Amino Acids and Peptides. XXV.[1] Preparation of Fibronectin-Related Peptide Poly(ethylene glycol) Hybrids and Their Inhibitory Effect on Experimental Metastasis

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Hybrids of fibronectin-related peptides [Arg–Gly–Asp (RGD), Arg–Gly–Asp–Ser (RGDS)] and poly(ethylene glycol) (PEG) were prepared and their inhibitory effects on experimental metastasis in mice were examined. The inhibitory effect of RGD was markedly potentiated by hybrid formation with poly(ethylene glycol) #6000. As to inhibitory effect, RGD was more potent than RGDS and RGD PEG hybrids were superior to RGDS PEG hybrids. Hybrid formation with PEG #6000 was more effective than that with PEG #4000.

Key words fibronectin; metastasis inhibitor; PEGylation; poly(ethylene glycol) hybrid; tumor metastasis

Fibronectin, a cell adhesion glycoprotein consisting of A and B chains, has been found on the cell surface, in the extracellular matrix, in the plasma, and in other body fluids.[2] The Arg–Gly–Asp–Ser (RGDS) sequence exists at positions 1493—1496 in the human plasma fibronectin A chain,[3] and Arg–Gly–Asp (RGD) and RGDS were reported to be inhibitors of platelet aggregation[4] and experimental metastasis.[5] Clinical application of these peptides as metastasis inhibitors has been examined, but there are many problems in the clinical use of peptides because of their short half-life in blood.

Since poly(ethylene glycol) (PEG) is stable, bioinert and only weakly immunogenic, it seems to be a promising canditate for a drug-carrier. Many studies have reported that some enzyme-PEG hybrids have prolonged half-life in blood, improved solubility and lower immunogenicity.[6] PED-oligopeptide hybrids may thus potentiate and prolong the activity of peptides themselves. In the preceding communication,[7] we reported that RGD-aminoPEG (aPEG) hybrid exhibited a potent inhibitory effect on lung metastasis in mice. Here we describe in detail the preparation of RGDS hybrids and their inhibitory effect on experimental metastasis. For preparation of PEG hybrids, two different types of PEG, #4000 [4 K; molecular weight (M.W.), 3000—3700, average M.W., 3350] and #6000 (6 K; M.W., 6700—9000, average M.W., 7850) were converted to corresponding aPEGs according to the procedure reported by Pillai and Mutter.[8] First, RGD-aPEG hybrid was prepared as shown in Fig. 1. Z(OMe)–Gly–ONp and H–Asp(OBzl)–OH[9] were allowed to react to give a protected dipeptide, which was purified by silica gel column chromatography. The dipeptide was treated with trifluoroacetic acid (TFA) to remove the Z (OMe) group and then reacted with Boc–Arg(Tos)–OH[10] by the mixed anhydride method[11] to give a protected tripeptide, Boc–Arg(Tos)–Gly–Asp(OBzl)–OH. The tripeptide was then reacted with aPEG by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) method.[12] The tripeptide-aPEG hybrid was treated with HF[13] to remove all protecting groups and the product was purified by Sephadex G-25 column chromatography and HPLC. The preparative HPLC profile of RGD-aPEG 6K is shown in Fig. 2. The synthetic PEG hybrids each showed a broad peak on HPLC, probably reflecting heterogeneous molecular weight of PEG.

RGD contents of the hybrids 4 K and 6 K, calculated from the average recovery of amino acids in the respective acid hydrolysates, were 278 μmol/g and 115 μmol/g respectively.

RGD (H–Arg–Gly–Asp–$NH_2 \cdot 2HCl$) was prepared by

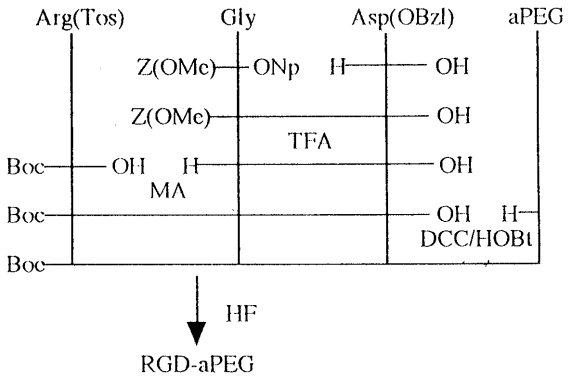

Fig. 1. Synthetic Scheme for RGD-aPEG
MA = mixed anhydride method.

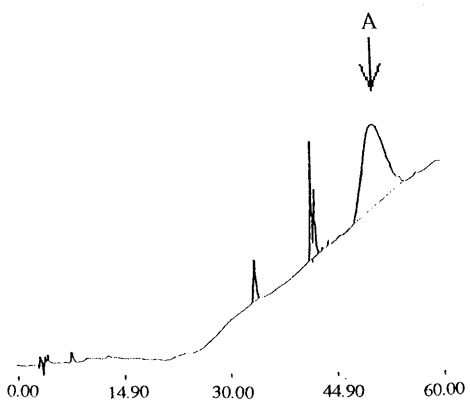

Fig. 2. Preparative HPLC Profile of RGD-aPEG6K
Column, YMC AQ-303 (20 × 250 mm). Flow rate, 12 ml/min. Solvent, A) 0.1% TFA/H_2O; B) 0.1% TFA/CH_3CN. Gradient, A/B: 75/25→75/25→50/50 (0 min→15 min→50 min). RGD-aPEG4K was obtained in peak A.

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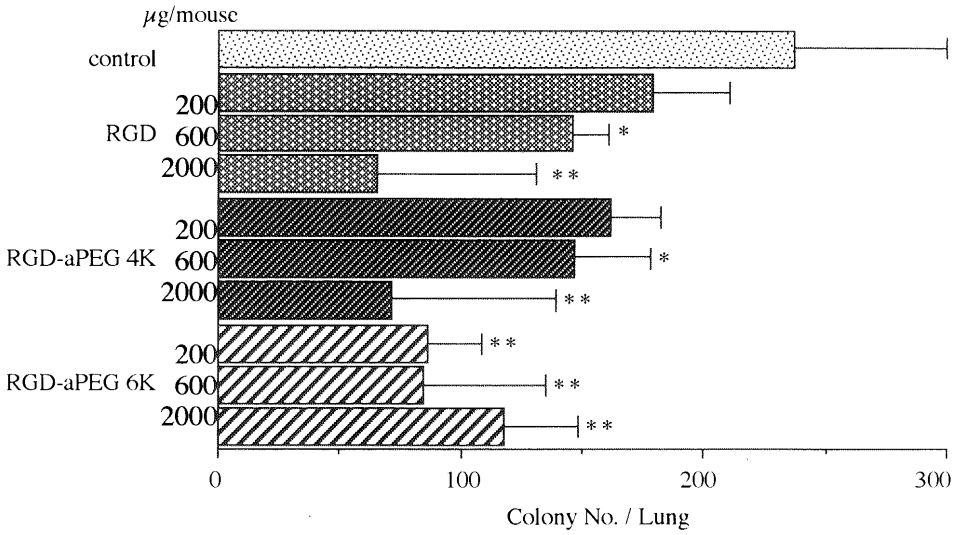

Fig. 3. Inhibitory Effect of RGD-aPEG Conjugates on the Formation of Lung Metastasis

B16-BL6 cells (1×10^5/0.2 ml) were injected i.v. with or without admixing with various concentrations of aPEG-RGD into five mice per group. Lung tumor colonies were examined 21 d later. Values are the mean ± S.D. * $p<0.05$, ** $p<0.01$ compared with untreated control by Student's t-test.

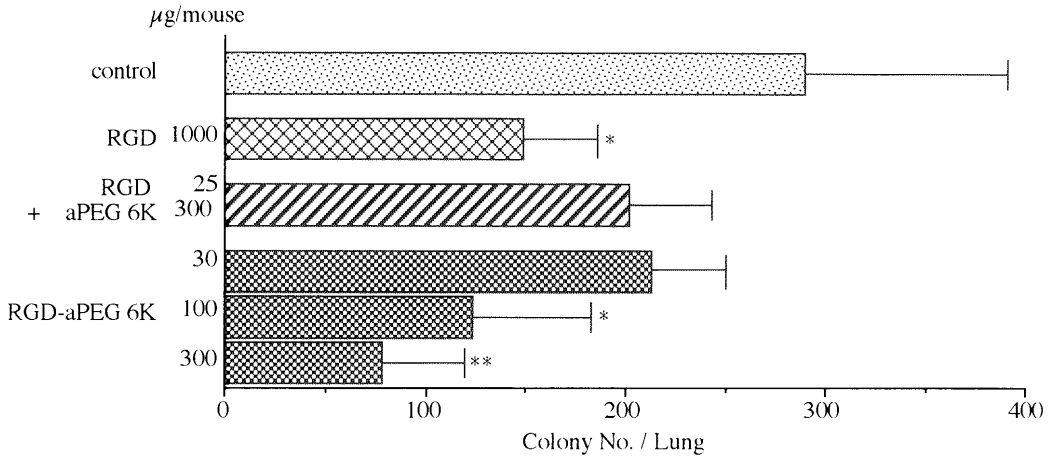

Fig. 4. Inhibition of Lung Colonization by RGD-aPEG

The tumor colonization assay was carried out as described in Fig. 2. Values are the mean ± S.D. * $p<0.05$, ** $p<0.005$ compared with uncreated control by Student's t-test.

the solid-phase method. To compare the inhibitory effect of the hybrids on experimental metastasis of B16 melanoma BL6 with that of RGD in mice, each hybrid was mixed with tumor cells in serum-free MEM; single-cell suspensions of 1×10^5 cells were injected into the lateral tail vein of syngeneic C57BL/6 male mice at 6 weeks of age. Three weeks later, the animals were killed, and the lungs were excised and fixed in 10% formaldehyde. The number of surface melanoma colonies was counted macroscopically. PEGs 4K and 6K were found to have no inhibitory effect on experimental metastasis as already reported.[14] Trifluoroacetic acid is cytotoxic and synthetic hybrids were purified by HPLC using TFA-containing eluent. So, after purification by HPLC, the hybrids were converted to hydrochlorides by repeated lyophilization from HCl-containing water. The inhibition of metastasis was not due to their cytotoxicity, because the incubation of B16 melanoma cells with these hybrids and RGD at 3 mg/ml for 1 h did not affect subsequent cell growth (data not shown).

As shown in Fig. 3, RGD and hybrids 4K and 6K inhibited lung metastasis. RGD and hybrid 4K exhibited their inhibitory effect at a dose of 600 μg (RGD content, 167 nmol)/mouse: hybrid 6K, at 200 μg (RGD content, 23 nmol)/mouse. The inhibitory effect of hybrid 6K at a dose of 200 μg/mouse was nearly equal to that at 2000 μg (4.78 μmol)/mouse of RGD. The reason why hybrid 6K did not show a dose-dependent effect was not clear. We speculated that the concentration of hybrid 6K might have been too high. Therefore, the effect of hybrid 6K was further examined at lower concentrations.

As shown in Fig. 4, diluted hybrid 6K inhibited metastasis dose-dependently. The inhibitory effect of 100 μg nearly equaled that of 1000 μg of RGD. One thousand μg of RGD·2HCl (M.W., 418) is about 2.4 μmol and 100 μg of RGD-aPEG6K contains about 11.5 nmol of RGD. Thus, it can be said that the inhibitory effect of hybrid 6K is 10 times and more than 200 times as potent as that of RGD in terms of weight and molar ratios, respectively. A mixture of RGD (25 μg, 60 nmol) and

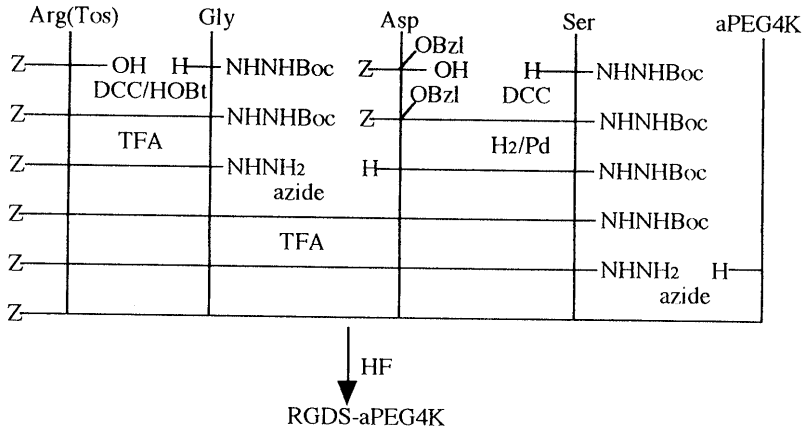

Fig. 5. Synthetic Scheme for RGDS-aPEG4K

aPEG 6 K (300 μg) was less active than 300 μg of RGD-aPEG 6 K hybrid, which contains about 35 nmol of RGD. This indicates that the presence of the covalent bond between RGD and aPEG is necessary for potentiation of the inhibitory effect of RGD.

Next, RGDS-aPEG 4 K hybrid was prepared as shown in Fig. 5. Z–Asp(OBzl)–OH and H–Ser–NHNHBoc[15] were coupled by the DCC method, followed by hydrogenation to remove the Z and benzyl groups. Since Asp(OBzl)–Ser binding is reported to be subject to imido formation,[16] removal of the benzyl group at an early step is reasonable. Z–Arg(Tos)–OH and H–Gly–NHNHBoc[17] were then coupled by the DCC/HOBt method, followed by TFA treatment to remove the Boc group. The resulting dipeptide hydrazide was coupled with H–Asp–Ser–NHNHBoc by the azide method to give a tetrapeptide, which was treated with TFA. The resulting tetrapeptide hydrazide, Z–Arg(Tos)–Gly–Asp–Ser–$NHNH_2$, was reacted with aPEG 4 K by the azide method to give a hybrid, followed by HF treatment. The product was purified by LH 20 column chromatography and HPLC. The RGDS content of the hybrid was 330 μmol/g.

As shown in Fig. 6, RGDS-aPEG 6 K was prepared using β-cyclohexyl aspartate[Asp(OcHx)][18] which was more stable than β-benzyl aspartate to imide formation. Z–Arg(Tos)–Gly–OBzl was prepared by the mixed anhydride method and was converted to the hydrazide by hydrazine treatment. Boc–Asp(OcHx)–OH and H–Ser(Bzl)–OH were also coupled by the mixed anhydride method, followed by TFA treatment to remove the Boc group. The dipeptides were allowed to react by the azide method and the resulting tetrapeptide was coupled with aPEG 6 K by the DCC/HOBt method. The protecting groups on the hybrid were removed by HF treatment and the resulting material was purified by Sephadex G-25 column chromatography and HPLC. The RGDS content of the hybrid 6 K was 163 μmol/g. The standard sample, H–Arg–Gly–Asp–Ser–NH_2 (RGDS) 2HCl, was prepared by the solid-phase method. The inhibitory effect of hybrids 4 K and 6 K are shown in Fig. 7.

RGDS showed a weak inhibitory effect at a dose of 300 μg/mouse. The synthetic aPEG hybrids also showed inhibitory effects, but RGDS-aPEG4 K showed no potention by hybrid formation in terms of weight ratio. The inhibitory effect at 1000 μg of hybrid 4 K corresponded

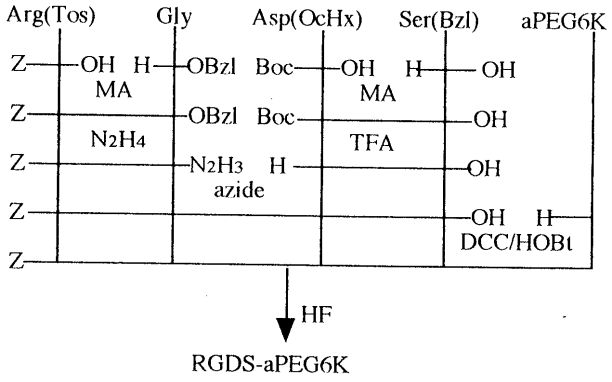

Fig. 6. Synthetic Scheme for RGDS-aPEG6K

to that at 300 μg of RGDS (300 μg of RGDS is about 0.59 μmol and 1000 μg of hybrid 4 K contains about 0.46 μmol of RGDS). Thus it might be said that the effect of RGDS was slightly potentiated by hybrid formation in terms of molar ratio. RGDS-aPEG 6 K exhibited a nearly equal effect at doses of 300 and 1000 μg/mouse. As described above, RGD-aPEG 6 K also did not show a dose-dependent effect at doses of 200, 600 and 2000 μg, but dose-dependence was seen at lower concentrations. Dose-dependence of RGDS-aPEG 6 K also be observed at lower concentrations. These results might be explained by the limited inhibitory effect of these inhibitors. The inhibitory effects of RGD-aPEG 6 K and RGDS-aPEG hybrids were compared at the dose of 300 μg/mouse and the results are shown in Fig. 8.

When the inhibitory effects of RGD and RGDS were compared with those of the hybrids, it was clear that the effects of RGD and RGDS were potentiated by hybrid formation. RGDS did not show an inhibitory effect at this dose, but RGD did. These results indicate that the inhibitory effect of RGD is more potent than that of RGDS. RGDS-aPEG 4 K did not show an inhibitory effect, but RGDS-aPEG 6 K did. Similar results had been observed when RGD-aPEG hybrids were examined (Fig. 7). The RGD and RGDS contents of the respective hybrids 6 K are less than those of the respective hybrids 4 K. These results indicate that hybrid formation with PEG 6 K is more effective than that with PEG 4 K. The inhibitory effect of RGD-aPEG 6 K is more potent than that of RGDS-aPEG6K and, as described already, the

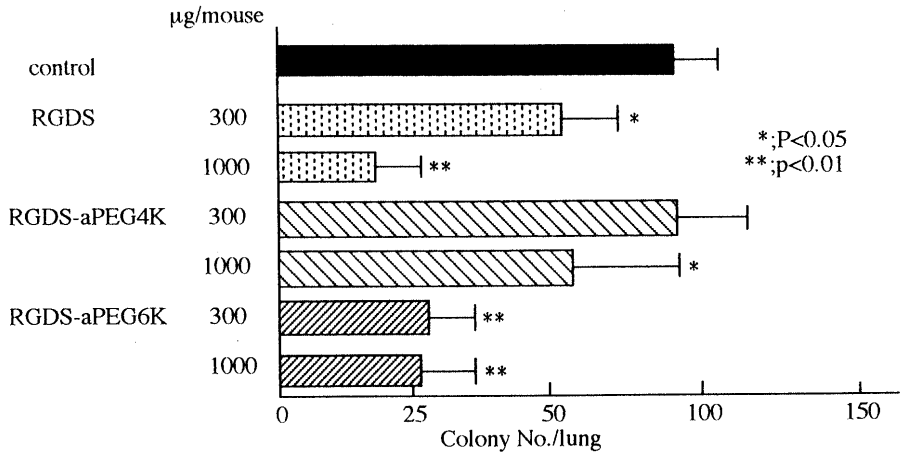

Fig. 7. Effect of RGDS-aPEGs on Lung Metastasis

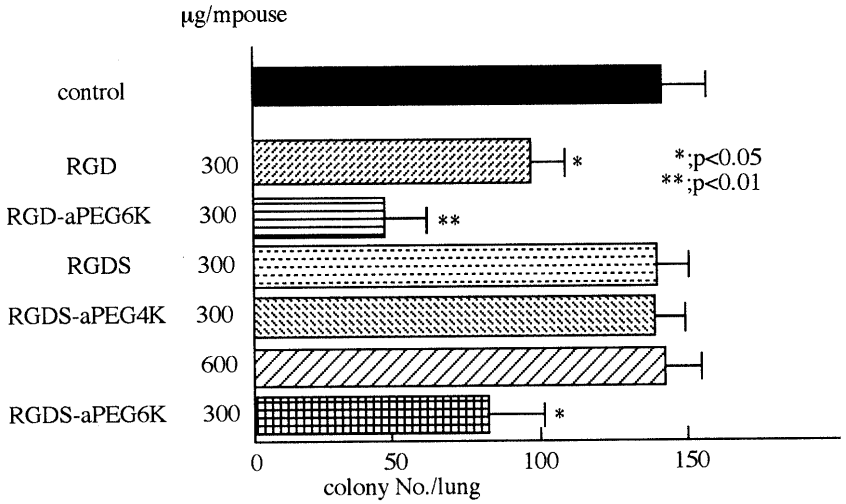

Fig. 8. Comparison of Inhibitory Effects of RGDS-aPEG and RGD-aPEG on Lung Metastasis

peptide contents of RGD-aPEG 6K and RGDS-aPEG 6K were 115 μmol/g and 163 μmol/g respectively. These results also indicate that the inhibitory effect of RGD and its hybrid are more potent than those of RGDS and its hybrid.

The reason why the inhibitory effect of RGD was potentiated by hybrid formation with PEG is not clear, but we speculate that the stability of RGD in blood may be increased by hybrid formation. We reported that YIGSR-aPEG hybrid was hydrolyzed by aminopeptidase M and chymotrypsin more slowly than the peptide itself.[19] The PEG moiety may prevent hydrolysis of the peptide portion by the enzyme. Recently, studies on the mechanisms of the enhanced inhibitory effect of YIGSR-PEG hybrid were done and the results have been submitted for publication.[20]

Experimental

Melting points are uncorrected. Solvent systems for ascending thin-layer chromatography (TLC) on Silica gel G (type 60, Merck) are indicated as follows: Rf^1 = BuOH–AcOH–H_2O (4 : 1 : 5, upper phase), Rf^2 = BuOH–pyridine–AcOH–H_2O (4 : 1 : 1 : 2), Rf^3 = $CHCl_3$–MeOH–H_2O (8 : 3 : 1, lower phase), Rf^4 = AcOEt–benzene (1 : 1), Rf^5 = $CHCl_3$–AcOH–MeOH (90 : 2 : 8). Synthetic peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h and PEG-peptide hybrids were hydrolyzed for 48 h. Amino acid compositions of acid hydrolysates were determined with a Hitachi 835 amino acid analyzer. Peptide content in a hybrid was calculated from average recovery of amino acids in an acid hydrolysate of the hybrid. Rotations were measured with a JASCO DIP-360 polarimeter. RP-HPLC was conducted with a Waters 600 apparatus equipped with a YMC Pack AQ-ODS-5 column, with a mixture of 0.1% TFA-containing CH_3CN/H_2O as an eluent. RGD and RGDS were prepared by the solid-phase method as reported.[19] The inhibitory effects of the hybrids were examined as reported.[21] PEGs #4000 (M.W. 3000—3700) and #6000 (M.W. 6700—9000) were purchased from Nacalai Tesque Co., Ltd. They were converted to aPEG according to the procedure reported by Pillai and Mutter.[8]

Z(OMe)-Gly-ONp Z(OMe)–Gly–OH (5 g, 21 mmol) and *p*-nitrophenol (3.06 g, 22 mmol) were reacted in dioxane by the DCC method in the usual manner. The product was recrystallized from EtOH. Yield 4.97 g (66%), mp 87—88 °C, Rf^3 0.90, Rf^4 0.72, Rf^5 0.83. *Anal.* Calcd for $C_{17}H_{16}N_2O_7$: C, 56.67; H, 4.47; N, 7.78. Found: C, 56.77; H, 4.39; N, 7.64.

Z(OMe)-Gly-Asp(OBzl)-OH Z(OMe)–Gly–ONp (3.64 g, 10 mmol) and H–Asp(OBzl)–OH (1.11 g, 5 mmol) were reacted in a mixture of DMF (30 ml), water (10 ml) and DIEA (0.87 ml, 5 mmol) for 6 h. The solvent was evaporated off and the residue was extracted with AcOEt and 5% citric acid. The organic layer was washed with 5% citric acid and water. The solvent was removed *in vacuo* and the residue was purified by silica gel column chromatography. The desired material was obtained in the 3% MeOH/$CHCl_3$ eluate. Yield 1.4 g (67%), amorphous powder, Rf^3 0.76. $[\alpha]_D^{27}$ +13.8° (c = 1.0, MeOH). *Anal.* Calcd for $C_{22}H_{24}N_2O_8$: C, 59.45; H, 5.44; N, 6.30. Found: C, 59.22; H, 5.54; N, 6.09.

Boc-Arg(Tos)-Gly-Asp(OBzl)-OH Boc–Arg(Tos)–OH (2.21 g, 5.16 mmol) was reacted with H–Gly–Asp(OBzl)–OH [prepared from 1.53 g (3.44 mmol) of its Z(OMe) derivative by TFA treatment] by the mixed anhydride method in the usual manner.[11] The product was purified by silica-gel column chromatography. Yield 1.21 g (51%), amorphous

powder, Rf^3 0.81, $[\alpha]_D^{27}$ +8.2° (c=1.0, dioxane). Amino acid ratios in an acid hydrolysate: Arg 0.99; Gly 1.00; Asp 0.97 (average recovery 86%). *Anal.* Calcd for $C_{31}H_{42}N_6O_{10}S$: C, 53.90; H, 6.13; N, 12.17. Found: C, 53.69; H, 6.30; N, 12.01.

RGD-aPEG 4K DCC (206 mg, 1 mmol) was added to a DMF/DCM solution (1/1, 10 ml) of Boc–Arg(Tos)–Gly–Asp(OBzl)–OH (691 mg, 1 mmol), HOBt (135 mg, 1 mmol) and aPEG 4K (amino content 0.52 meq/g, 490 mg) at −5 °C. The mixture was stirred for 24 h at room temperature and the solvent was removed *in vacuo*. The residue was purified by Sephadex LH20 column (3 × 150 cm) chromatography using MeOH/DCM (1/1) as an eluent. Yield 570 mg. The whole was treated with 5% anisole/HF (15 ml) at −10 °C for 40 min. The HF was removed and the residue was extracted with water. The aqueous solution was washed with ether and lyophilized. The residue was purified by Sephadex G-25 column (3 × 150 cm) chromatography using water as an eluent, followed by HPLC. Yield 182 mg, Rf^3 0.64. Amino acid ratios in an acid hydrolysate: Arg 0.91; Gly 1.00; Asp 0.94. The RGD content of the hybrid was determined by calculation of the average recovery of amino acids in an acid hydrolysate: 278 μmol/g. The hybrid was converted to hydrochloride by repeated lyophilization from HCl-containing water.

RGD-aPEG 6K Prepared from Boc–Arg(Tos)–Gly–Asp(OBzl)–OH (690 mg, 1 mmol) and aPEG 6K (amino content 0.186 meq/g, 640 mg) by the same procedure as described above. Yield 155 mg. Fluffy powder, Rf^3 0.65. Amino acid ratios in an acid hydrolysate: Arg 0.87; Gly 1.00; Asp 0.84. The RGD content of the hybrid was 115 μmol/g. The hybrid was converted to hydrochloride by repeated lyophilization from HCl-containing water.

Z–Asp(OBzl)–Ser–NHNHBoc Z–Asp(OBzl)–OH (5 g, 14 mmol) and H–Ser–NHNHBoc [prepared from its Z derivative (4.9 g, 14 mmol) by hydrogenation] were coupled in DCM (20 ml) by the DCC method in the usual manner. The product was recrystallized from AcOEt. Yield 4.3 g (55%), mp 129—131 °C, Rf^5 0.65, $[\alpha]_D^{27}$ −8.1° (c=1.0, DMF). Amino acid ratio in an acid hydrolysate: Asp 1.00, Ser 0.82 (average recovery 83%). *Anal.* Calcd for $C_{27}H_{34}N_4O_9$: C, 58.06; H, 6.13; N, 10.03. Found: C, 57.81; H, 6.04; N, 9.83.

Z–Arg(Tos)–Gly–NHNHBoc Z–Arg(Tos)–OH (2.08 g, 4.5 mmol) and H–Gly–NHNHBoc [prepared from 1.57 g (4.9 mmol) of its Z derivative by hydrogenation] were reacted by the DCC/HOBt method in DMF in the usual manner. The product was purified by silica gel column (2.0 × 28 cm) chromatography. The desired material was obtained in the 3% MeOH/$CHCl_3$ eluate. Yield 1.33 g (63%), mp 93—96 °C, Rf^5 0.25, $[\alpha]_D^{27}$ −2.2° (c=1.0, MeOH). Amino acid ratio in an acid hydrolysate: Arg 0.90, Gly 1.00 (average recovery 81%). *Anal.* Calcd for $C_{28}H_{39}N_7O_8S$: C, 53.07; H, 6.20; N, 15.47. Found: C, 53.37; H, 6.37; N, 15.68.

Z–Arg(Tos)–Gly–Asp–Ser–NHNHBoc Z–Arg(Tos)–Gly–NHNHBoc (1.7 g, 2.7 mmol) was treated with 5% anisole/TFA (10 ml) for 1 h. A mixture of ether and petroleum ether was added and the resulting precipitate was washed with the same mixture 5 times by decantation. The precipitate was dried *in vacuo* and dissolved in DMF (10 ml). HCl/dioxane (5.5 N, 1.61 ml) and isoamyl nitrite (0.42 ml, 2.97 mmol) were added at −20 °C and the mixture was stirred for 5 min, followed by neutralization with NMM. Z–Asp(OBzl)–Ser–NHNHBoc (1.8 g, 3.24 mmol) was hydrogenated to remove the Z and Bzl groups. The hydrogenated material was dissolved in DMF (10 ml) and the solution was combined with the azide solution described above. The reaction mixture was kept at pH 8 by addition of NMM and stirred for 40 h in a cold room. The solvent was removed *in vacuo* and the residue was extracted with AcOEt. The AcOEt layer was washed with 5% citric acid and saturated NaCl solution. It was dried over Na_2SO_4, the solvent was removed and $CHCl_3$ was added. The resulting gelatinous material was collected and dissolved in MeOH. The solution was concentrated and combined with chilled AcOEt to give a precipitate. The material was lyophilized from dioxane to give a fluffy powder. Yield 1.6 g (70%), Rf^3 0.45, $[\alpha]_D^{30}$ −25.2° (c=1.0, MeOH). Amino acid ratios in an acid hydrolysate: Arg 0.88, Gly 1.00, Asp 0.96, Ser 0.78 (average recovery 76%). *Anal.* Calcd for $C_{35}H_{49}N_9O_{13}S \cdot 1.5H_2O$: C, 49.03; H, 6.04; N, 14.70. Found: C, 49.32; H, 6.14; N, 14.93.

RGDS-aPEG 4K Z–Arg(Tos)–Gly–Asp–Ser–NHNHBoc (1.1 g, 1.3 mmol) was treated with 10% anisole/TFA at 0 °C for 1 h and chilled ether was added. The resulting precipitate was collected, washed with ether and dissolved in DMF (10 ml). The solution was combined with 5.5 N HCl/dioxane (0.71 ml) and isoamyl nitrite (0.2 ml, 1.5 mmol) at −20 °C and the whole was stirred for 5 min. The solution was neutralized with NMM and combined with a DMF solution (10 ml) of aPEG 4K (amino content 0.485 meq/g, 1 g). The whole was stirred at pH 8.0 for 20 h in a cold room. Additional azide (the same amount) was added and the reaction mixture was further stirred for 14 h. The mixture was concentrated and the insoluble precipitate was removed by filtration. The solution was applied on LH-20 column (3 × 150 cm) and fractions (15 g each) 21—26 were pooled. The solvent was removed *in vacuo* and the residue was purified again by LH-20 column (3 × 150 cm) chromatography using MeOH/DCM (1/1) as an eluent. Yield 1.02 g. The material was treated with HF containing anisole (1 ml) and *m*-cresol (1 ml) at −10 °C for 40 min and purified by LH-20 column chromatography (eluent: DCM/MeOH, 1/1) and HPLC. The hybrid was converted to the hydrochloride by repeated lyophilization from HCl-containing water. Yield 320 mg, Rf^3 0.56. Amino acid ratios in an acid hydrolysate: Arg 0.82, Gly 1.00, Asp 0.87, Ser 0.89. RGDS content: 330 μmol/g.

Boc–Asp(OcHx)–Ser–OH · CHA Boc–Asp(OcHx)–OH (2.2 g, 7 mmol) and H–Ser(Bzl)–OH · TFA (2 g, 6.5 mmol) were coupled by the mixed anhydride method in the usual manner.[11] The product was purified by silica gel column chromatography and the desired material was obtained in the 1% MeOH/$CHCl_3$ eluate. The material was crystallized in ether by addition of cyclohexylamine. Yield 2.28 g (55%), mp 157—160 °C, Rf^5 0.60, $[\alpha]_D^{27}$ +2.4° (c=1.0, DMF). Amino acid ratios in an acid hydrolysate: Asp 1.00, Ser 0.87 (average recovery 85%). *Anal.* Calcd for $C_{25}H_{36}N_4O_8 \cdot C_6H_{13}N$: C, 62.92; H, 8.35; N, 7.10. Found: C, 62.95; H, 8.27; N, 7.09.

Z–Arg(Tos)–Gly–$NHNH_2$ Z–Arg(Tos)–OH (4.62 g, 10 mmol) and H–Gly–OBzl tosylate (3.4 g, 10 mmol) were reacted by the mixed anhydride method in the usual manner. The product was purified by silica gel column chromatography and the desired material was obtained in the 1% MeOH/$CHCl_3$ eluate. 5.2 g, mp 76—77 °C, Rf^5 0.5. The dipeptide benzyl ester was converted to the hydrazide in MeOH (50 ml) by addition of hydrazine hydrate (2.43 ml, 50 mmol). The hydrazide was precipitated by addition of ether and washed with ether. Yield 4.2 g (78%), mp 171—173 °C, Rf^5 0.12, $[\alpha]_D^{30}$ +2.6° (c=1.0, DMF). Amino acid ratios in an acid hydrolysate: Arg 1.02, Gly 1.00 (average recovery 83%). *Anal.* Calcd for $C_{23}H_{31}N_7O_6S$: C, 51.77; H, 5.86; N, 18.37. Found: C, 51.52; H, 5.87; N, 18.33.

Z–Arg(Tos)–Gly–Asp(OcHx)–Ser(Bzl)–OH Boc–Asp(OcHx)–Ser(Bzl)–OH (1.4 g, 2.81 mmol) was treated with 5% anisole/TFA (5 ml) at 0 °C for 1 h and was precipitated by addition of ether. The precipitate was dried and then dissolved in DMF (8 ml). Z–Arg(Tos)–Gly–$NHNH_2$ (1.5 g, 2.81 mmol) was dissolved in DMF (10 ml) and combined with 5.5 N HCl/dioxane (1.7 ml) and isoamyl nitrite (0.44 ml, 3 mmol) at −20 °C. The mixture was stirred for 10 min and combined with the H–Asp(OcHx)–Ser(Bzl)–OH solution described above. The whole was stirred at pH 8 for 40 h in a cold room. The solvent was removed *in vacuo* and the residue was washed with 5% citric acid, followed by purification on an LH-20 column (3 × 150 cm) with MeOH/DCM (1/1). The product was further purified by silica gel column chromatography using the lower phase of a mixture of $CHCl_3$/MeOH/water (8/3/1) as an eluent. The material was washed with water and dried. Yield 1.6 g (65%), mp 96—98 °C, Rf^3 0.55, $[\alpha]_D^{30}$ +3.7° (c=1.0, MeOH). Amino acid ratios in an acid hydrolysate: Arg 0.98, Gly 1.00, Asp 0.89, Ser 0.80 (average recovery 76%). *Anal.* Calcd for $C_{43}H_{55}N_7O_{12}S$: C, 57.77; H, 6.20; N, 10.97. Found: C, 57.82; H, 6.16; N, 10.91.

RGDS-aPEG 6K Z–Arg(Tos)–Gly–Asp(OcHx)–Ser(Bzl)–OH (420 mg, 471 μmol) and aPEG 6K (amino content 0.174 meq/g, 900 mg) were dissolved in DCM (15 ml) and combined with 1 M HOBt/DMF (0.47 ml). The mixture was cooled to −20 °C and combined with 1 M DCC/DCM (0.47 ml). The whole was stirred in a cold room and the same amounts of the peptide, DCC and HOBt were further added after 15 h and 40 h. The reaction mixture was stirred for 48 h in total and the solvent was removed *in vacuo*. The residue was purified by LH-20 column (3 × 150 cm) chromatography using a mixture of MeOH and DCM (1/1) as an eluent. The product (1.1 g) was treated with 10% anisole/HF (20 ml) at −10 °C for 40 min. The product was purified by Sephadex G-25 column (3 × 150 cm) chromatography and HPLC. Yield 200 mg. Rf^3 0.66. Amino acid ratios in an acid hydrolysate: Arg 1.08, Gly 1.00, Asp 0.98, Ser 0.94. RGDS content: 163 μmol/g.

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References and Notes

1) Standard abbreviations for amino acids, peptides and protecting groups are used [*Eur. J. Biochem.*, **138**, 9 (1984)]; other abbreviations include: DCC = dicyclohexylcarbodiimide, DCM = dichloromethane, DMF = dimethylformamide, TFA = trifluoroacetic acid, HOBt = 1-hydroxybenzotriazole, Z(OMe) = *p*-methoxybenzyloxycarbonyl, aPEG = amino-poly(ethylene glycol), MEM = minimum essential medium, NMM = *N*-methylmorphorine.
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