A conjugate from a laminin-related peptide, Tyr-Ile-Gly-Ser-Arg, and chitosan: efficient and regioselective conjugation and significant inhibitory activity against experimental cancer metastasis †,[1]

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A laminin-related antimetastatic peptide was conjugated with chitosan, and antimetastatic activity of the peptide–chitosan conjugate was assayed. Chitosan was converted to its organosoluble derivative, 6-*O*-trityl-chitosan, in 3 steps, and then coupled with the peptide portion, Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH (βAla; β-alanine), which contains a spacer amino acid at the carboxy-terminus. The product was treated with $CHCl_2CO_2H$ to afford the desired conjugate, Ac-Tyr-Ile-Gly-Ser-Arg-βAla–chitosan. The peptide was introduced to every 6.3 glucosamine residues. The conjugate proved to have higher inhibitory activity against experimental lung metastasis of B16BL6 melanoma cells in mice than did the parent peptide.

Introduction

Laminin, a 900 kDa trimeric glycoprotein in the basement membrane, is known to be involved in the invasion and metastasis of tumour cells.[2–4] A synthetic pentapeptide corresponding to a partial sequence of the laminin β1 chain, H-Tyr-Ile-Gly-Ser-Arg-NH_2, was reported to inhibit experimental metastasis formation.[5] It was also reported that peptides containing the Tyr-Ile-Gly-Ser-Arg (YIGSR) sequence inhibited angiogenesis and tumour growth.[6] YIGSR-related peptides have thus attracted attention as potential candidates for the development of anticancer and antimetastatic agents, and a number of modifications have been attempted to enhance their activity and clinical utility. In particular, conjugation of the YIGSR peptide with polymeric materials appears to offer promise in developing highly active antimetastatic agents. For instance, Kawasaki *et al.*[7,8] reported that hybrid compounds of the YIGSR peptide and a bioinert polymer, PEG, exhibited a much higher antimetastatic activity and stability to enzymic degradation than did the parent peptide.

An amino polysaccharide, chitosan, is highly attractive as a polymeric carrier for bioactive peptides. Chitosan can be readily obtained from chitin, which consists of *N*-acetyl-D-glucosamine and occurs to a great extent in shells of crustaceans, by simple deacetylation.[9,10] Owing to their low toxicity, biodegradability, and abundance, their applications in various fields including their use as drug carriers are being studied extensively.[11] Since chitosan has an amino function in every glucosamine residue, peptide molecules introduced at these amino groups would be ordered in a comb-like fashion, whereas the peptide–PEG conjugate is a linear molecule. A comb-like arrangement might be more advantageous than a linear one to increase the local concentration of the peptide. Furthermore, the degree of substitution (d.s.) of the chitosan-conjugate would be controlled over a wider range than that of linear conjugates on demand. The biodegradability and extremely low toxicity of chitosan are also attractive in view of its possible use as a therapeutic agent for humans. Under these circumstances, we have focused our attention on the potential of chitosan for use in conjugation with the YIGSR peptide. Chitosan is, however, insoluble in common reaction media, and is thus rather intractable. We report here the efficient and regioselective preparation of the YIGSR–chitosan conjugate, as shown in Fig. 1, *via* organosoluble chitosan derivatives, and its enhanced inhibitory activity on experimemtal cancer metastasis.

Results and discussion

Preparation of YIGSR–chitosan conjugates

To avoid two possible side-reactions in the direct conjugation of YIGSR with chitosan, *i.e.*, epimerization and δ-lactam formation of the carboxy-terminal Arg,[12] βAla was employed as a spacer molecule. The peptide moiety containing the spacer amino acid, Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH **1**, was synthesized by the conventional solution method as outlined in Scheme 1. Protecting groups employed were as follows: Boc for α-amino functions; benzyl (Bzl) for the carboxy group of βAla and the hydroxy function of Ser; *p*-tolylsulfonyl (Tos) for the

† Amino acids used in this study are of L-configuration, except β-alanine. Abbreviations used in this report for amino acids, peptides, and their derivatives are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature: *Biochem. J.*, 1984, **219**, 345. The following additional abbreviations are used: AcOEt, ethyl acetate; β-Ala, β-alanine; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; 2,6-Cl_2Bzl, 2,6-dichlorobenzyl; DPPA, diphenylphosphoryl azide; d.s., degree of substitution; GPC, gel-permeation chromatography; TFMSA, trifluoromethanesulfonic acid; Tos, *p*-tolylsulfonyl; Trt, trityl; WSCI, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride.

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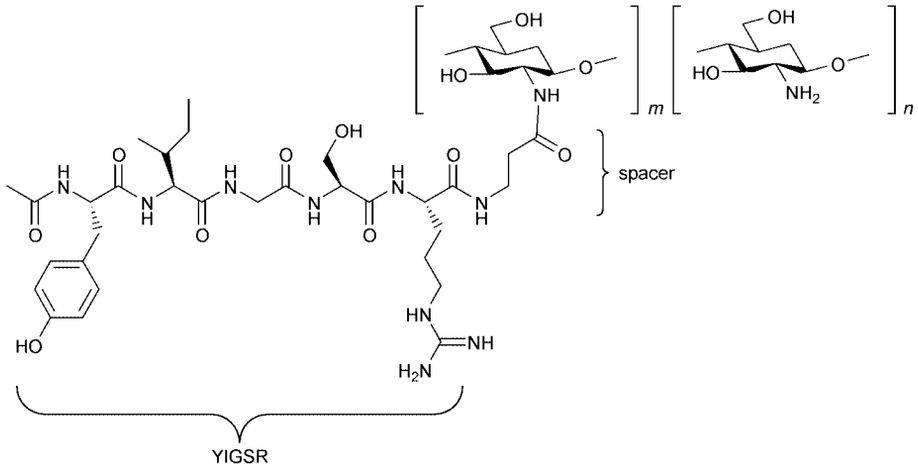
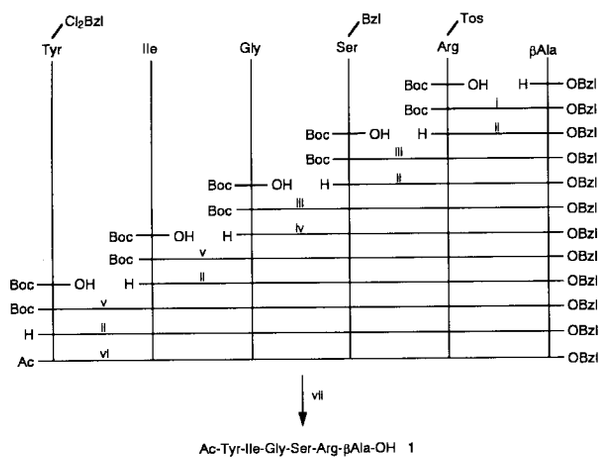


Fig. 1 Structure of the YIGSR–chitosan conjugate.



Scheme 1 Synthetic scheme for peptide **1**. *Reagents and conditions*: (i) DPPA, Et_3N, DMF; (ii) TFA, anisole; (iii) BOP, Et_3N, DMF; (iv) 4 mol dm^{-3} HCl in AcOEt; (v) BOP, HOBT, Et_3N, DMF; (vi) Ac_2O, DMF; (vii) 1 mol dm^{-3} TFMSA–thioanisole in TFA, *m*-cresol.

guanidino function of Arg; 2,6-dichlorobenzyl (2,6-Cl_2Bzl) for the hydroxy function of Tyr. Boc-Arg(Tos)-OH was coupled with H-βAla-OBzl by diphenylphosphoryl azide[13] (DPPA) to minimize δ-lactam formation.[14] The other couplings were performed with the aid of benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP)[15] or BOP–HOBT. After acetylation of the terminal amino function with acetic anhydride, all protecting groups were removed with 1 mol dm^{-3} trifluoromethanesulfonic acid (TFMSA)–thioanisole in TFA containing *m*-cresol.[16] After purification with HPLC, peptide **1** was converted to its hydrochloride to prevent the undesirable acylation of the guanidino function of Arg during the coupling with chitosan.

DCC-mediated coupling of peptide **1** with chitosan suspended in DMA failed to yield the peptide–chitosan conjugate, due to the heterogeneous conditions used. However, regioselective introduction of the peptide at the amino function of chitosan has been accomplished using an organosoluble chitosan derivative, 6-*O*-trityl (Trt)-chitosan,[17] which has a protective group at the reactive C-6 hydroxy function. 6-*O*-Trt-chitosan was prepared as reported previously;[17] chitosan was treated with phthalic anhydride and then chlorotriphenylmethane to give *N*-phthaloyl-6-*O*-Trt-chitosan, whose phthaloyl group was selectively removed with hydrazine hydrate to afford 6-*O*-Trt-chitosan. The synthetic scheme for the chitosan conjugate from 6-*O*-Trt-chitosan is shown in Scheme 2. To couple peptide **1** with 6-*O*-Trt-chitosan, the following three reaction conditions were attempted: (1) peptide **1** (1 equiv. for -NH_2 of the chitosan derivative), 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (WSCI, 1.2 equiv.); (2) peptide **1** (0.5 equiv.), DPPA (0.5 equiv.), $EtNPr^i_2$ (0.5 equiv.); (3) peptide **1** (1 equiv.), DPPA (1.2 equiv.), $EtNPr^i_2$ (1 equiv.). All coupling reactions were carried out in a homogeneous DMA solution. The products were then treated with $CHCl_2CO_2H$ to remove the Trt group. The IR spectra of the products, **2a** (WSCI method), **2b** (DPPA method, 0.5 equiv.), and **2c** (DPPA method, 1 equiv.), demonstrated the regioselective introduction of the peptide at the amino function of chitosan; clear amide bands and no ester bands were observed. The d.s.-values of conjugates were determined by amino acid analysis of their acid hydrolysates, and are listed in Table 1. The DPPA-mediated condensation at an equivalent ratio gave the highest d.s. so far examined. Recently, it was reported that either of DPPA, diisopropylcarbodiimide–HOBT, WSCI, and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate[18] failed to conjugate the YIGSR peptide with water-soluble chitosan in a mixture of DMF and water.[19] Our data clearly indicate that the efficient and regioselective conjugation was accomplished under mild reaction conditions, proving the effectiveness of our conjugation strategy using 6-*O*-protected chitosan.

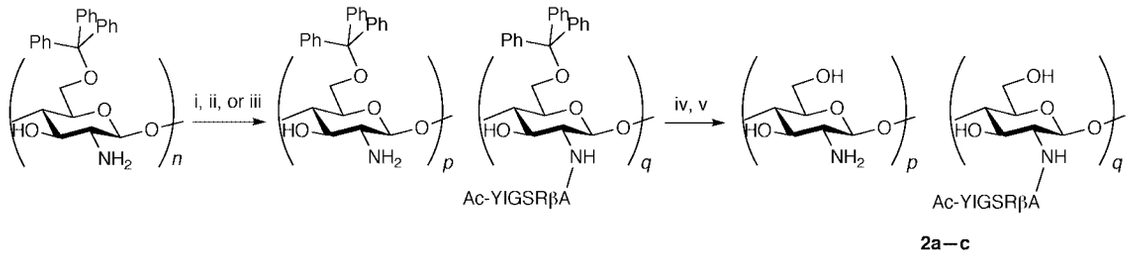
Table 1 D.s. of conjugates **2a–c**

	Coupling method	Peptide/-NH_2	D.s.
2a	WSCI	1.0	0.10
2b	DPPA	0.5	0.13
2c	DPPA	1.0	0.16

Antimetastatic activity

The inhibitory activity of Ac-Tyr-Ile-Gly-Ser-Arg-βAla–chitosan **2c**, as well as those of chitosan **3**‡ and Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH **1**, on experimental lung metastasis was examined with B16BL6 melanoma cells in mice. As shown in Table 2, conjugate **2c** showed a significant inhibitory activity at a dose of 0.08 mg $mouse^{-1}$, whereas chitosan **3** did not show any inhibition even at 1.0 mg $mouse^{-1}$. Injection of 1.0 mg $mouse^{-1}$ of peptide **1** also decreased the number of colonies in the lung to ≈50% of the control. Previously, 0.3 mg $mouse^{-1}$ of the YIGSR–PEG conjugate was reported to decrease the number of colonies to ≈50% of the control.[7,8] Based on the d.s., 0.08 mg of chitosan-conjugate **2c** contains 0.04 μmol of the YIGSR peptide, while 0.3 mg of the PEG-conjugate contains 0.05 μmol peptide, and 1.0 mg of peptide **1** corresponds to 1.2 μmol. Thus

‡ To obtain chitosan with a molecular mass similar to that of the conjugate, chitosan **3** was regenerated from 6-*O*-Trt-chitosan with $CHCl_2CO_2H$ as described in the Experimental section.



Scheme 2 Synthetic scheme for conjugates **2a**–**c**. *Reagents and conditions*: (i) **1** (1 equiv. for -NH_2 of chitosan), WSCI (1.2 equiv.), DMA (rt, overnight); (ii) **1** (0.5 equiv.), DPPA (0.5 equiv.), $EtNPr^i_2$ (0.5 equiv.), DMA (rt, overnight); (iii) **1** (1 equiv.), DPPA (1.2 equiv.), $EtNPr^i_2$ (1 equiv.), DMA (rt, overnight); (iv) $CHCl_2CO_2H$ (rt, 20 min × 2); (v) Sephadex G-25 column chromatography (3% aq. AcOH) or dialysis (3% aq. AcOH). βA = βAla.

Table 2 Antimetastatic activity of peptide **1**, conjugate **2c**, and chitosan **3**

	Dose			
	mg mouse^{-1}	μmol peptide mouse^{-1}	Number of colonies	% Inhibition
Control			220 ± 33.6	
Chitosan **3**	1.0		265 ± 37.0	
Peptide **1**	0.1	0.12	141 ± 47.2	36
	1.0	1.2	108 ± 24.7	51
Conjugate **2c**	0.08	0.04	104 ± 35.5	53
	0.24	0.12	77.8 ± 27.2	65

chitosan-conjugate **2c** has been confirmed to have a more potent antimetastatic activity than free peptide **1** and a comparable activity with the PEG-conjugate.

It is noteworthy that conjugate **2c** exhibited higher activity than did free peptide **1**. Generally, conjugation of small bioactive peptides with polymers has a possible drawback; a large polymer portion may hinder the peptide–receptor interaction. The results of an antimetastatic assay in this study clearly indicate that the chitosan molecule does not severely hinder the YIGSR portion from the interaction with the laminin receptor. In previous reports,[8,20] the YIGSR–PEG conjugate was demonstrated to be much more stable against enzymic degradation than the free peptide. This might be one reason for the high antimetastatic activity of the PEG-conjugate. Although the chitosan-conjugate is structurally different from the PEG-conjugate, a similar effect of protection against enzymic digestion is expected and may be partly responsible for the high activity of **2c**. It is also notable that chitosan-conjugate **2c** with d.s. 0.16 showed an antimetastatic potency comparable to that of the PEG-conjugate, whose two termini are completely substituted with the peptide.[8] Chitosan, unlike PEG, may be conjugated with the YIGSR peptide at a higher d.s. than that of **2c**, if reaction conditions are appropriately controlled. The chitosan conjugate with a high d.s. is undoubtedly favourable for the increase in the local concentration of the peptide, and might possibly inhibit cancer metastasis more potently. Appropriate reaction conditions to control the d.s. of the peptide–chitosan conjugate are now under examination in our laboratories.

Conclusions

The Ac-Tyr-Ile-Gly-Ser-Arg-βAla–chitosan conjugate has been successfully synthesized on the basis of regioselective modification strategy of chitosan. Organosoluble 6-*O*-Trt-chitosan was useful to enable the regioselective introduction of the YIGSR peptide at the amino function of chitosan under mild conditions, and would be applicable to conjugation with other bioactive peptides. Conjugate **2c** has been shown to exhibit higher antimetastatic activity than the parent peptide. This indicates that chitosan is promising as a polymeric carrier for bioactive peptides. The results obtained here would facilitate the utilization of peptide–chitosan conjugates in medicinal applications.

Experimental

R_f-values of TLC (Kieselgel 60 F_{254}, Merck) refer to $CHCl_3$–MeOH–AcOH (90 : 8 : 2), and spots were detected with UV light and/or staining with 0.1% ninhydrin in acetone. Optical rotations were measured with a JASCO DIP-370 polarimeter, and $[a]_D$-values are in units of 10^{-1} deg cm^2 g^{-1}. HPLC was conducted with a WATERS 600 system, and A and B in the mobile phase system refer to 0.05% TFA aq. and 0.05% TFA in MeCN, respectively. The amino acid compositions of acid hydrolysates were determined with a WATERS PicoTAG amino acid analyser. The IR spectra were recorded on a JASCO IRS-700 instrument. Gel-permeation chromatography (GPC) was carried out with a JASCO 880-PU connected with a Shodex RI detector SE-61 [column TSK-GMPWXK × 2, solvent 0.1% lactic acid (1.0 cm^3 min^{-1})] using pullulan as reference.

Silica gel (WAKO Gel C-200) for column chromatography was purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). DMF, DMA, and $EtNPr^i_2$ were distilled from ninhydrin before use. HOBT used was its hydrate. Boc-Arg-(Tos)-OH·4/5AcOEt, DPPA, TFA, anisole, and 4 mol dm^{-3} HCl in AcOEt were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Chitosan was prepared from shrimp chitin in the usual manner.[21] WSCI was purchased from Peptide Institute, Inc. (Osaka, Japan). Other reagents were of reagent grade and used without purification.

Boc-Arg(Tos)-βAla-OBzl

To an ice-cooled solution of Boc-Arg(Tos)-OH·4/5AcOEt (5.3 g, 10 mmol) and H-βAla-OBzl·TosOH (3.5 g, 10 mmol) in DMF containing Et_3N (1.4 cm^3, 10 mmol) were added DPPA (2.2 cm^3, 10 mmol) and Et_3N (1.4 cm^3, 10 mmol). The mixture was stirred for 4 h under cooling with ice. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed successively with 5% aq. $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated. The oily residue in $CHCl_3$ (10 cm^3) was applied to a silica gel (100 g) column, which was equilibrated with $CHCl_3$ and eluted with $CHCl_3$ (500 cm^3) and then with 1.5% MeOH in $CHCl_3$ (1.9 dm^3). The solvent of the effluent (0.9–2.4 dm^3) was removed by evaporation to give a slightly yellowish amorphous powder (4.6 g, 78%); $[a]_D^{25}$ +3.5 (*c* 0.5 in DMF); R_f 0.43 (Found: C, 54.6; H, 6.49; N, 10.9. $C_{28}H_{39}N_5O_7S·1.6H_2O$ requires C, 54.4; H, 6.88; N, 11.3%). The amino acid ratio of the acid hydrolysate was βAla : Arg 1.00 : 1.09 (average recovery 108%).

Boc-Ser(Bzl)-Arg(Tos)-βAla-OBzl

To an ice-cooled solution of H-Arg(Tos)-βAla-OBzl·TFA [prepared from Boc-Arg(Tos)-βAla-OBzl (4.0 g, 6.8 mmol), TFA (8.0 cm^3), and anisole (2.0 cm^3) in the usual manner] in DMF

(100 cm^3) were added Boc-Ser(Bzl)-OH (2.4 g, 8.1 mmol), BOP (3.6 g, 8.1 mmol), and Et_3N (3.2 cm^3, 23 mmol). The mixture was stirred at rt for 2 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed successively with 5% aq. $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated. The oily residue in $CHCl_3$ (30 cm^3) was applied to a silica gel (100 g) column, which was equilibrated with $CHCl_3$ and eluted with $CHCl_3$ (0.6 dm^3) and then with 1% MeOH in $CHCl_3$ (2.8 dm^3). The solvent of the effluent (1.4–3.4 dm^3) was removed by evaporation to give a slightly yellowish amorphous powder (3.3 g, 63%); $[a]_D^{25}$ +1.7 (*c* 0.5 in DMF); R_f 0.50 (Found: C, 57.4; H, 6.48; N, 10.4. $C_{38}H_{50}N_6O_9S·1.5H_2O$ requires C, 57.5; H, 6.73; N, 10.6%). The amino acid proportions of the acid hydrolysate were βAla : Arg : Ser 1.00 : 0.89 : 0.83 (average recovery 90.7%).

Boc-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl

To an ice-cooled solution of H-Ser(Bzl)-Arg(Tos)-βAla-OBzl·HCl [prepared from Boc-Ser(Bzl)-Arg(Tos)-βAla-OBzl (3.3 g, 4.25 mmol) and 4 mol dm^{-3} HCl in AcOEt (11 cm^3) in the usual manner] in DMF (80 cm^3) were added Boc-Gly-OH (0.89 g, 5.1 mmol), BOP (2.3 g, 5.1 mmol), and Et_3N (2.0 cm^3, 14.45 mmol). The mixture was stirred at rt for 2 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed successively with 5% aq. $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated. The oily residue in $CHCl_3$ (10 cm^3) was applied to a silica gel (80 g) column, which was equilibrated with $CHCl_3$ and eluted successively with $CHCl_3$ (0.2 dm^3), 1% MeOH in $CHCl_3$ (0.5 dm^3), and then 2% MeOH in $CHCl_3$ (1.8 dm^3). The solvent of the effluent (1.0–2.5 dm^3) was removed by evaporation to give a slightly yellowish amorphous powder (2.9 g, 83%); $[a]_D^{25}$ +2.5 (*c* 0.5 in DMF); R_f 0.35 (Found: C, 56.1; H, 6.36; N, 11.0. $C_{40}H_{53}N_7O_{10}S·2H_2O$ requires C, 55.9; H, 6.68; N, 11.4%). The amino acid proportions of the acid hydrolysate were βAla : Arg : Ser : Gly 1.00 : 0.97 : 0.80 : 1.19 (average recovery 99.0%).

Boc-Ile-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl

To an ice-cooled solution of H-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl·TFA [prepared from Boc-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl (2.9 g, 3.6 mmol), TFA (4.0 cm^3), and anisole (1.0 cm^3) in the usual manner] in DMF (80 cm^3) were added Boc-Ile-OH·1/2H_2O (0.94 g, 3.9 mmol), HOBT (0.6 g, 3.9 mmol), BOP (1.7 g, 3.9 mmol), and Et_3N (1.6 cm^3, 11.4 mmol). The mixture was stirred at rt for 2 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed successively with 5% $NaHCO_3$ and water, dried over Na_2SO_4, and evaporated. Diethyl ether was added to the gelatinous residue, which was collected by filtration, washed with diethyl ether, and reprecipitated from EtOH–diethyl ether (1 : 10 *v*/*v*; 110 cm^3); yield 2.8 g (83%); mp range 65–73 °C; $[a]_D^{25}$ −1.6 (*c* 0.5 in DMF); R_f 0.47 (Found: C, 58.9; H, 7.04; N, 11.5. $C_{46}H_{64}N_8O_{11}S·0.25H_2O$ requires C, 58.7; H, 6.90; N, 11.9%). The amino acid proportions of the acid hydrolysate were βAla : Arg : Ser : Gly : Ile 1.00 : 0.90 : 0.63 : 0.96 : 1.00 (average recovery 102%).

Boc-Tyr(Cl_2Bzl)-Ile-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl

To an ice-cooled solution of H-Ile-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl·TFA [prepared from Boc-Ile-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl (1.00 g, 1.1 mmol), TFA (4.0 cm^3), and anisole (1.0 cm^3) in the usual manner] in DMF (50 cm^3) were added Boc-Tyr(Cl_2Bzl)-OH (0.56 g, 1.3 mmol), HOBT (0.20 g, 1.3 mmol), BOP (0.57 g, 1.3 mmol), and Et_3N (0.5 cm^3, 3.6 mmol). The mixture was stirred at rt for 2 h. After removal of the solvent, EtOH–water (1 : 1 *v*/*v*; 50 cm^3) was added to the residue to afford a precipitate, which was collected by filtration, washed with EtOH–water (1 : 1 *v*/*v*), and recrystallized from EtOH (30 cm^3); yield 1.1 g (79%); mp range 185–190 °C; $[a]_D^{25}$ −0.1 (*c* 0.5 in DMF); R_f 0.58 (Found: C, 58.9; H, 6.23; N, 9.68. $C_{62}H_{77}Cl_2N_9O_{13}S·0.2H_2O$ requires C, 59.0; H, 6.18; N, 9.68%). βAla : Arg : Ser : Gly : Ile : Tyr 1.00 : 0.81 : 0.57 : 0.94 : 0.91 : 0.95 (average recovery 96.0%).

Ac-Tyr(Cl_2Bzl)-Ile-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl

To an ice-cooled solution of H-Tyr(Cl_2Bzl)-Ile-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl·HCl [prepared from Boc-Tyr(Cl_2Bzl)-Ile-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl (1.08 g, 0.86 mmol), TFA (4.0 cm^3), and anisole (1.0 cm^3) in the usual manner] in DMF (50 cm^3) containing Et_3N (0.12 cm^3, 0.86 mmol) was added acetic anhydride (0.10 g, 0.94 mmol). The mixture was stirred at rt overnight. After removal of the solvent, water was added to the residue to afford a precipitate, which was collected by filtration and washed successively with water, EtOH, and *n*-hexane; yield 0.76 g (74%); mp range 216–221 °C; $[a]_D^{25}$ −1.6 (*c* 0.5 in DMF); R_f 0.58 (Found: C, 58.5; H, 5.96; N, 10.1. $C_{59}H_{71}Cl_2N_9O_{12}S·0.6H_2O$ requires C, 58.5; H, 6.00; N, 10.4%).

Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH·HCl 1

To an ice-cooled solution of Ac-Tyr(Cl_2Bzl)-Ile-Gly-Ser(Bzl)-Arg(Tos)-βAla-OBzl (0.24 g, 0.20 mmol) in TFA (3.8 cm^3) containing thioanisole (0.59 cm^3, 5.0 mmol) and *m*-cresol (0.17 cm^3) was added TFMSA (0.44 cm^3, 5.0 mmol). The mixture was stirred at an ice-bath temperature for 30 min, and then at rt for 90 min. Diethyl ether (50 cm^3) and water (20 cm^3) were added to the reaction mixture. The water layer was washed with diethyl ether (50 cm^3 × 5), neutralized with $NaHCO_3$, and applied to a Sephadex G-10 column (2.8 × 48 cm), which was equilibrated and eluted with 3% aq. AcOH. Individual fractions (11 cm^3) were collected, and the desired fractions (# 11–20) were combined and lyophilized to give a white fluffy powder (174 mg). After purification by preparative HPLC [column YMC-D-ODS (20 × 250 mm), A : B 90 : 10 to 40 : 60 in 60 min (10 cm^3 min^{-1})], successive lyophilization from 0.01 mol dm^{-3} aq. HCl gave the title compound as a white powder (145 mg, 98%); t_R 15.87 min [99.4%, column YMC-R-ODS (4.6 × 250 mm), A : B 95 : 5 to 20 : 80 in 45 min (1.0 cm^3 min^{-1})]; *m*/*z* (FAB) 708 (MH^+).

Coupling of peptide 1 with 6-*O*-Trt-chitosan

WSCI method. To an ice-cooled solution of 6-*O*-Trt-chitosan (200 mg, 0.50 mmol equiv. NH_2-) and **1** (372 mg, 0.50 mmol) in DMA (5 cm^3) was added WSCI (0.12 g, 0.60 mmol). The mixture was stirred at rt overnight, and then poured into ice-cooled diethyl ether (400 cm^3) to give an off-white precipitate, which was collected by filtration and washed successively with water (10 cm^3 × 5) and acetone (10 cm^3 × 5); yield 267 mg. The IR spectrum of the product was almost identical with that of the product obtained by the DPPA method (1 equiv.) (see below).

DPPA method (0.5 equiv.). To a solution of 6-*O*-Trt-chitosan (40 mg, 0.10 mmol equiv. NH_2-) and **1** (37 mg, 0.05 mmol) in DMA (2 cm^3) were added DPPA (11 mm^3, 0.05 mmol) and 1 mol dm^{-3} $EtNPr^i_2$ in DMF (50 mm^3, 0.05 mmol). The mixture was stirred at rt overnight. After removal of the solvent, the residue was dissolved in MeOH (10 cm^3). The solution was poured into ice-cooled diethyl ether (200 cm^3) to give an off-white precipitate, which was collected by centrifugation and washed successively with diethyl ether (50 cm^3) and water (15 cm^3 × 5); yield 38 mg. The IR spectrum of the product was almost identical with that of the product by the DPPA method (1 equiv.).

DPPA method (1 equiv.). To a solution of 6-*O*-Trt-chitosan (200 mg, 0.50 mmol equiv. NH_2-) and **1** (372 mg, 0.50 mmol) in DMA (5 cm^3) were added DPPA (0.13 cm^3, 0.60 mmol) and 1 mol dm^{-3} $EtNPr^i_2$ in DMF (0.50 cm^3, 0.50 mmol). The mixture was stirred at rt overnight. After removal of the solvent,

the residue was dissolved in MeOH (10 cm^3). The solution was poured into ice-cooled diethyl ether (200 cm^3) to give an off-white precipitate, which was collected by filtration, washed successively with diethyl ether (200 cm^3) and water (200 cm^3), and reprecipitated from MeOH–diethyl ether (1 : 10 *v*/*v*; 220 cm^3); yield 245 mg; ν_{max}(KBr)/cm^{-1} 3386, 1655, 1517, 1489, 1447, 1207, 1156–1000, 914, 761, 700.

Ac-Tyr-Ile-Gly-Ser-Arg-βAla–chitosan (2a–c)

2c. The 6-*O*-Trt-chitosan-conjugate obtained by the DPPA method (1 equiv.) (240 mg) was dissolved in $CHCl_2CO_2H$ (10 cm^3) and the solution was stirred at rt for 1 h. The solution was poured into ice-cooled diethyl ether (200 cm^3) to give an off-white precipitate, which was collected by centrifugation and washed with diethyl ether (20 $cm^3 \times 5$). This was treated again with $CHCl_2CO_2H$ (10 cm^3) as described above. The crude product in 3% aq. AcOH (15 cm^3) was applied to a Sephadex G-25 column (2.8 × 90 cm), which was equilibrated and eluted with 3% aq. AcOH. Individual fractions (7 cm^3) were collected, and the desired fractions (# 30–46) were combined and lyophilized to give an off-white fluffy powder (140 mg), ν_{max}(KBr)/cm^{-1} 3388, 1654, 1541, 1406, 1251 and 1151–1000. Number-average molecular mass (determined by GPC): 22.2×10^3. The amino acid proportions in the acid hydrolysate were Tyr : Ile : Gly : Ser : Arg : βAla 0.76 : 0.87 : 1.00 : 1.00 : 0.97 : 0.78 (peptide content 0.49 mmol g^{-1}).

Conjugates **2a** and **2b** were prepared by similar methods to that of **2c**. **2a**: Yield 160 mg. The amino acid proportions in the acid hydrolysate were Tyr : Ile : Gly : Ser : Arg : βAla 0.83 : 0.97 : 1.00 : 0.97 : 0.98 : 1.01 (peptide content 0.35 mmol g^{-1}). **2b**: Yield 20 mg. The amino acid proportions in the acid hydrolysate were Tyr : Ile : Gly : Ser : Arg 0.94 : 0.90 : 1.00 : 1.12 : 0.92 (peptide content 0.40 mmol g^{-1}). βAla was not determined. The IR spectra of **2a** and **2b** were almost identical with that of **2c**.

Chitosan regenerated from 6-*O*-Trt-chitosan 3

A solution of 6-*O*-Trt-chitosan (500 mg) in $CHCl_2CO_2H$ (20 cm^3) was sitirred at rt for 1 h, and poured into ice-cooled diethyl ether (200 cm^3) to give an off-white precipitate, which was collected by centrifugation and washed with diethyl ether (20 $cm^3 \times 5$). The crude product in 3% aq. AcOH (15 cm^3) was applied to a Sephadex G-25 column (2.8 × 90 cm), which was equilibrated and eluted with 3% aq. AcOH. Individual fractions (7 cm^3) were collected, and the desired fractions (# 31–46) were combined and lyophilized to give an off-white fluffy powder (240 mg), ν_{max}(KBr)/cm^{-1} 1643, 1384, 1151–1000. Number-average molecular mass (determined by GPC): 13.8×10^3.

Antimetastatic activity

B16BL6 melanoma cells (1.5×10^5/100 mm^3) were inoculated intravenously into C57BL6 mice, and then **1**, **2c**, or **3** was injected intravenously. Mice were killed 2 weeks after the tumour inoculation and tumour colonies of lungs were counted with a stereoscopic microscope. Each value represents the mean ± S.E. ($n = 5$). Details of the assay procedure were described previously.[7,8,20]

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