# Amino Acids and Peptides. XXI.[1] Laminin-Related Peptide Analogs Including Poly(Ethylene Glycol) Hybrids and Their Inhibitory Effect on Experimental Metastasis

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**Laminin-related peptides, Tyr–Ile–Gly–Ser–Arg analogs, were prepared and their inhibitory effects on experimental metastasis were examined. Of the amino acids in the Tyr–Ile–Gly–Ser–Arg sequence, L-Arg was very important and Ile was not essential for the inhibitory effect. To obtain a potent inhibitor of metastasis, hybrids of Tyr–Ile–Gly–Ser–Arg–Gly and 2 types of poly(ethylene glycol) were prepared. The inhibitory effects of the hybrids were more potent than that of Tyr–Ile–Gly–Ser–Arg–Gly.**

**Keywords** laminin; metastasis inhibitor; poly(ethylene glycol); hybrid; poly(ethylene glycol)hybrid; polymer hybrid

Laminin is a glycoprotein which is classified as a cell adhesion protein. It promotes the adhesion and growth of epithelial and tumor cells. It consists of three peptide chains ( A, B1 and B2) and partial sequences of the B1 chain, Tyr–Ile–Gly–Ser–Arg (YIGSR) and Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg (CDPGYIGSR), were found to be inhibitors of experimental metastasis in mice by Iwamoto *et al.*[2] We prepared YIGSR analogs and examined their inhibitory effect on experimental metastasis in mice. In the preceding communication,[4] we reported that the inhibitory effect of the hybrid of poly(ethylene glycol) (PEG) and YIGSRG was more potent than that of YIGSRG. Here we present full details of that work, and additional studies on the preparation and the inhibitory effect of YIGSR analogs. First, YIGSR, CDPGYIGSR, $(CDPGYIGSR)_2$ (the disulfide-bonded dimer of CDPGYIGSR),[2] YIGS, IGSR, YIGSRCD, VCDPGYIGSRCD, and YIGSE[2] were prepared by the solid phase method.[5] The α-amino group was protected with a *tert*-butyloxycarbonyl (Boc) group or *p*-methoxybenzyloxycarbonyl group, which was removable by trifluoroacetic acid (TFA) treatment. The following groups were used for side chain protection: *p*-methylbenzyl group for Cys, benzyl group for Tyr and Ser, tosyl or nitro group for Arg, and cyclohexyl group for Asp and Glu. Final deprotection was performed by HF treatment[6] and products were purified by reverse phase high performance liquid chromatography (RP-HPLC). $(CDPGYIGSR)_2$ was prepared by air oxidation of CDPGYIGSR in aqueous solution at pH 8 and its molecular weight was examined by mass spectroscopy. The products were converted to the hydrochlorides and their inhibitory effects were examined. Each synthetic peptide (1 mg) was mixed with B16-F10 melanoma cells and the mixture was injected into the tail vein of mice. Three weeks later, the mice were killed and the numbers of surface melanoma colonies on the lungs were counted macroscopically. The results are shown in Fig. 2. YIGSR and CDPGYIGSR exhibited an inhibitory effect as reported.[2] The inhibitory effects of YIGSRCD and VCDPGYIGSRCD were expected to be more potent than those of YIGSR and CDPGYIGSR, but in fact these chain-extended peptides were less active than YIGSR and CDPGYIGSR. The reason is not clear, but the extension of the peptide chain might change the conformation and ionic charge and these changes might result in decreased activity. The dimer of CDPGYIGSR was less active than the monomer. IGSR and YIGSE did not show any inhibitory effect but YIGS showed a weak effect. Tyr may have a more important role in the inhibitory effect compared with Arg.

Next, YIGSDR (DR = dextro Arg), YCGSR, $(YCGSR)_2$ (the disulfide-bonded dimer of YCGSR), and $(YIGSR)_2K$ were similarly prepared by the solid phase method. B16-BL6 melanoma cells were used to examine the

–Leu–Ala–Cys–Val–Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg
–Cys–Asp–Asp–

Fig. 1. Amino Acid Sequence of Mouse Laminin B1 Chain 921—936[3]

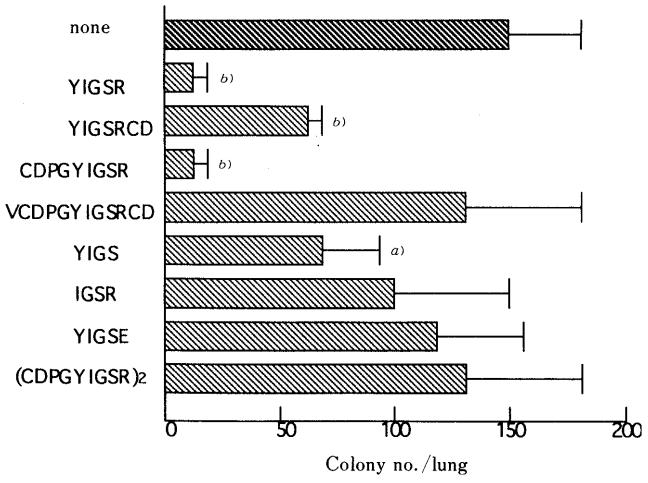


Fig. 2. Inhibitory Effect of Synthetic Peptides on Experimental Metastasis in Mice

B16-F10 melanoma cells ($1 \times 10^5$/0.2 ml) were injected i.v. with or without admixing with 1 mg of peptide into five mice per group. Lung tumor colonies were examined 21 d later. Values are the mean ± S.D. *a)* $p<0.01$, *b)* $p<0.001$.

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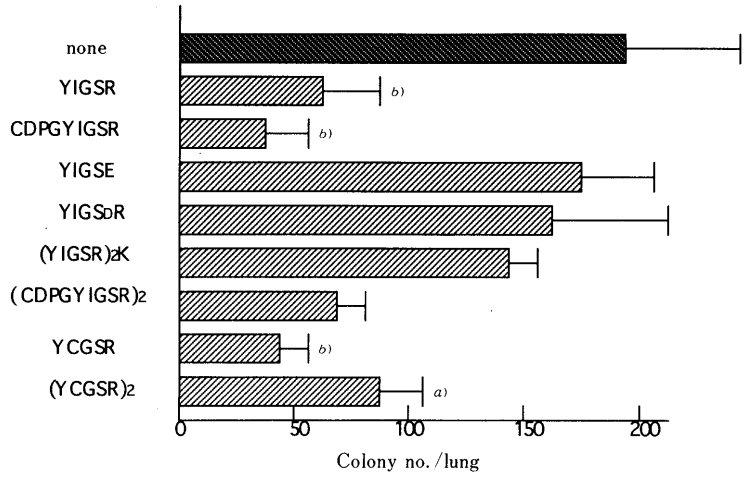


Fig. 3. Effect of Synthetic Peptides on Lung Tumor Colnization

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

inhibitory effect of these synthetic peptides on experimental metastasis since they were more metastatic than B16-F10. The inhibitory effects of the synthetic peptides are shown in Fig. 3.

YIGSE also did not show an inhibitory effect on B16-BL6 melanoma cells at 1 mg dose. YIGSR, CDPGYIGSR, $(CDPGYIGSR)_2$ were inhibitory and $(CDPGYIGSR)_2$ was less effective than CDPGYIGSR. YIGSDR was not effective. This result suggested a very important role of L-Arg for the inhibitory effect. YCGSR and $(YCGSR)_2$ were inhibitory, indicating that Ile was not essential for the inhibitory effect. As with CDPGYIGSR, YCGSR was more effective than its dimer, $(YCGSR)_2$. Recently it was reported that a thiol group might exist in $\mu$-opioid receptor and enkephalin analogs containing an activated thiol group bind to the receptor through a disulfide bond.[7] Similarly, a functional group which reacts with the thiol group of CDPGYIGSR and YCGSR may exist in the receptor of YIGSR analogs. The dimer through the Lys residue, $(YIGSR)_2K$, was not effective.

To obtain a more potent inhibitor, other types of inhibitor were considered. Two types of potent inhibitors have been reported: cyclic YIGSR[8] and polymers of YIGSR and RGD.[9] Since these peptides have specifically cyclic and polymeric forms, we prepared a cyclic YIGSR analog and polymer-bound YIGSR analogs. Since we found that Ile was not essential for the inhibitory effect, YCGSRC was prepared by the solid phase method and oxidized it to form a cyclic peptide through an intramolecular disulfide bond. PEG is a polymer which has many advantages as a drug carrier. It is stable, weakly toxic, soluble in aqueous and organic solvents, weakly immunogenic, and has little effect on the conformation of peptides. Though many PEG hybrids of proteins have been studied to improve the stability and the activity of the proteins,[10] small-peptide hybrids have not been investigated. Therefore, we prepared PEG hybrids of YIGSRG and examined their inhibitory effect on metastasis. PEG#4000 (4K, M.W. 3000—3700) and PEG#6000 (6K, M.W. 6700—9000) were converted to amino-poly(ethylene glycol) [aPEG, $H_2N$–$CH_2CH_2(OCH_2CH_2)_n$–$NH_2$] according to the procedure reported by Pillai and Mutter.[11] aPEGs were purified by Dowex 50 column chromatography. YIGSRG–aPEG was prepared as shown in Fig. 4. $Gly^6$ was introduced as a spacer. Fmoc–YIGSRG–OH was prepared by the solid phase method starting from chloromethyl resin. For protection of $\alpha$-amino groups, a Boc group was used for Ile, Gly, Ser and Arg. Fmoc–Tyr(OBzl)–OH was used at the final coupling on resin and Fmoc–Tyr(Bzl)–Ile–Gly–Ser(Bzl)–Arg($NO_2$)–Gly-resin was treated with HF to give Fmoc–YIGSRG–OH. The Fmoc hexapeptide was purified by HPLC and converted to its hydrochloride. Then the peptide was coupled with aPEG (4K and 6K) by the diphenylphosphoryl azide (DPPA) method.[12] The Fmoc group on Tyr was removed by piperidine treatment to give YIGSRG–aPEG. The product was purified by HPLC. Peptide content of each hybrid was calculated from the amino acid content in an acid hydrolysate. The peptide contents of hybrids 4K and 6K were 0.34 and 0.16 mmol/g respectively. Prior to the examination of the inhibitory effect of hybrids 4K and 6K, the inhibitory effect of PEG itself was examined and the result is shown in Fig. 5. PEG 4K and PEG 6K (2 mg) did not show any inhibitory effect on metastasis. The inhibitory effects of the cyclic peptide and hybrid 4K are shown in Fig. 6. The effects of YIGSR

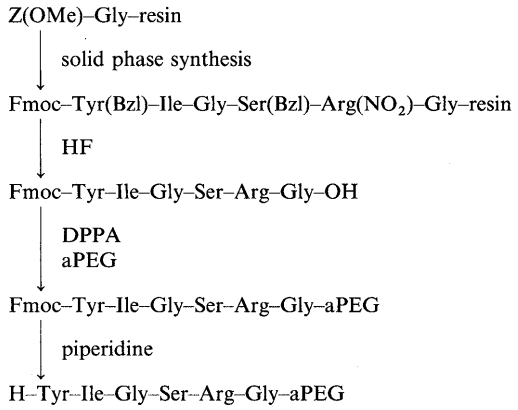


Fig. 4. Synthetic Scheme for YIGSRG–aPEG

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and YIGSRG were not distinctive. The cyclic peptide was less active than expected, but the hybrid 4K exhibited potent inhibitory effect. The effect of 0.3 mg of hybrid 4K was comparable to that of 1.0 mg of YIGSR or CDPGYIGSR.

The effects of hybrids 4K and 6K were compared and the results are shown in Fig. 7. The inhibitory effects of hybrids 4K and 6K were almost equal, and the effects of 300 $\mu$g of hybrids 4K and 6K were equivalent to that of 600 $\mu$g of YIGSRG. Considering the molecular weight of the hybrids, the inhibitory effect of hybrid 6K was about twice as potent as that of hybrid 4K in term of molecular ratio. The peptide content of the hybrid 6K is 0.16 mmol/g, so 300 $\mu$g of the hybrid 6K contains 0.048 $\mu$mol of YIGSRG. Since 600 $\mu$g of YIGSRG·2HCl is 0.829 $\mu$mol, it can be said that the inhibitory effect of YIGSRG is potentiated about 17-fold by the hybrid formation. Why the inhibitory effect of the PEG hybrids was more potent than that of YIGSRG is not clear, but presumably one reason is slower enzymatic degradation of the YIGSRG portion. Enzymatic degradation of the YIGSRG moiety may be prevented by PEG. YIGSRG was easily hydrolyzed by aminopeptidase M but hydrolysis of the YIGSRG portion of the PEG hybrid 4K by the enzyme was very slow. Hydrolysis of the hybrid 6K by $\alpha$-chymotrypsin was also slower than that of YIGSRG. PEG with its flexible conformation does not prevent the binding of the hybrid to the receptor, and its bulk in the hybrid can stabilize the binding between the YIGSRG portion and the receptor.

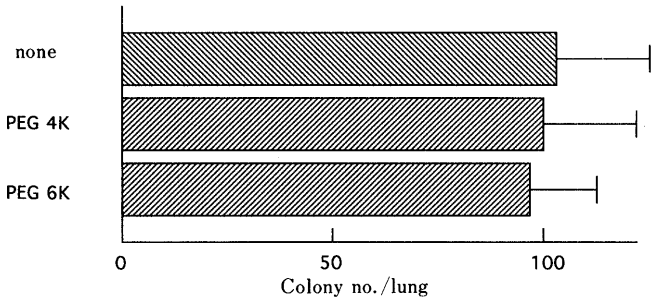


Fig. 5. Effect of PEG on B16 Melanoma BL6 Cell Lung Metastasis

B16-BL6 cells ($1 \times 10^5$/0.2 ml) were injected i.v. with or without admixing with 2 mg of PEG into five mice per group. Lung tumor colonies were examined 21 d later. Values are the mean ± S.D.

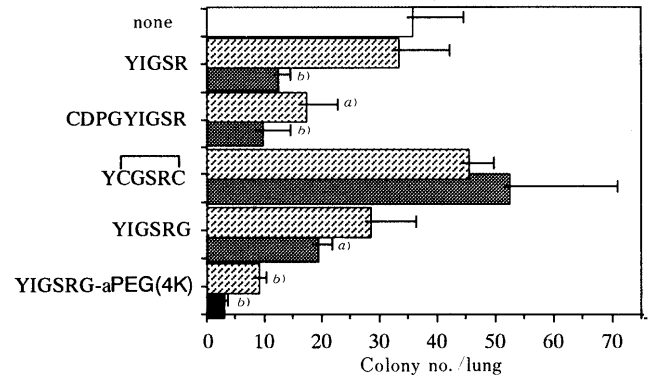


Fig. 6. Inhibitory Effect of Synthetic Peptides on Experimental Metastasis in Mice

B16-BL6 cells ($1 \times 10^5$/0.2 ml) were injected i.v. with or without admixing with 0.3 mg (▨), 1.0 mg (▩), 2.0 mg (■) of peptide into five mice per group. Lung tumor colonies were examined 21 d later. Values are the mean ± S.D. *a)* $p<0.05$, *b)* $p<0.01$ compared with untreated control (MEM) by Student's *t* test.

**Experimental**

Solvent systems for ascending thin-layer chromatography 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–$H_2O$ (90 : 8 : 3, lower phase). 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. RP-HPLC was conducted with a Waters 600 on a YMC Pack AQ-ODS-5 column using gradient systems of $CH_3CN/H_2O$ containing 0.1% TFA. FAB-MS were measured on a VG Analytical ZAV-SE spectrometer. PEG was purchased from Nacalai Tesque, Inc.

**A) Peptide Synthesis by the Solid Phase Method. General Procedure** *p*-Methylbenzhydrylamine resin and chloromethylated resin were purchased from Peptide Institute, Inc. The following amino acid derivatives were used; Z(OMe)–Gly–OH, Boc–Tyr(Bzl)–OH, Boc–Ser(Bzl)–OH, Boc–Arg(Tos)–OH, Boc–Ile–OH, Boc–Glu(OBzl)–OH. The synthetic protocol for solid-phase peptide synthesis is shown below. Reactions were checked by using the ninhydrin test.[13]

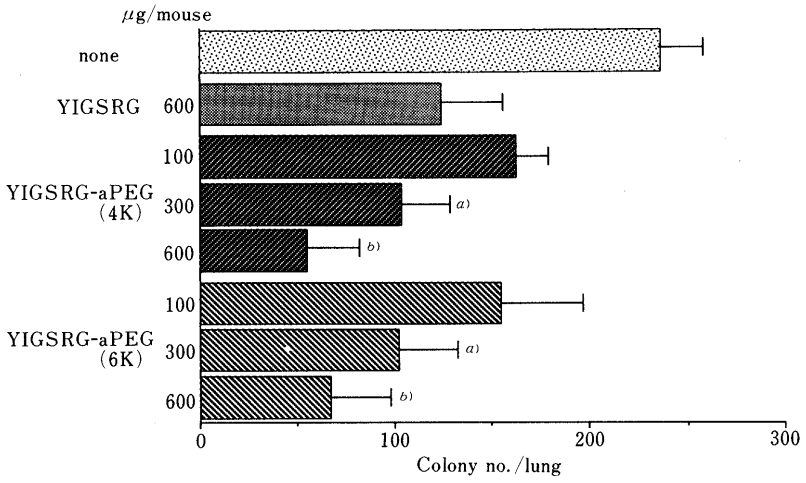


Fig. 7. Inhibitory Effect of YIGSRG–aPEG Conjugates on the Formation of Lung Tumors

B16-BL6 cells ($1 \times 10^5$/0.2 ml) were injected i.v. with or without admixing with various concentrations of peptide into five mice per group. Lung tumor colonies were examined 21 d later. Values are the mean ± S.D. *a)* $p<0.005$, *b)* $p<0.001$ compared with untreated control by Student's *t* test.

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| step | reagents | reaction time | |
|---|---|---|---|
| 1 | NMM/DCM | 10 min | ×2 |
| 2 | DCM | 3 min | ×3 |
| 3 | Boc- or Z(OMe)-amino acid (2eq) in DMF (or DCM) 1 M DCC/DCM (2 eq) | 120 min | |
| 4 | 50% MeOH/DCM | 5 min | ×3 |
| 5 | DCM | 2 min | 1 |
| 6 | 50% TFA/DCM, anisole | 2 min | 1 |
| | | 45 min | 1 |
| 7 | DCM | 3 min | ×4 |

A 1 M HOBt/DMF solution (2 eq) was added when Boc–Arg(Tos)–OH was activated. Final deprotection was performed by HF treatment. The product was purified by RP-HPLC. Yields were calculated from crude deblocked material. The peptides purified by HPLC were converted to their hydrochlorides by lyophilization from HCl-containing water.

**H–Tyr–Ile–Gly–Ser–Arg–$NH_2$** Yield 26%, hygroscopic powder, $Rf^1$ 0.05, $Rf^2$ 0.12, $[\alpha]_D^{25}$ −18.0° ($c$=1.0, $H_2O$). MS $m/z$ 594 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ile 0.97, Gly 1.00, Ser 0.88, Arg 0.94 (average recovery 80.3%).

**H–Tyr–Ile–Gly–Ser–$NH_2$** Yield 24%, hygroscopic powder, $Rf^1$ 0.10, $Rf^2$ 0.27, $[\alpha]_D^{25}$ −3.8° ($c$=1.0, $H_2O$). MS $m/z$: 438 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Tyr 0.97, Ile 0.94, Gly 1.00, Ser 0.98 (average recovery 83%).

**H–Ile–Gly–Ser–Arg–$NH_2$** Yield 28%, hygroscopic powder, $Rf^1$ 0.02, $Rf^2$ 0.05, $[\alpha]_D^{25}$ −8.0° ($c$=1.0, $H_2O$). MS $m/z$: 431 $(M+1)^+$. Amino acid ratios in an acid hydrolysate: Ile 0.96, Gly 1.00, Ser 0.91, Arg 0.99 (average recovery 92%).

**H–Tyr–Ile–Gly–Ser–Arg–Cys–Asp–$NH_2$** Yield 10%, hygroscopic powder, $Rf^2$ 0.05, $[\alpha]_D^{25}$ −60.4° ($c$=1.0, $H_2O$). MS $m/z$: 813 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Tyr 0.92, Ile 0.91, Gly 1.00, Ser 0.97, Arg 0.99, Cys 0.91, Asp 1.01 (average recovery 84%).

**H–Tyr–Ile–Gly–Ser–Glu–$NH_2$** Yield 24%, hygroscopic powder, $Rf^2$ 0.02, $[\alpha]_D^{25}$ −19.4° ($c$=1.0, $H_2O$). MS $m/z$: 567 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Tyr 1.04, Ile 0.95, Gly 1.00, Ser 0.95, Glu 1.07 (average recovery 85%).

**H–Tyr–Cys–Gly–Ser–Arg–$NH_2$** Yield 26%, hygroscopic powder, $Rf^2$ 0.03, $[\alpha]_D^{25}$ −14.9° ($c$=1.0, $H_2O$). MS $m/z$: 584 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Tyr 0.97, Cys 0.83, Gly 1.00, Ser 1.02, Arg 1.04 (average recovery 85%).

**(H–Tyr–Cys–Gly–Ser–Arg–$NH_2$)$_2$** H–Tyr–Cys–Gly–Ser–Arg–$NH_2$·2HCl (70 mg) was dissolved in $H_2O$ (10 ml) and the solution was adjusted to pH 8 by addition of 3% $NH_4OH$. The mixture was stirred for 24 h and lyophilized. The residue was purified by HPLC and converted to its hydrochloride in the usual manner. Yield 73%, hygroscopic powder, $Rf^2$ 0.02, $[\alpha]_D^{25}$ −28.5° ($c$=1.0, $H_2O$). MS $m/z$: 1166 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Tyr 0.86, Cys 0.86, Gly 1.00, Ser 0.96, Arg 1.00 (average recovery 84%).

**H–Tyr–Ile–Gly–Ser–D–Arg–$NH_2$** Yield 10%, hygroscopic powder, $Rf^2$ 0.12, $[\alpha]_D^{25}$ −8.0° ($c$=1.0, $H_2O$). MS $m/z$: 594 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Tyr 0.95, Ile 0.96, Gly 1.00, Ser 1.00, Arg 1.02 (average recovery 76%).

**(H–Tyr–Ile–Gly–Ser–Arg)$_2$–Lys–$NH_2$** Yield 14%, $Rf^2$ 0.05, $[\alpha]_D^{25}$ −28.5° ($c$=1.0, $H_2O$). MS $m/z$: 1300 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Tyr 0.82, Ile 1.02, Gly 1.00, Ser 0.89, Arg 1.00, Lys 0.48 (average recovery 81%).

**H–Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg–$NH_2$** Yield 11%, hygroscopic powder, $Rf^2$ 0.02, $[\alpha]_D^{25}$ −53.3° ($c$=1.0, $H_2O$). MS $m/z$: 967 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Cys 0.89, Asp 0.94, Pro 0.98, Gly 2.00, Tyr 0.95, Ile 0.98, Ser 0.95, Arg 0.97 (average recovery 81%).

**(H–Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg–$NH_2$)$_2$** H–Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg–$NH_2$·2HCl (70 mg) was dissolved in $H_2O$ (7 ml) and the solution was adjusted to pH 8 by addition of 3% $NH_4OH$. Stirring was continued for 24 h and the solvent was removed by lyophilization. The residue was purified by HPLC and converted to its hydrochloride in the usual manner. Yield 57 mg (81%), hygroscopic powder, $Rf^2$ 0.02, $[\alpha]_D^{25}$ −61.3° ($c$=1.0, $H_2O$). MS $m/z$: 1931 ($M^+$+1). Amino acid ratios in an acid hydrolysate: Cys 0.91, Asp 0.95, Pro 1.02, Gly 2.00, Tyr 0.94, Ile 0.95, Ser 0.98, Arg 1.00 (average recovery 81%).

**H–Val–Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg–Cys–Asp–$NH_2$** Yield 83 mg (12%), hygroscopic powder, $Rf^2$ 0.02, $[\alpha]_D^{25}$ −57.4° ($c$=1.0, $H_2O$). MS $m/z$: 1283($M^+$). Amino acid ratios in an acid hydrolysate: Val 0.96, Cys 1.67, Asp 1.98, Pro 1.06, Gly 2.00, Tyr 0.85, Ile 1.03, Ser 0.89, Arg 0.99 (average recovery 77%).

**Fmoc–Tyr–Ile–Gly–Ser–Arg–Gly–OH** Z(OMe)–Gly–OH was introduced on chloromethyl resin by the cesium salt method.[14] Fmoc–Tyr(Bzl)–Ile–Gly–Ser(Bzl)–Arg($NO_2$)–Gly-resin was prepared in the usual manner. The peptide-resin (4.4 g) was treated with HF (44 ml) containing 2.5% $m$-cresol and 2.5% anisole at 0°C for 90 min. The HF was removed *in vacuo* and the product was extracted with 50% dioxane. The resin was removed by filtration and the filtrate was lyophilized. The residue was washed with MeOH and collected by centrifugation.

The residue was purified by LH20 column (3×160 cm) chromatography using DMF as an eluent. The DMF was removed in vacuo and the residue was further purified by HPLC. Yield 290 mg (19%), amorphous powder, $Rf^1$ 0.55, $[\alpha]_D^{18}$ −14.9° ($c$=1.0, MeOH). MS $m/z$: 875 ($M^+$+1). *Anal.* Calcd for $C_{43}H_{55}N_9O_{11}\cdot CF_3COOH\cdot 2H_2O$: C, 52.77; H, 5.92; N, 12.31. Found: C, 52.84; H, 5.63; N, 12.21. Amino acid ratios in an acid hydrolysate: Tyr 0.95, Ile 0.99, Gly 2.00, Ser 0.96, Arg 0.96 (average recovery 84%). For the next coupling reaction, the peptide was converted to its hydrochloride by lyophilization from dioxane/$H_2O$ containing HCl.

**B) Peptide Synthesis by the Solution Method. H–Tyr–Ile–Gly–Ser–Arg–Gly–aPEG** DPPA (32 μl, 0.15 mmol) and 10% $Et_3N$ in DMF (210 μl, 0.15 mmol) were added to a DMF solution (7 ml) of Fmoc–Tyr–Ile–Gly–Ser–Arg–Gly–OH (137 mg, 0.15 mmol) and aPEG (#4K, 150 mg, 84 mmol; #6K, 365 mg, 99 mmol) at −5 °C, and the mixture was stirred for 24 h in a cold room. The solvent was removed *in vacuo*. The residue was dissolved in 50% MeOH/DCM and the solution was passed through a Sephadex LH 20 column (3×162 cm). Each fraction (6 ml) was checked for absorbance at 270 nm. After removal of the solvent, the residue was dissolved in a mixture of DMF (2 ml) and piperidine (1 ml). The mixture was stirred for 20 min and the solvent was removed *in vacuo*. The residue was extracted with $H_2O$ and the extract was washed with ether, followed by lyophilization. The residue was purified by Sephadex LH20 column chromatography using 50% MeOH/DCM as an eluent, followed by HPLC.

YIGSRG–aPEG (4K): Yield 42 mg (19%), fluffy powder, $Rf^3$ 0.63. Amino acid ratios in an acid hydrolysate: Tyr 0.89, Ile 0.91, Gly 2.00, Ser 0.94, Arg 0.97. Peptide content: 0.34 mmol/g.

YIGSRG–aPEG (6K): Yield 47 mg (11%), fluffy powder, $Rf^3$ 0.63. Amino acid ratios in an acid hydrolysate: Tyr 0.87, Ile 0.88, Gly 2.00, Ser 0.79, Arg 0.93. Peptide content: 0.16 mmol/g.

**C) Preparation of aPEG** PEG #4000 and PEG #6000 were converted to the corresponding aPEG according to the procedure reported by Pillai and Mutter.[11] The aPEG (10 g) was dissolved in $H_2O$ (100 ml) and the solution was passed through a Dowex 50 ($H^+$) column (6×30 cm). After washing with $H_2O$, the column was washed with 50% MeOH and $H_2O$. Finally the aPEG was eluted with 3% $NH_4OH$. The eluate was evaporated to give a white solid. Both aPEG 4K and 6K gave almost the same $Rf$ value: $Rf^3$ 0.62. Each aPEG was titrated with 0.01 N HCl using methyl red as an indicator. Amino content: aPEG 4K 0.56 meq/g, aPEG 6K 0.27 meq/g.

**D) Enzymatic Hydrolysis of YIGSRG and Its aPEG Hybrids** The peptide (or hybrid) and the enzyme (10 ml) were dissolved in 0.2 M Tris buffer (pH 7.9, 0.1 ml) and the solution was shaken at 37 °C for 24 h. Then 0.1 N HCl (1 ml) was added and the solution was filtered with a Chromatodisc 13A (0.45 μm, Kurabo). An aliquot of the filtrate was examined with an amino acid analyzer. The following enzyme solutions were used: aminopeptidase M (Boehringer Mannheim GmbH, 5 mg/ml) and α-chymotrypsin (Sigma, 10 units/ml).

YIGSRG: YIGSRG (485 μg and 241 μg) was digested by aminopeptidase M and α-chymotrypsin, respectively. Amino acid ratios in an aminopeptidase M digestion: Tyr 1.37, Ile 1.68, Gly 2.00, Ser 1.72, Arg 1.36 (average recovery 82%). α-Chymotrypsin digest: recovery of Tyr 17.8%.

YIGSRG–aPEG: The hybrid 4K (1.048 mg) was digested by aminopeptidase M. The peptide portion was almost unaffected by the enzyme. Amino acid ratios in an aminopeptidase M hydrolysate: Tyr 5.44, Ile 1.50, Gly 2.00, Ser 5.18, Arg 2.40 (average recovery 1.5%). Traces of some other amino acids (Ala, Cys, Leu, Orn *etc.*) were also detected. These were derived from the enzyme.

The hybrid 6K (3.058 mg) was also digested by α-chymotrypsin: recovery of Tyr 2.6%.

We dedicate this paper to Professor Yoshifumi Maki on the occasion of his retirement from Gifu Pharmaceutical University.

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**References and Notes**

1) Standard abbreviations are used for amino acids, protecting groups, and peptides [*Eur. J. Biochem.*, **138**, 9 (1984)]. Other abbreviations include: Mts = mesitylenesulfonyl, DMF = dimethylformamide, TFA = trifluoroacetic acid, DCM = dichloromethane, NMM = *N*-methylmorpholine.
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