBzl)], was purified with preparative hplc (t_R 19.5 min, gradient 1% B for 5 min, then 1 → 80% B in 30 min; R_f 0.76); it was then treated with liquid HF for 1.5 h at 0 °C. Ether was added and the precipitate was filtered off and washed in the filter with ether.

Characterization of the Diketopiperazines

c(Arg(Tos)-Asp(OMe)). ^{1}H NMR: δ 8.00 [1H, s, lactam N*H* (Asp)], 7.96 [1H, s, lactam N*H* (Arg)], 7.64 and 7.28 (2x2H, d, $J = 7.2$ Hz, Ar*H*s in Tos), 6.87 and 6.62 (1H and 2H, 2xbr s, guanidino N*H*s), 4.23 [1H, t, $J = 5.4$ Hz, α-C*H* (Asp)], 3.90 [1H, t, $J = 4.8$ Hz, α-C*H* (Arg)], 3.60 (3H, s, OCH_3), 3.08 [2H, q, $J = 4.2$ Hz, δ-CH_2 (Arg)], 2.75 [1H, dd, $J = 16.4$ Hz and 5.4 Hz, β-CH_AH$_B$ (Asp)], 2.69 [1H, dd, $J = 16.4$ Hz and 5.4 Hz, β-CH$_A$$H_B$ (Asp)], 2.35 (3H, s CH_3 in Tos), 1.69 [2H, m, β-CH_2 (Arg)], 1.50 [2H, br s, γ-CH_2 (Arg)]; ^{13}C NMR: δ 171.2, 168.7, 168.0, 157.7, 142.8, 141.9, 129.8, 126.4, 54.5, 52.3, 52.0, 41.3, 37.6, 31.3, 25.2, 21.8. HR-MS was found for $C_{18}H_{25}N_5O_6S$ [M + H$^+$] 440.15868 (error: 3.9 ppm). R_f 0.49.

c(Arg(Tos)-Asp(OcHex)). ^{1}H NMR: δ 8.12 [1H, s, lactam N*H* (Asp)], 8.10 [1H, s, lactam N*H* (Arg)], 7.63 and 7.28 (2x2H, d, $J = 8.3$ Hz, Ar*H*s in Tos), 7.03, 6.77 and 6.57 (3x1H, 3xbr s, guanidino N*H*s), 4.66 [1H, tt $J = 9.0$ Hz and 3.9 Hz, 1-C*H* (cHex)], 4.21 [1H, t, $J = 5.1$ Hz, α-C*H* (Asp)], 3.91 [1H, t, $J = 4.8$ Hz, α-C*H* (Arg)], 3.05 [2H, qa, $J = 4.3$ Hz, δ-CH_2 (Arg)], 2.64 [2H, d, $J = 5.5$ Hz, β-CH_2 (Asp)], 2.33 (3H, s CH_3 in Tos), 1.65 [6H, overlapping br m, β-CH_2 (Arg) and 2-CH_2 (cHex)], 1.46 [2H, br s, γ-CH_2 (Arg)], 1.40-1.26 [4H, br m, 3-CH_2 (cHex)], 1.22 [2H, m, 4-CH_2 (cHex)]; ^{13}C NMR: δ 170.1, 168.9, 168.4, 157.5, 141.9 (two coalesced lines), 129.9, 126.4, 73.0, 54.4, 51.8, 41.1, 31.9, 29.8, 28.6, 25.7, 25.4, 21.7.

c(Arg(Tos)-Asp(O^{t}Bu)). Hplc t_R 23 min, gradients: 1% B for 5 min, then 1–40% B in 30 min. R_f 0.67.

c(Arg(Tos)-Asp(OBzl)). ^{1}H NMR: δ 8.16 [2H, s, lactam N*H*s (Asp and Arg)], 7.63 and 7.28 (2x2H, d, $J = 8.3$ Hz, Ar*H*'s in Tos), 7.39–7.30 [5H, overlapping m's, C_6H_5 (Bzl)], 7.01, 6.81 and 6.58 (3x1H, 3xbr s, guanidino N*H*s), 5.09 [2H, s, CH_2 (Bzl)], 4.26 [1H, t, $J = 5.1$ Hz, α-C*H* (Asp)], 3.92 [1H, t, $J = 4.8$ Hz, α-C*H* (Arg)], 3.04 [2H, q, $J = 4.3$ Hz, δ-CH_2 (Arg)], 2.77 [2H, d, $J = 5.6$ Hz, β-CH_2 (Asp)], 2.33 (3H, s CH_3 in Tos), 1.65 [2H, br m, β-CH_2 (Arg)], 1.46 [2H, br s, γ-CH_2 (Arg)]; ^{13}C NMR: δ 171.7, 168.6, 168.3, 157.5, 142.5, 142.0, 137.0, 129.9, 129.3, 128.8, 128.7, 126.5, 66.7, 54.6, 51.8, 41.0, 37.3, 29.8, 25.4, 21.8. R_f 0.62. MS was found for the title compound $C_{24}H_{29}N_5O_6S$ [M + H$^+$] 515.9 and [M + H]$^+$ = 547.9 (H-Arg(Tos)-Asp(OBzl)-OMe) and [M + H]$^+$ = 575.9 (For-Arg(Tos)-Asp(OBzl)-OMe) as impurities.

c(Arg(Tos)-Glu(OMe)). ^{1}H NMR: δ 8.16 (2H, s, lactam N*H*s), 7.62 and 7.28 (2x2H, d, $J = 7.2$ Hz, Ar*H*s in Tos), 7.03, 6.73 and 6.56 (3x1H, 3xbr s, guanidino N*H*s), 3.86 [1H, t, $J = 5.1$ Hz, α-C*H* (Glu)], 3.80 [1H, t, $J = 4.8$ Hz, α-C*H* (Arg)],

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3.57 (3H, s, OCH_3), 3.04 [2H, q, $J = 4.1$ Hz, δ-CH_2 (Arg)], 2.37 [2H, m, γ-CH_2 (Glu)], 1.97 and 1.88 [2x1H, 2xm, β-C$H_A H_B$ (Glu)], 2.33 (3H, s CH_3 in Tos), 1.62 [2H, m, β-CH_2 (Arg)], 1.44 [2H, br s, γ-CH_2 (Arg)]; ^{13}C NMR: δ 173.6, 168.8, 168.4, 157.5, 141.9 (two coalesced lines), 129.9, 126.4, 54.6, 54.0, 52.2, 41.0, 31.1, 30.0, 29.2, 25.4, 21.7. HR-MS was found for $C_{19}H_{27}N_5O_6S$ [M + H$^+$] 454.17569 (error: 0.8 ppm).

c(Arg(Tos)-Glu(O^{t}Bu)). ^{1}H NMR: δ 8.15 [1H, s, lactam N*H* (Arg)], 8.13 [1H, s, lactam N*H* (Glu)], 7.63 and 7.28 (2x2H, d, $J = 7.2$ Hz, Ar*H*s in Tos), 7.03, 6.82 and 6.58 (3x1H, 3xbr s, guanidino N*H*s), 3.85 [1H, t, $J = 5.1$ Hz, α-C*H* (Glu)], 3.80 [1H, t, $J = 4.8$ Hz, α-C*H* (Arg)], 3.06 [2H, q, $J = 4.3$ Hz, δ-CH_2 (Arg)], 2.26 [2H, m, γ-CH_2 (Glu)], 1.92 and 1.83 [2x1H, 2xm, β-C$H_A H_B$ (Glu)], 2.33 (3H, s CH_3 in Tos), 1.66 and 1.59 [2x1H, 2xm, β-C$H_A H_B$ (Arg)], 1.44 [2H, br s, γ-CH_2 (Arg)], 1.38 (9H, s, CH_3 in ^{t}Bu); δ_C 172.5, 168.8, 168.5, 157.5, 142.4, 142.0, 129.9, 126.4, 80.7, 54.5, 54.1, 41.0, 31.4, 31.2, 29.3, 28.6, 25.4, 21.7. HR-MS was found for $C_{22}H_{33}N_5O_6S$ [M + H$^+$] 496.22434 (error: 2.7 ppm). R_f 0.72, t_R 22.5 min, hplc gradients: 1% B for 5 min, then 1 → 40% B in 30 min.

c(Arg(Tos)-Glu(OcHex)). ^{1}H NMR: δ 8.15 [1H, s, lactam N*H* (Arg)], 8.13 [1H, s, lactam N*H* (Glu)], 7.63 and 7.28 (2x2H, d, $J = 7.2$ Hz, Ar*H*s in Tos), 7.03, 6.82 and 6.58 (3× 1H, 3xbr s, guanidino N*H*s), 3.85 [1H, t, $J = 5.1$ Hz, α-C*H* (Glu)], 3.80 [1H, t, $J = 4.8$ Hz, α-C*H* (Arg)], 3.06 [2H, q, $J = 4.3$ Hz, δ-CH_2 (Arg)], 2.26 [2H, m, γ-CH_2 (Glu)], 1.92 and 1.83 [2x1H, 2xm, β-C$H_A H_B$ (Glu)], 2.33 (3H, s CH_3 in Tos), 1.66 and 1.59 [2x1H, 2xm, β-C$H_A H_B$ (Arg)], 1.44 [2H, br s, γ-CH_2 (Arg)], 1.38 (9H, s, CH_3 in ^{t}Bu); ^{13}C NMR: δ 172.5, 168.8, 168.5, 157.5, 142.4, 142.0, 129.9, 126.4, 80.7, 54.5, 54.1, 41.0, 31.4, 31.2, 29.3, 28.6, 25.4, 21.7. HR-MS was found for $C_{24}H_{35}N_5O_6S$ [M + H$^+$] 522.2395 (error: 1.7 ppm).

c(Arg-Asp). Hplc t_R 3.7 min, gradient: 1% B for 5 min, then 1 → 40% B in 30 min. MS was calculated for $C_{10}H_{17}N_5O_4$ (271.2), and [M + H$^+$] 272.2 was found.

c(Arg-Glu). Hplc t_R 3.5 min, gradient: 1% B for 5 min, then 1–40% B in 30 min. MS was calculated for $C_{11}H_{19}N_5O_4$ (285.3), and [M + H$^+$] 286.3 was found.

c(Arg-Asp(Phe-OH)). Hplc t_R 3 min, gradient: 1% B for 5 min, then 1 → 40% B in 30 min. MS was calculated for $C_{19}H_{26}N_6O_5$ (418.4), and [M + H$^+$] 419.2 was found.

Inhibition of *in Vitro* Binding of Fibrinogen to Isolated $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ Integrin Assay

The binding affinities of the synthesized compounds to integrin $\alpha_{IIb}\beta_3$ were characterized by a solid phase competitive displacement assay as described [8]. Human fibrinogen (100 mg) was dissolved in aqueous NaCl (0.3 M, 5 ml) at 30 °C and then further diluted with 0.1 M $NaHCO_3$(aq) to a final concentration of 1 mg/ml. Biotin *N*-hydroxysuccinimide ester (2 mg) was dissolved in *N*,*N*-dimethylformamide (2 ml) and added to 6 ml of fibrinogen solution. The reaction mixture was incubated for 90 min at 30 °C and dialyzed for 3 h at RT against buffer 1 (3 l, 20 mM Tris, 150 mM NaCl, pH=7.4). After dialysis, the solution was centrifuged for 5 min at 5400 rpm and Tween 20 (0.005%) was added (stock solution). Human integrin (10µl of $\alpha_{IIb}\beta_3$ or 5µl of $\alpha_v\beta_3$ solution, purchased from Calbiochem) were diluted in 10.2 ml of buffer 2 (20 mM Tris, 150 mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 1 mM $MnCl_2$, pH = 7.4) and adsorbed to 96-well (100 µl/well) high-binding microtiter plates (Greiner, Lumitrac 600) overnight at 4 °C. The remaining integrin solution was thrown away and nonspecific receptor-binding sites were blocked with 1% BSA in buffer 2 (200 µl/well). Following incubation for 1 h at RT, the plates were washed twice with buffer 3 (buffer 2 containing 0.1% of Tween 20). The potential antagonists were serially diluted with buffer and solutions added (50 µl/well) at the same time with biotinylated fibrinogen (50 µl/well, 1 : 10 dilution of stock solution in buffer 2) to each well. The plates were incubated for 2 h at RT and then washed twice with buffer 3. In each well, peroxidase-conjugated antibiotin goat antibody (100 µl/well, 1 : 1000 dilution of purchased solution in buffer 3 + 0.1% of BSA) was added and incubated for another hour. The microtiter plates were washed with buffer 3 three times. Finally, chemiluminescence substrate (50 µl/well) was added and the luminescence detected with a GENios (Tecan Group AG) multimode research reader 3 times in the 10 min period. Positive controls received no inhibitors while negative controls received no ligands, c(RGDfV) was used as the internal standard. The assays were performed at least in triplicate. The mean experimental data were fitted to the sigmoid model and IC_{50} values were calculated from the dose-response curve (OriginPro, OriginLab®, Version 7.5).

RESULTS

Semiempirical Optimization of Diketopiperazine Structures (AM1)

Xray studies revealed that the Arg-Gly-Asp sequence in c(RGDfV) adopts a stretched overall conformation when bound to $\alpha_v\beta_3$ integrin receptor (Figure 1) [9]. While arginine side chain is oriented to Asp^{218} of integrin α_v subunit, the Asp side chain makes two hydrogen bonds with Ser^{121} and Ser^{123} of integrin β_3 subunit, respectively. Based on Xray data it is known that in the 'receptor bound form' of c(RGDfV), the distance between the δ-guanidino group of arginine and the β-carboxyl group of aspartic acid is equal to 13.72 Å. We investigated whether our peptidomimetics, c[Arg-Asp], c[Arg-Glu] and c[Arg-Asp(Phe-OH)] diketopiperazines, can adopt the desired distance between the appropriate functional groups in an extended conformation. Geometry optimizations at the AM1 level of theory were performed for all three molecules and it was found that neither c[Arg-Asp] nor c[Arg-Glu] can adopt the required distance (13.72 Å) between the appropriate functional groups. The achieved interatomic distances are 12.8 and 13.2 Å for c[Arg-Asp] and c[Arg-Glu] respectively, while the distance between the δ-guanidino group of arginine and the α-carbonyl group of phenylalanine in c[Arg-Asp(Phe-OH)] can be easily stretched up to the optimal value of 13.72 Å (Figure 2).

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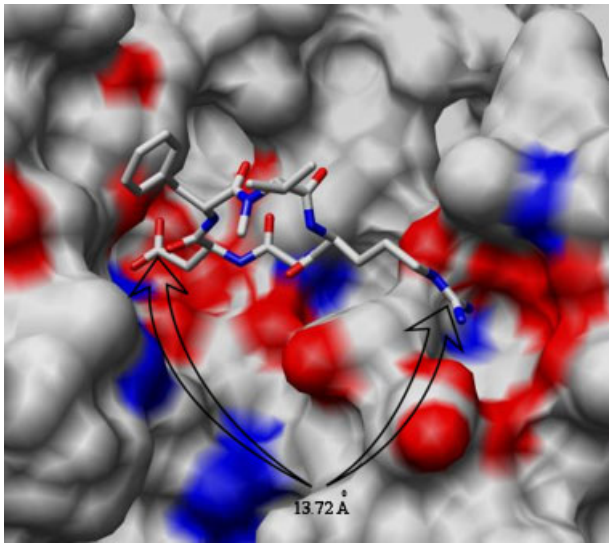

Figure 1 c(RGDfV) pentapeptide bound to $\alpha_v\beta_3$ integrin receptor. Arginine side chain is oriented to Asp^{218} of integrin α_V subunit while the side chain of Asp makes hydrogen bonds both with Ser^{121} and Ser^{123} of integrin β_3 subunit. The distance between these two functional groups is 13.7 Å.

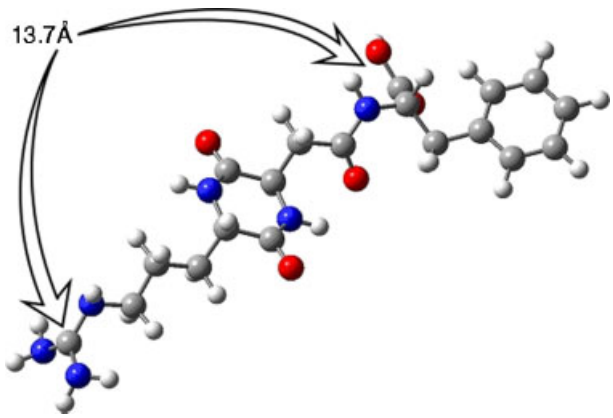

Figure 2 c[Arg-Asp(Phe-OH)] in its extended conformation can easily adopt the distance of 13.7 Å between the δ-guanidino group of arginine and the α-carbonyl group of phenylalanine, which was found between the functional groups in c(RGDfV) bound to $\alpha_v\beta_3$ integrin receptor.

Synthesis

For the solution phase synthesis of Arg-Glu and Arg-Asp diketopiperazines the common Boc/Bzl protecting strategy was chosen, which allows further derivatization of the cyclic dipeptides after selective deprotection of the functional groups. Accordingly Boc-Arg(Tos)-Glu(OBzl)-OMe and Boc-Arg(Tos)-Asp(OBzl)-OMe were prepared, the *N*-terminal Boc group was removed and cyclization of H-Arg(Tos)-Glu(OBzl)-OMe.HCl and H-Arg(Tos)-Asp(OBzl)-OMe.HCl was performed by the usual NH_3/methanol method [4]. Surprisingly enough, MS data in both cases indicated the formation of diketopiperazine methylester. Accordingly it seemed that cyclization happened through the γ-carboxyl group of the glutamic acid and the β-carboxyl group of the aspartic acid, forming an eight- and a seven-membered ring, respectively. As the formation of these cycles instead of the usual preferred six-membered ring system was unlikely, NMR measurements were performed in the case of both compounds.

For each cyclodipeptide discussed here 1,4-diazocine-dione and 1,4-diazonine-dion ring systems can be ruled out on the basis of ^{1}H-^{13}C-HMBC spectra in which both lactam carbonyl signals exhibit cross peaks with the sharp singlets of the lactam NH groups, but do not give correlation with the signals of β and/or γ protons in the Asp and Glu residue. The latter proton signals have correlation with β or γ carbonyl signal which can be identified through its cross peak with the proton signals of the methoxycarbonyl group. The presence of the same diketopiperazine ring system in both products is also evidenced by the same ^{15}N-NMR resonance (116 ppm on the scale adjusted for the reference signal of liquid ammonia) obtained by ^{1}H-^{15}N-HMBC measurements pointing to the same degree of amide conjugation, which should be different in larger rings. Racemization (ca 15%) was detected on the α-CH (Asp) as supported by the following additional ^{1}H-NMR signals of diketopiperazine and Asp residue: 8.22 N*H* (lactam); 4.15 [t, $J = 4.7$ Hz, α-C*H* (Asp)]; 3.78 [t, $J = 5.5$ Hz, α-C*H* (Arg)]; 2.78 and 2.72 [2xdd, $J = 16.7$ Hz and 4.7 Hz, β-CH_AH_B (Asp)].

From the data of the NMR spectra it became clear that the six-membered diketopiperazine ring was formed and transesterification of the benzyl to the methyl ester occurred at the γ- and β-carboxyl groups respectively.

As the transesterification was proved, the question arose whether it happened before or after cyclization. To answer this question H-Arg(Tos)-Asp(OBzl)-OMe.HCl was dissolved in NH_3/methanol and samples were taken from the solution at certain time intervals and analyzed on a thin layer of silica gel. The ninhydrin positive spot on TLC of the dipeptide ester disappeared after 1 h and two new ninhydrin negative, chlor/tolidine positive spots appeared. After 4 h only one spot was present, the R_f value of this corresponded to that of the c[Arg(Tos)-Asp(OMe)] identified previously by NMR. From this observation it was concluded that transesterification took place mainly after ring closure. This finding was also supported by NMR measurement, performed following the interruption of the cyclization reaction after 1 h by evaporating NH_3/methanol.

No transesterification was found, when side chain β- or γ-carboxyl groups were protected with *tert*-butyl ester or cyclohexyl ester groups.

However we were keen on the proper cyclization of H-Arg(Tos)-Asp(OBzl)-OMe.HCl in solution and tried it with the Suzuki method [6]. Accordingly, the dipeptide ester HCl was treated with an equivalent amount of *N*-methyl morpholine and heated in *sec*-butanol in the

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presence of a catalytic amount of acetic acid. After 3 h boiling, a brown multicomponent reaction mixture was formed. To avoid the presence of base from the reaction mixture, the deprotection of the Boc group was carried out with the aid of formic acid and after heating the product in *sec*-butanol-toluene mixture [7], c[Arg(Tos)-Asp(OBzl)] was obtained as the main product. Its structure was proved by NMR and MS; however, in the MS spectrum, the signals of H-Arg(Tos)-Asp(OBzl)-OMe and For-Arg(Tos)-Asp(OBzl)-OMe could also be detected.

Because of the above mentioned complications in the solution phase synthesis, c(Arg-Asp) and c(Arg-Glu) were prepared finally on solid phase. Fmoc-Asp(OBu^t)-OH and Fmoc-Glu(OBu^t)-OH respectively, were coupled to H-Arg(Tos)-*O*-PAM with the aid of DIC/HOBt. Diketopiperazine formation from Fmoc-Asp(OBu^t)-Arg(Tos)-*O*-PAM was tried as previously described in the literature [10] in 50% piperidine/DMF; however, after 24 h, remarkable racemization occurred, although the protected diketopiperazine formed nearly in quantitative yield. Therefore Fmoc group was first removed cautiously with 0.1 M TBAF/DMF; and after neutralization, the peptide resin stood in 0.1 M acetic acid/DCM solution for 2 days. The side chain protecting groups were removed in liquid HF resulting in c(Arg-Asp) and c(Arg-Glu) diketopiperazines. For the preparation of [Arg-Asp(Phe-OH)] the *tert*-butyl ester group was removed from c[Arg(Tos)-Asp(OBu^t)] and phenylalanine benzyl ester was acylated with c[Arg(Tos)-Asp] followed by liquid HF treatment.

Receptor Binding

The binding affinity of the diketopiperazines was tested on two integrin receptors, namely on the $\alpha_v\beta_3$ (vitronectin, Figure 3) and on the $\alpha_{IIb}\beta_3$ (fibrinogen, Figure 4). Selective binding on the $\alpha_v\beta_3$ is shown by all the three compounds; the lowest IC_{50} value, 0.74 μM was obtained in the case of c[Arg-Asp(Phe-OH)]. Much less affinity was shown toward the $\alpha_{IIb}\beta_3$ integrin receptor; here only c[Arg-Asp(Phe-OH)] has a measurable IC_{50} value of 159 μM which is three orders of magnitude less than that in the vitronectin assay.

DISCUSSION

The integrin receptor selectivity of RGD-peptides is determined by the distance between the arginin guanidino and the aspartic acid β-carboxyl group. So we supposed that c(Arg-Asp), c(Arg-Glu) and c[Arg-Asp(Phe-OH)] – where this critical distance is increasing – may serve as RGD peptidomimetics. The absence of glycine, which is considered to be essential for the integrin receptor binding of the RGD sequence, is curious in these mimetics.

Geometry optimizations of the above molecules revealed that in their extended conformation the distance between the carbon atom of the δ-guanidino group of arginine and the carboxyl group of the appropriate diketopiperazines are 12.8, 13.2 and 13.7 Å, respectively. As according to the crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in

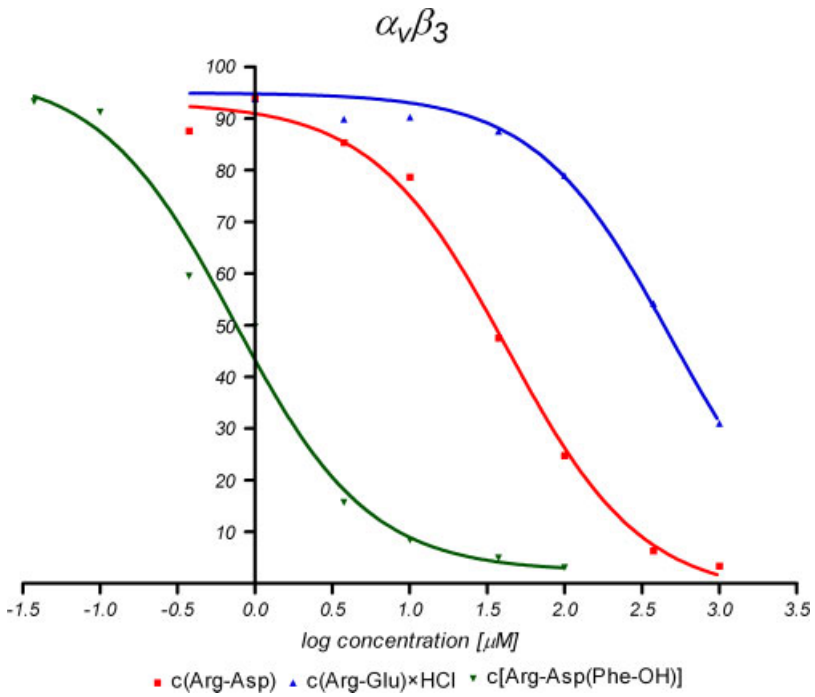

Figure 3 Inhibition of binding of vitronectin to isolated $\alpha_v\beta_3$ integrin receptor with c(Arg-Asp) IC_{50} 42 μM; c(Arg-Glu) IC_{50} 480 μM and c[Arg-Asp(Phe-OH)] IC_{50} 0.74 μM.

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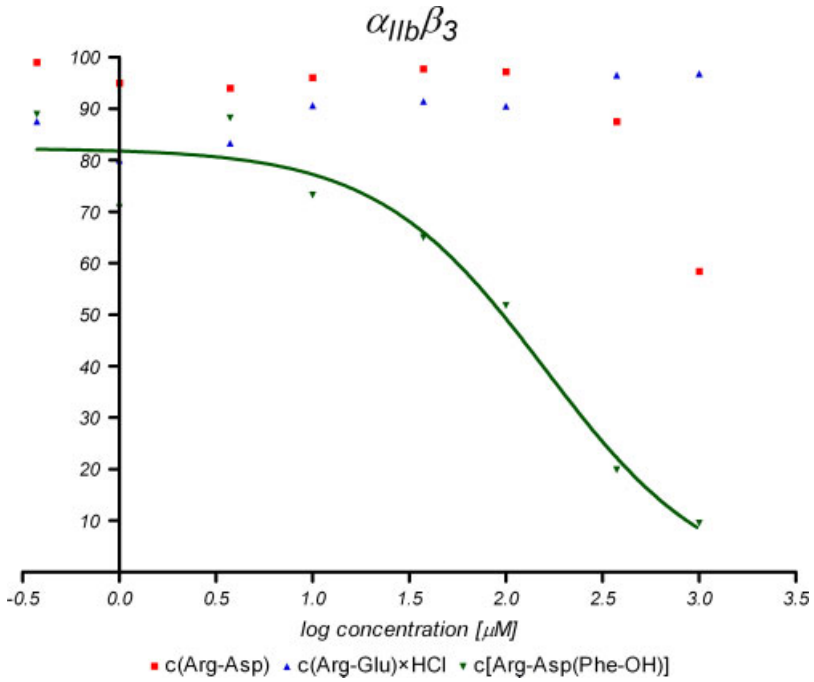

Figure 4 Inhibition of binding of fibronectin to isolated $\alpha_{IIb}\beta_3$ integrin receptor with c(Arg-Asp); c(Arg-Glu) and c[Arg-Asp(Phe-OH)] IC_{50} 159 μM.

complex with the c(RGDfV) ligand, the distance between the carbon atom of the Arg δ–guanidino group and of the Asp β-carbonyl group is 13.72, the synthesis and receptor binding investigation of these models seemed to be reasonable.

For the preparation of the new diketopiperazines the solid phase method proved to be the best. In solution the Fischer method cannot be used for diketopiperazine formation in case of dipeptide methyl esters containing glutamic acid or aspartic acid in the *C*-terminal position with benzyl ester side chain protection, because transesterification of the benzyl ester group occurs, and the removal of the side chain methyl ester group by saponification from the protected diketopiperazine induces ring opening at the same time. Neither did the Suzuki method prove to be successful for the solution phase cyclization of H-Arg(Tos)-Asp(OBzl)-OMe.HCl. Recently similar difficulties were reported by Ivanov and his coworkers in the acetic acid catalyzed cyclization reaction of aspartic acid or glutamic acid containing dipeptide benzyl esters [11]. c[Arg(Tos)-Asp(OBzl)] can only be prepared in solution after cautious deprotection of the Boc group with formic acid – to avoid the hydrolysis of benzyl ester – followed by heating the dipeptide formate in *sec*-butanol-toluene mixture.

The diketopiperazines show different affinity to the two members of the β_3 integrin receptor group. In both cases, receptor affinity of the compounds is growing in the following order c(Arg-Glu)<c(Arg-Asp)<c[Arg-Asp(Phe-OH)]; however, all of them show much higher binding to the $\alpha_v\beta_3$ than to the $\alpha_{IIb}\beta_3$ integrin receptor. In agreement with the data obtained by the geometry optimization of the diketopiperazine structures, the highest affinity was found in the case of c[Arg-Asp(Phe-OH)] with an IC_{50} value of 159 μM for the $\alpha_{IIb}\beta_3$ and of 0.74 μM for the $\alpha_v\beta_3$ receptor. The lower binding to the fibrinogen receptor is easy to understand as it is known that distance data – between the terminal basic (amino, guanidine etc.) and carboxylic groups – within the range of 12–15 Å are optimal in the $\alpha_{IIb}\beta_3$ integrin receptor antagonists [12].

Although in the extended conformation of c[Arg-Asp(Phe-OH)] the distance between the side chain functional groups is the same as that in the case of the $\alpha_v\beta_3$ receptor bound c(RGDfV) and it contains an aromatic moiety, there is still a great difference in their IC_{50} value, indicating that proper receptor binding requires other structural elements as well.

These results indicate that our new diketopiperazines, in-spite of the omission of glycine from the RGD sequence, show α_v selectivity in the β_3 integrin receptor group and as such they are possible candidates for further RGD mimetics by derivatization either on the carboxyl or on the guanidino group.

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