Synthesis of Cancer Peptide Antigen-Lipid A Analog Conjugates for Synthetic Vaccines

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Received October 22, 1998; accepted January 12, 1999

Conjugates 6 and 7 of cancer peptide antigen with *N*-tetradecanoyl L-serine-β-alanine-containing D-glucosamine derivative structurally related to lipid A as an immunoadjuvant were synthesized for the development of totally synthetic vaccines against cancers. The mitogenic activities of compounds 6 and 7 were stronger than that of lipid A analog 3.

Key words lipid A analog; mitogenic activity; immunoadjuvant; synthetic vaccine; cancer peptide antigen

Lipid A is well known as being responsible for the expression of many of the biological activities: endotoxicity, adjuvanticity, antitumor activity and so on, of lipopolysaccharide (LPS) of gram-negative bacteria.[1] Among the various synthetic lipid A analogs, D-glucosamine-4-phosphate analogs of the non-reducing unit of lipid A showed many of the biological activities of LPS.[2] With the aim of enhancing its potentially beneficial immunostimulatory properties, numerous acyclic analogs related to lipid A partial structure have been synthesized.[3] We previously reported that *N*-tetradecanoyl-L-serine-linked lipid A analog (**2**) structurally similar to the lipid A disaccharide backbone exhibited potent mitogenic activity.[4] In addition, compound **3** with β-alanine introduced into compound **2** as a spacer possessed the same activity as **2**.[5]

Synthetic antigens generally have poor immunogenicity in the absence of immunological adjuvants.[6] However, the most popular adjuvant used in laboratory animals, Freund's complete adjuvant, is too toxic and unacceptable for humans. Therefore, development of totally synthetic vaccines against cancers or human immunodeficiency virus (HIV) using the synthetic immunoadjuvants, *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) and lipopeptide analog, has been attempted.[7] One of the merits of synthetic vaccines is that they are safer and more versatile than a whole or viral protein vaccine. We recently have reported the development of completely synthetic vaccines (**4**, **5**) against cancers which consist of the lipid A analog (**3**) as a synthetic immunoadjuvant , covalently coupled to Tn (α-D-GalNAc-(1$\rightarrow$*O*)-Ser) and Sialyl Tn (α-D-Neu5Ac-(2$\rightarrow$6)-α-D-GalNAc-(1$\rightarrow$*O*)-Ser) epi-

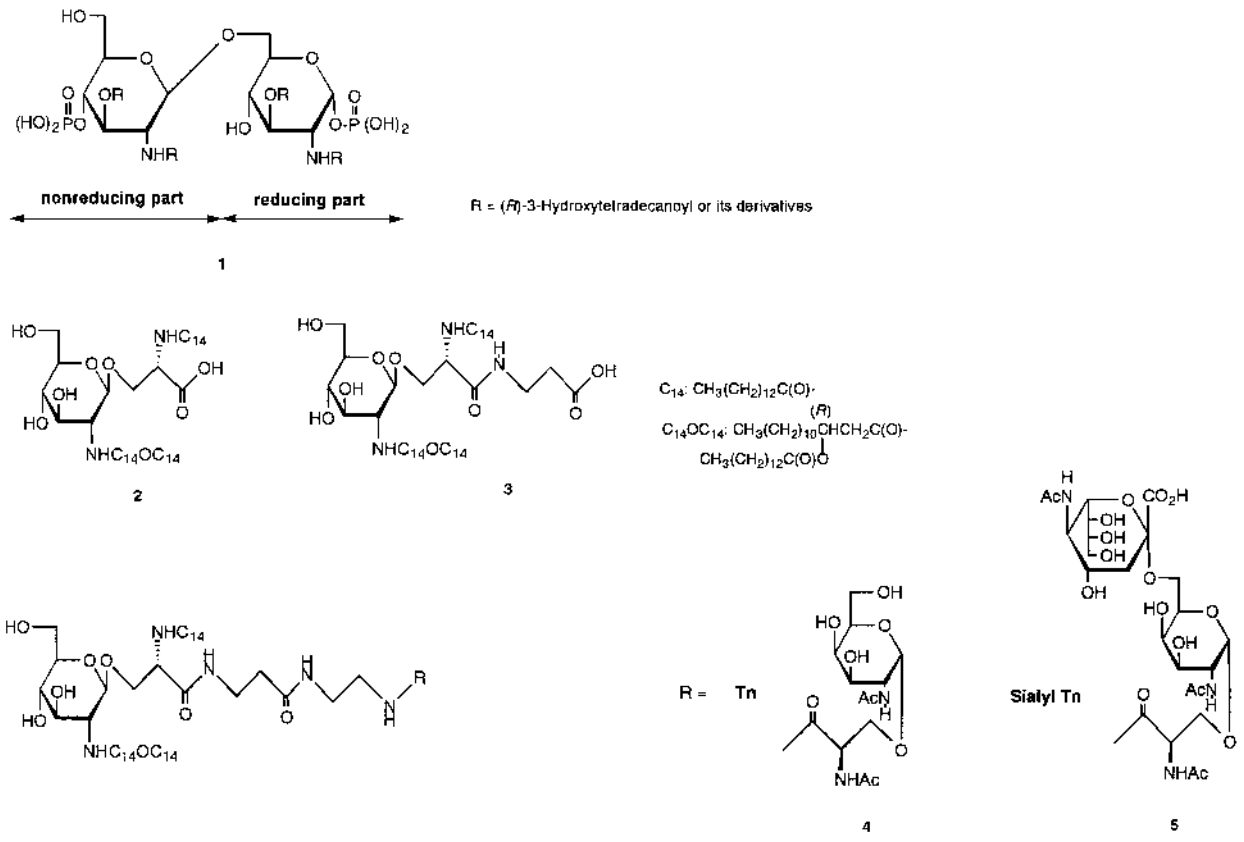

Fig. 1

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topes for antigens without the addition of other macromolecular carriers or adjuvants, respectively.[8] In this paper, we describe the synthesis of conjugates **6** and **7** containing of cancer peptide antigens, identified as tumour-associated peptide antigens on the surface of cancer cells, covalently attached to a *N*-tetradecanoyl-L-seryl-β-alanine-containing D-glucosamine derivative (**3**) structurally similar to lipid A, and their biological effects.

Synthesis First, synthesis of leukemia cancer gene derived peptide antigen derivative (**8**)[9] and melanoma *MAGE*-3 derived peptide epitope (**9**)[10] was accomplished manually by a stepwise liquid-phase procedure shown in Charts 1 and 2, respectively.

The assembly of the synthetic adjuvant–cancer peptide antigen conjugates is shown in Chart 3. Compound **3** was coupled with *p*-nitrophenol in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) in *N*,*N*-dimethylformamide (DMF) to give the *p*-nitrophenyl ester (**10**) [FAB-MS *m/z*: 1106 $(M+H)^+$, 1128 $(M+Na)^+$] in 55% yield. The active ester (**10**) was condensed with **8** and **9** in the presence of diisopropylethylamine (DIEA) to give **6** [FAB-MS *m/z*: 1797 $(M+Na)^+$] and **7** [FAB-MS *m/z*: 2030 $(M+Na)^+$] in 69% and 35%, respectively, after purification by chromatography on a column of silica gel and then Sephadex LH-20, followed by lyophilization from H_2O suspension.

Biological Activity In a preliminary examination of mitogenic activities towards the splenocytes of C3H/He mice,[11] conjugates **6** and **7** exhibited potent mitogenic activities in comparison with lipid A analog **3** (Fig. 3.). It is speculated that lipid A conjugates **6** and **7** may amplify peptide antigen-specific immune responses in mice.

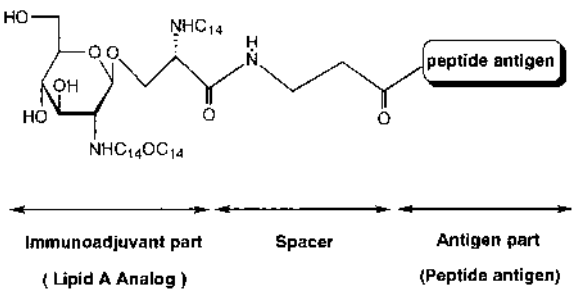

Fig. 2. Structure of Synthetic Vaccine

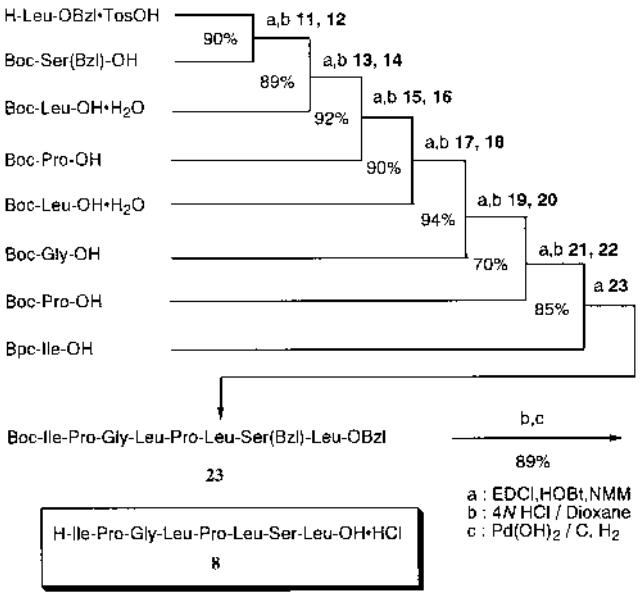

Chart 1

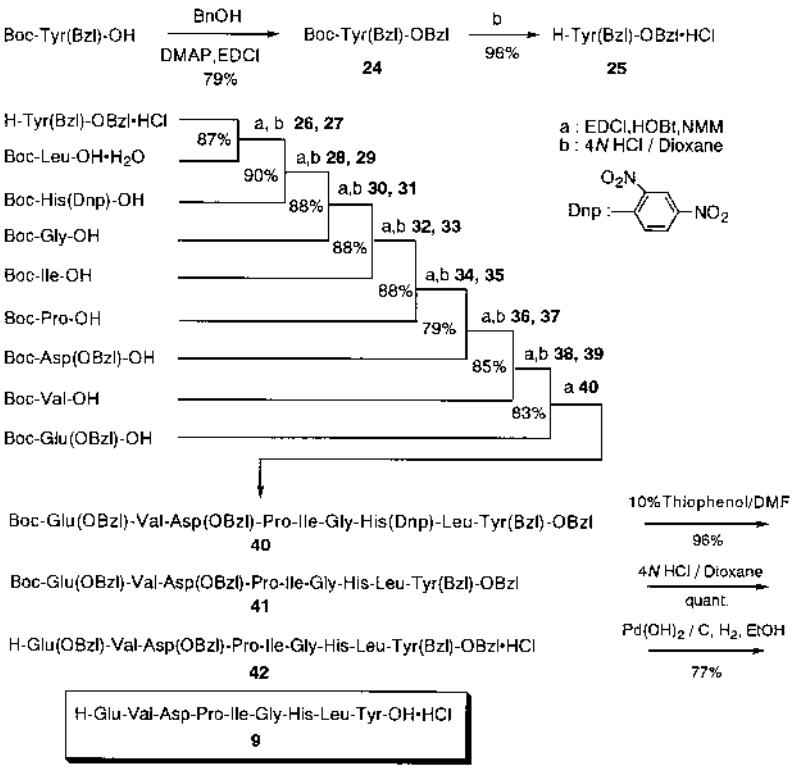

Chart 2

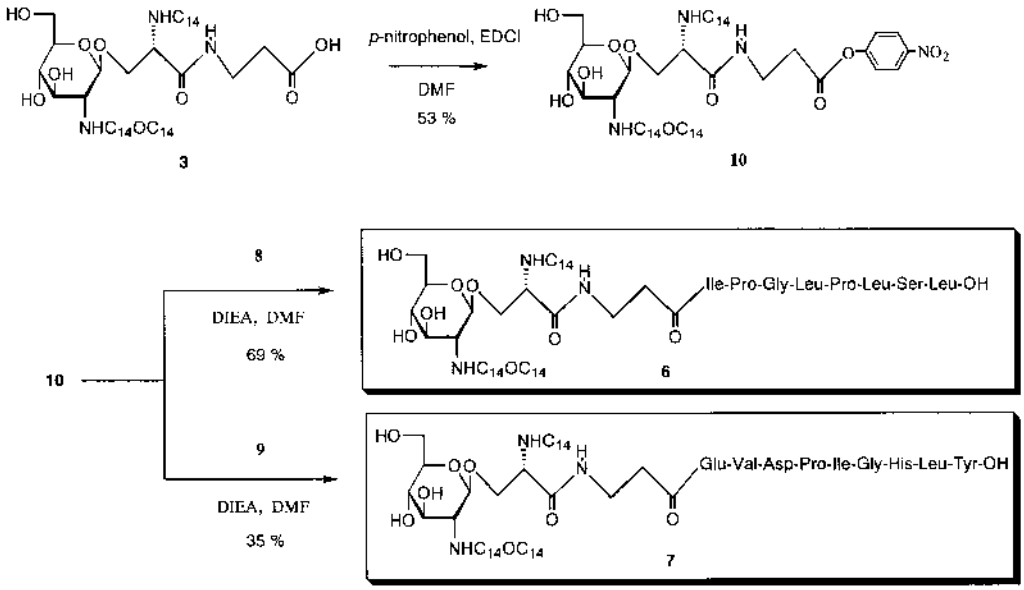

Chart 3

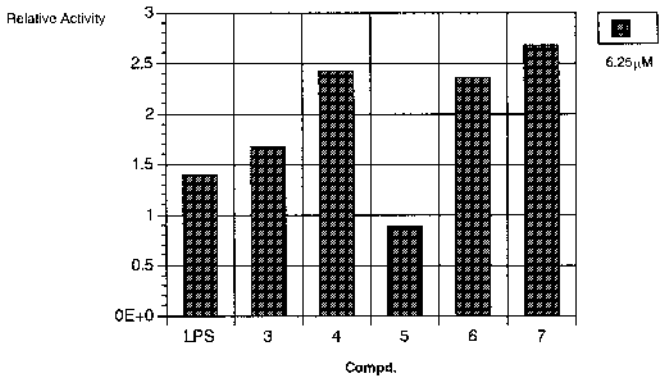

Fig. 3. Mitogenic Activities of Synthetic Vaccines
LPS: *S. typhimurium* LT-2 LPS.

Experimental

All melting points are uncorrected. Optical rotations were measured with a JASCO DIP-140 digital polarimeter. IR spectra were recorded on a JASCO IR-810 spectrometer. ^{1}H NMR spectra were recorded with a JEOL JNM-EX 270 (270 MHz) spectrometer. ^{1}H chemical shifts are given in ppm relative to Me_4Si (δ=0) in $CDCl_3$ or CD_3OD as internal standards at ambient temperature. The abbreviations of signal patterns are as follows: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Fast atom bombardment (FAB) mass spectra were obtained with a JEOL JMS SX-102 mass spectrometer in the positive ion mode using NBA matrix. Column chromatography was performed on Silica Gel 60 (70—230 mesh, Merck) and Sephadex LH-20 (Pharmacia). Thin-layer chromatography (TLC) was performed on aluminum sheets coated with Silica Gel 60F_{254}(Merck). The spots were visualized by spraying the plates with 5% aqueous sulfuric acid in MeOH and then heating.

***N-tert*-Butoxycarbonyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester (11)** To a solution of L-leucine benzyl ester *p*-toluenesulfonate (2.00 g, 5.08 mmol), *N*-methylmorpholine (NMM) (0.52 g, 5.08 mmol), *tert*-butoxycarbonyl-*O*-benzyl-L-serine (1.50 g, 5.08 mmol) and 1-hydroxybenzotriazole (HOBt) (0.78 g, 5.08 mmol) in DMF (10 ml) were added EDCl (0.98 g, 5.08 mmol) and NMM (0.52 g, 5.08 mmol) at 0 °C and the mixture was stirred for 18 h at room temperature under argon. After removal of the solvent, the residue was dissolved in CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$,10% aqueous citric acid and saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by silica gel column chromatography (hexane–EtOAc 3 : 1) to give syrupy **11** (2.28 g, 90%), $[\alpha]_D$ +8.2° (c=0.56, $CHCl_3$). IR (film): 3318, 1741, 1666, 1497cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.85 (3H, d, J=6.3 Hz, –CH_3), 0.88 (3 H, d, J=5.9 Hz, –CH_3), 1.44 (9 H, s, *tert*-Bu), 1.49—1.69 (3 H, m, C$\underline{H}_2$C$\underline{H}$(CH_3)$_2$), 3.48—3.94 (2 H, m, CH_2O–), 4.29 (1H, br s, CH–), 4.51 (2H, s, OC$\underline{H}_2$Ph), 4.56—4.69 (1H, m, =CH–), 5.15 (2H, s, COOC$\underline{H}_2$Ph), 5.42 (1H, br s, NH), 6.99 (1H, br s, NH), 7.29—7.41 (10H, m, 2 Ph).

***O*-Benzyl-L-seryl-L-leucine Benzyl Ester Hydrochloride (12)** Compound **11** (2.20 g, 4.41 mmol) was treated with 4 N HCl dioxane (10 ml) at room temperature for 1 h. Removal of the solvent gave amorphous **12** (1.92 g, quant.), which was used for the subsequent condensation without further purification.

***N-tert*-Butoxycarbonyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester (13)** The reaction was carried out using **12** (1.92 g, 4.41 mmol) and *tert*-butoxycarbonyl-L-leucine H_2O (1.10 g, 4.41 mmol) in a manner similar to the preparation of **11** to give amorphous **13** (2.39 g, 89%), mp 105—107 °C, $[\alpha]_D$ −13.0° (c=0.52, $CHCl_3$). IR (film): 3276, 1735, 1639, 1544 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.85 (3H, d, J=5.9 Hz, –CH_3), 0.87 (3 H, d, J=6.3 Hz, –CH_3), 0.93 (3H, d, J=2.0 Hz, –CH_3), 0.95 (3H, d, J=2.3 Hz, –CH_3), 1.41 (9H, s, *tert*-Bu), 1.46—1.70 (6H, m, 2 C$\underline{H}_2$C$\underline{H}$(CH_3)$_2$), 3.48—3.97 (2H, m, CH_2O–), 4.09 (1H, br s, =CH–), 4.51 (2H, s, OC$\underline{H}_2$Ph), 4.53—4.62 (1H, m, =CH–), 4.82 (1H, br s, =CH–), 5.15 (2H, s, COOC$\underline{H}_2$Ph), 6.90 (1H, d, J=7.2 Hz, NH), 7.08 (1H, d, J=8.2 Hz, NH), 7.28—7.37 (10H, m, 2 Ph). *Anal.* Calcd for $C_{34}H_{49}N_3O_7$: C, 66.75; H, 8.07; N, 6.87. Found: C, 66.71; H, 8.12; N, 6.98.

L-Leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester Hydrochloride (14) The reaction was carried out using **13** (2.30 g, 3.76 mmol) in a manner similar to the preparation of **12** to give amorphous **14** (2.06 g, quant.), which was used for the subsequent condensation without further purification.

***N-tert*-Butoxycarbonyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester (15)** The reaction was carried out using **14** (2.06 g, 3.76 mmol) and *tert*-butoxycarbonyl-L-proline (0.89 g, 4.13 mmol) in a manner similar to the preparation of **11** to give **15** (2.46 g, 92%), after purification by silica gel column chromatography (hexane–EtOAc 1 : 3). mp 79—81 °C, $[\alpha]_D$ −58.9° (c=0.50, $CHCl_3$). IR (film): 3280, 1743, 1637, 1542 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.85—0.94 (12H, m, 4 CH_3), 1.46 (9H, s, *tert*-Bu), 1.59—1.88 (6H, m, 2 C$\underline{H}_2$C$\underline{H}$(CH_3)$_2$), 1.88—2.37 (4H, m, Pro), 3.38—3.50 (2H, m, Pro), 3.69—3.95 (2H, m, CH_2O–), 4.22 (1H, br s, =CH–), 4.32 (1H, br s, =CH–), 4.49 (2H, s, OC$\underline{H}_2$Ph), 4.58—4.68 (2H, m, 2 =CH–),5.14 (2H, s, COOC$\underline{H}_2$Ph), 7.09 (1H, br d, J=7.2 Hz, NH), 7.16 (1H, br d, J=8.2 Hz, NH), 7.29 (5H, s, Ph), 7.34 (5H, s, Ph), 7.42 (1H, br s, NH). *Anal.* Calcd for $C_{39}H_{56}N_4O_8$: C, 66.08; H, 7.96; N, 7.90. Found: C, 65.90; H, 8.12; N, 7.61.

L-Prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester Hydrochloride (16) The reaction was carried out using **15** (2.36 g, 3.33 mmol) in a manner similar to the preparation of **12** to give amorphous **16** (2.01 g, 94%), which was used for the subsequent condensation without further purification.

***N-tert*-Butoxycarbonyl-L-leucyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester (17)** The reaction was carried out using **16** (2.01 g,

3.12 mmol) and *tert*-butoxycarbonyl-L-leucine H_2O (0.85 g, 3.43 mmol) in a manner similar to the preparation of **11** to give amorphous **17** (2.30 g, 90%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 30 : 1), mp 59—62 °C, $[\alpha]_D$ −63.0° (c=0.50, $CHCl_3$). IR (film): 3290, 1742, 1645, 1528 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.83—0.98 (18H, m, 6–CH_3), 1.44 (9H, s, *tert*-Bu), 1.21—1.72 (9H, m, 3C$\underline{H}_2$C$\underline{H}$(CH_3)$_2$), 1.80—2.30 (4H, m, Pro), 3.48—3.60 (2H, m, Pro), 3.66—4.01 (2H, m, CH_2O–), 4.25—4.32 (1H, m, =CH–), 4.40—4.62 (4H, m, 47 =CH–), 4.50 (2H, br s, OC$\underline{H}_2$Ph), 5.15 (2H, br s, COOC$\underline{H}_2$Ph), 6.97 (1H, d, J=6.9 Hz, NH), 7.10 (1H, d, J=8.6 Hz, NH), 7.30 (5H, s, Ph), 7.35 (5H, s, Ph). *Anal.* Calcd for $C_{45}H_{67}N_5O_9\cdot 1/4H_2O$: C, 65.39; H, 8.23; N, 8.47. Found: C, 65.36; H, 8.22; N, 8.62.

L-Leucyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester Hydrochloride (18) The reaction was carried out using **17** (2.22 g, 2.70 mmol) in a manner similar to the preparation of **12** to give amorphous **18** (2.05 g, quant.), which was used for the subsequent condensation without further purification.

***N*-*tert*-Butoxycarbonylglycyl-L-leucyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester (19)** The reaction was carried out using **18** (2.05 g, 2.70 mmol) and *tert*-butoxycarbonylglycine (0.52 g, 2.97 mmol) in a manner similar to the preparation of **11** to give amorphous **19** (2.23 g, 94%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 20 : 1), mp 70—73 °C, $[\alpha]_D$ −61.2° (c=0.50, $CHCl_3$). IR (film): 3280, 1718, 1628, 1533 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.84—0.97 (18H, m, 6 –CH_3), 1.44 (9H, s, *tert*-Bu), 1.48—1.72 (9H, m, 3 C$\underline{H}_2$C$\underline{H}$(CH_3)$_2$), 1.85—2.22 (4H, m, Pro), 3.51—3.57 (2H, m, Pro), 3.76—3.98 (2H, m, CH_2O–), 3.83 (2H, s, –CH_2–), 4.28—4.38 (1H, m, =CH–), 4.47—4.64 (3H, m, 3 =CH–), 4.51 (2H, s, OC$\underline{H}_2$Ph), 4.78—4.88 (1H, m, =CH–), 5.15 (2H, br s, COOC$\underline{H}_2$Ph), 6.93 (1H, d, J=6.9 Hz, NH), 7.10 (1H, d, J=8.3 Hz, NH), 7.30 (5H, s, Ph), 7.35 (5H, s, Ph). *Anal.* Calcd for $C_{47}H_{70}N_6O_{10}\cdot 1/2H_2O$: C, 63.56; H, 8.06; N, 9.46. Found: C, 63.51; H, 8.00; N, 9.76.

Glycyl-L-leucyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester Hydrochloride (20) The reaction was carried out using **19** (2.15 g, 2.45 mmol) in a manner similar to the preparation of **12** to give amorphous **20** (2.00 g, quant.), which was used for the subsequent condensation without further purification.

***N*$^{\alpha}$-*tert*-Butoxycarbonyl-L-prolyl-glycyl-L-leucyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester (21)** The reaction was carried out using **20** (2.00 g, 2.45 mmol) and *tert*-butoxycarbonyl-L-proline (0.58 g, 2.70 mmol) in a manner similar to the preparation of **11** to give amorphous **21** (1.68 g, 70%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 15 : 1), mp 80—83 °C, $[\alpha]_D$ −63.0° (c=0.53, $CHCl_3$). IR (film): 3280, 1742, 1649, 1530 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.86—0.92 (18H, m, 6 –CH_3),1.46 (9H, s, *tert*-Bu), 1.50—1.74 (9H, m, 3 C$\underline{H}_2$C$\underline{H}$(CH_3)$_2$), 1.84—2.15 (8H, m, 2 Pro), 3.40—3.60 (4H, m, 2Pro), 3.76—4.02 (2H, m, CH_2O–), 3.98 (2H, s, –CH_2–), 4.21—4.26, 4.30—4.36 (each 1H, m, =CH–), 4.51 (2H, s, OC$\underline{H}_2$Ph), 4.52—4.62 (3H, m, 3 =CH–), 4.76 (1H, m, =CH–), 5.15 (2H, br s, COOC$\underline{H}_2$Ph), 7.00 (2H, br s, 2 NH), 7.10—7.14 (2H, m, 2NH), 7.30 (5H, s, Ph), 7.35 (5H, s, Ph). *Anal.* Calcd for $C_{52}H_{77}N_7O_{11}\cdot H_2O$: C, 62.82; H, 8.01; N, 9.86. Found: C, 62.96; H, 7.88; N, 9.77.

L-Prolyl-glycyl-L-leucyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester Hydrochloride (22) The reaction was carried out using **21** (1.60 g, 1.64 mmol) in a manner similar to the preparation of **12** to give amorphous **22** (1.50 g, quant.), which was used for the subsequent condensation without further purification.

***N*$^{\alpha}$-*tert*-Butoxycarbonyl-L-isoleucyl-L-prolyl-glycyl-L-leucyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Benzyl Ester (23)** The reaction was carried out using **22** (1.50 g, 1.64 mmol) and *tert*-butoxycarbonyl-L-isoleucine (0.42 g, 1.80 mmol) in a manner similar to the preparation of **11** to give amorphous **23** (1.51 g, 85%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 20 : 1), mp 100—102 °C, $[\alpha]_D$ −45.4° (c=0.50, $CHCl_3$). IR (film): 3280, 1740, 1625, 1524 cm^{-1}. *Anal.* Calcd for $C_{58}H_{88}N_8O_{12}\cdot H_2O$: C, 62.91; H, 8.19; N, 10.11. Found: C, 62.68; H, 8.08; N, 10.00.

L-Isoleucyl-L-prolyl-glycyl-L-leucyl-L-prolyl-L-leucyl-*O*-benzyl-L-seryl-L-leucine Hydrochloride (8) Compound **23** (0.23 g, 0.21 mmol) was treated with 4 N HCl–dioxane (10 ml) in a manner similar to the preparation of **12** to give the amino compound which was dissolved in EtOH (25 ml) and then was hydrogenated over $Pd(OH)_2$–C (0.10 g) for 6 h at 40 °C, then filtered and concentrated. The residue was purified by reverse phase high performance liquid chromatography (RP-HPLC) purification (column: Asahipak ODP-50 (0.6×25 cm). Eluent: A, 0.05% trifluoroacetic acid (TFA); B, CH_3CN, linear gradient from 25% to 45% B in 30 min. Detection: UV at 210 nm. Flow rate: 1.0 ml/min) to give amorphous **6** (0.155 g, 89%), $[\alpha]_D$ −108.2° (c=0.50, 3% AcOH). IR (film): 3304, 1646, 1536 cm^{-1}. Positive FAB-MS m/z: 810 $(M+H)^+$, 832 $(M+Na)^+$.

***N*$^{\alpha}$-*tert*-Butoxycarbonyl-*O*-benzyl-L-tyrosine Benzyl Ester (24)** To a solution of *tert*-butoxycarbonyl-*O*-benzyl-L-tyrosine (1.00 g, 2.69 mmol) and dimethylaminopyridine (DMAP) (33 mg, 0.27 mmol) and benzyl alcohol (0.35 g, 3.23 mmol) in CH_2Cl_2 (10 ml) was added EDCl (0.62 g, 3.23 mmol) at 0 °C and the mixture was stirred for 14 h at room temperature. The reaction mixture was washed with saturated aqueous $NaHCO_3$, 10% aqueous citric acid and saturated aqueous brine, dried ($MgSO_4$) and concentrated. The residue was crystallized from MeOH–ether–petroleum ether to give **24** (0.98 g, 79%), mp 85—86 °C. IR (film): 3420, 1705, 1699, 509 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.44 (9H, s, *tert*-Bu), 2.98 (2H, s, C$\underline{H}_2$PhO–), 4.57—4.64 (1H, m, =CH–), 5.02 (2H, s, OC$\underline{H}_2$Ph), 5.14 (2H, s, COOC$\underline{H}_2$Ph), 6.82—6.96 (4H, m, CH_2Ph$\underline{O}$–), 7.30—7.43 (10H, m, 2 Ph). *Anal.* Calcd for $C_{28}H_{31}NO_5$: C, 72.86; H, 6.77; N, 3.03. Found: C, 72.54; H, 6.93; N, 2.83.

***O*-Benzyl-L-tyrosine Benzyl Ester Hydrochloride (25)** The reaction was carried out using **24** (0.87 g, 1.88 mmol) in a manner similar to the preparation of **12** to give amorphous **25** (0.73 g, 98%), which was used for the subsequent condensation without further purification, mp 203—205 °C, $[\alpha]_D$ −17.1° (c=1.1, MeOH).

***N*$^{\alpha}$-*tert*-Butoxycarbonyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (26)** The reaction was carried out using **25** (0.68 g, 1.71 mmol) and *tert*-butoxycarbonyl-L-leucine. H_2O (0.43 g, 1.71 mmol) in a manner similar to the preparation of **11** to give syrupy **26** (0.85 g, 87%), after purification by silica gel column chromatography (hexane–EtOAc 4 : 1), mp 113—114 °C, $[\alpha]_D$ −7.1° (c=0.60, $CHCl_3$). IR (film): 3340, 1732, 1663, 1520 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (6H, d, J=4.9 Hz, 2 –CH_3), 1.43 (9H, s, *tert*-Bu), 1.61 (2H, s, C$\underline{H}_2$CH(CH_3)$_2$), 3.06 (2H, s, C$\underline{H}_2$PhO–), 4.08 (1H, br s, C$\underline{H}$(CH_3)$_2$), 4.80—4.87 (2H, m, 2 =CH–), 5.01 (2H, s, OC$\underline{H}_2$Ph), 5.12 (2H, s, COOC$\underline{H}_2$Ph), 6.48 (1H, d, J=7.6 Hz, NH), 6.81 (2H, d, J=8.6 Hz, –PhO–), 6.92 (2H, d, J=8.9 Hz, –PhO–), 7.26—7.44 (10H, m, 2 Ph). *Anal.* Calcd for $C_{34}H_{42}N_2O_6$: C, 71.06; H, 7.37; N, 4.87. Found: C, 71.84; H, 7.46; N, 4.83.

L-Leucyl-*O*-benzyl-L-tyrosine Benzyl Ester Hydrochloride (27) The reaction was carried out by using **26** (0.80 g,1.39 mmol) in a manner similar to the preparation of **12** to give amorphous **27** (0.71 g, quant.), which was used for the subsequent condensation without further purification.

***N*$^{\alpha}$-*tert*-Butoxycarbonyl-*N*^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (28)** The reaction was carried out using **27** (0.71 g, 1.39 mmol) and *tert*-butoxycarbonyl-*N*^{im}-2,4-dinitrophenyl-L-histidine. isopropyl amine·isopropanol (IPA) (0.67 g, 1.39 mmol) in a manner similar to the preparation of **11** to give amorphous **28** (1.10 g, 90%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 30 : 1), mp 85—88 °C, $[\alpha]_D$ −8.6° (c=0.52, $CHCl_3$). IR (film): 3264, 1736, 1643, 1508 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, d, J=5.9 Hz, –CH_3), 0.86 (3H, d, J=6.3 Hz, –CH_3), 1.45 (9H, s, *tert*-Bu), 1.50—1.78 (3H, m, C$\underline{H}_2$C$\underline{H}$(CH_3)$_2$), 2.94—3.10 (4H, m, C$\underline{H}_2$PhO–, C$\underline{H}_2$Im), 4.37—4.44 (1H, m, 2 =CH–), 4.72—4.80 (1H, m, =CH–), 4.99 (2H, s, OC$\underline{H}_2$Ph), 5.10 (2H, s, COOC$\underline{H}_2$Ph), 5.90 (1H, br s, NH), 6.80, 6.94 (each 2H, d, J=8.6 Hz, –PhO–), 6.85 (1H, s, imidazole), 7.22—7.42 (10H, m, 2 Ph), 7.44 (1H, s, imidazole), 7.62 (1H, d, J=8.9 Hz, –Ph(NO_2)$_2$), 8.45 (1H, dd, J=8.9, 2.7 Hz, –Ph(NO_2)$_2$), 8.78 (1H, d, J=2.7 Hz, –Ph(NO_2)$_2$). *Anal.* Calcd for $C_{46}H_{51}N_7O_{11}\cdot 1/2H_2O$: C, 62.29; H, 5.91; N, 11.05. Found: C, 62.52; H, 5.92; N, 10.97.

***N*^{im}-2,4-Dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester Hydrochloride (29)** The reaction was carried out using **28** (1.00 g, 1.14 mmol) in a manner similar to the preparation of **12** to give amorphous **29** (0.93 g, quant.), which was used for the subsequent condensation without further purification.

***N*$^{\alpha}$-*tert*-Butoxycarbonylglycyl-*N*^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (30)** The reaction was carried out using **29** (0.93 g, 1.14 mmol) and *tert*-butoxycarbonylglycine (0.20 g, 1.14 mmol) in a manner similar to the preparation of **11** to give amorphous **30** (0.94 g, 88%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 30 : 1), mp 165—168 °C, $[\alpha]_D$ −1.7° (c=0.53, $CHCl_3$). IR (film): 3264, 1746, 1636, 1536 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, d, J=5.9 Hz, –CH_3), 0.85 (3H, d, J=5.9 Hz, –CH_3),1.42 (9H, s, *tert*-Bu), 1.48—1.72 (1H, m, CH_2C$\underline{H}$(CH_3)$_2$), 1.94 (2H, br s, C$\underline{H}_2$CH(CH_3)$_2$), 2.88—3.15 (4H, m, C$\underline{H}_2$PhO–, C$\underline{H}_2$Im), 3.80 (2H, d, J=5.3 Hz, –CH_2–), 4.42—4.50 (1H, m, =CH–), 4.71—4.78 (each 1H, m, =CH–), 4.97 (2H, s, OC$\underline{H}_2$Ph), 5.09 (2H, s, COOC$\underline{H}_2$Ph), 5.33 (1H, br s, NH), 6.80, 6.98 (each 2H, d, J=8.2 Hz, –PhO–), 6.89 (1H, s, imidazole), 7.24—7.38 (10H, m, 2 Ph), 7.44 (1H, s, imidazole), 7.63 (1H, br s, NH), 7.67 (1H, d, J=8.9 Hz, –Ph(NO_2)$_2$), 7.76 (1H, br s, NH), 8.45 (1H, dd, J=8.6, 2.2 Hz, –Ph(NO_2)$_2$),

8.78 (1H, d, J=2.3 Hz, –Ph(NO_2)$_2$). *Anal.* Calcd for $C_{48}H_{54}N_8O_{12}\cdot H_2O$: C, 60.50; H, 5.92; N, 11.76. Found: C, 60.56; H, 5.75; N, 11.55.

Glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester Hydrochloride (31) The reaction was carried out using **30** (0.84 g, 0.90 mmol) in a manner similar to the preparation of **12** to give **31** (0.78 g, quant.) as amorphous powder, which was used for the subsequent condensation without further purification.

N^{α}-*tert*-Butoxycarbonyl-L-isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (32) The reaction was carried out using **31** (0.78 g, 0.90 mmol) and *tert*-butoxycarbonyl-L-isoleucine (0.23 g, 0.99 mmol) in a manner similar to the preparation of **11** to give amorphous **32** (0.83 g, 88%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 20 : 1), mp 189—190 °C, $[\alpha]_D$ +4.3° (c=0.54, $CHCl_3$). IR (film): 3276, 1735, 1639, 1540 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.76 (3H, d, J=5.6 Hz, –CH_3), 0.81 (3H, d, J=5.6 Hz, –CH_3), 0.85—0.91 (6H, m, 2 –CH_3), 1.40 (9H, s, *tert*-Bu), 1.12—1.88 (6H, m, –C$\underline{H}_2$C$\underline{H}$(C$\underline{H}_3$)$_2$, C$\underline{H}$(CH_3)C$\underline{H}_2$$CH_3$), 2.88—3.04 (4H, m, C$\underline{H}_2$PhO–, C$\underline{H}_2$Im), 4.05—4.14 (3H, m, –$CH_2$–, =CH–), 4.58—4.63 (each 1H, m, 2 =CH–), 4.90 (2H, s, OC$\underline{H}_2$Ph), 4.92—4.96 (1H, m, =CH–), 5.07 (2H, s, COOC$\underline{H}_2$Ph), 5.37 (1H, br s, NH), 6.75 (2H, d, J=8.6 Hz, –PhO–), 6.93 (2H, d, J=8.3 Hz, –PhO–), 6.86 (1H, s, imidazole), 7.21—7.34 (10H, m, 2 Ph), 7.56 (1H, s, imidazole), 7.70 (1H, d, J=8.9 Hz, –Ph(NO_2)$_2$), 7.98, 8.13 (each 1H, br s, NH), 8.36 (1H, d, J=6.6 Hz, –Ph(NO_2)$_2$), 8.69 (1H, d, J=2.3 Hz, –Ph(NO_2)$_2$). *Anal.* Calcd for $C_{54}H_{65}N_9O_{13}\cdot 3/2H_2O$: C, 60.32; H, 6.37; N, 11.72. Found: C, 60.49; H, 6.11; N, 11.57.

L-Isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester Hydrochloride (33) The reaction was carried out using **32** (0.75 g, 0.72 mmol) in a manner similar to the preparation of **12** to give amorphous **33** (0.70 g, quant.), which was used for the subsequent condensation without further purification.

N^{α}-*tert*-Butoxycarbonyl-L-prolyl-L-isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (34) The reaction was carried out using **33** (0.70 g, 0.72 mmol) and *tert*-butoxycarbonyl-L-proline (0.17 g, 0.79 mmol) in a manner similar to the preparation of **11** to give yellow amorphous **34** (0.72 g, 88%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 20 : 1), mp 118—121°. $[\alpha]_D$ −11.8° (c=0.58, $CHCl_3$). IR (film): 3272, 1746, 1637, 1539 cm^{-1}. *Anal.* Calcd for $C_{59}H_{72}N_{10}O_{14}\cdot H_2O$: C, 60.92; H, 6.41; N, 12.04. Found: C, 61.12; H, 6.34; N, 11.79.

L-prolyl-L-isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester Hydrochloride (35) The reaction was carried out by using **34** (0.67g, 0.59 mmol) in a manner similar to the preparation of **12** to give amorphous **35** (0.63 g, quant.).

N^{α}-*tert*-Butoxycarbonyl-L-aspartyl (β-benzyl ester)-L-prolyl-L-isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (36) The reaction was carried out using **34** (0.63 g, 0.59 mmol) and *tert*-butoxycarbonyl-L-aspartic acid β-benzyl ester (0.21 g, 0.64 mmol) in a manner similar to the preparation of **11** to give yellow amorphous **36** (0.62 g, 79%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 30 : 1), mp 151—154 °C, $[\alpha]_D$ −15.8° (c=0.58, $CHCl_3$). IR (film): 3272, 1735, 1639, 1540 cm^{-1}. *Anal.* Calcd for $C_{70}H_{83}N_{11}O_{17}\cdot 2H_2O$: C, 60.64; H, 6.32; N, 11.11. Found: C, 60.62; H, 6.21; N, 10.94.

L-Aspartyl (β-benzyl ester)-L-prolyl-L-isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester Hydrochloride (37) The reaction was carried out using **36** (0.56 g, 0.42 mmol) in a manner similar to the preparation of **11** to give amorphous **37** (0.53 g, quant.), which was used for the subsequent condensation without further purification.

N^{α}-*tert*-Butoxycarbonyl-L-valyl-L-aspartyl(β-benzyl ester)-L-prolyl-L-isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (38) The reaction was carried out using **37** (0.53 g, 0.42 mmol) and *tert*-butoxycarbonyl-L-valine (99 mg, 0.46 mmol) in a manner similar to the preparation of **11** to give yellow amorphous **38** (0.51 g, 85%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH 20 : 1), mp 176—177 °C, $[\alpha]_D$ −24.5° (c=0.52, $CHCl_3$). IR (film): 3270, 1737, 1639, 1541 cm^{-1}. *Anal.* Calcd for $C_{75}H_{92}N_{12}O_{18}\cdot 2H_2O$: C, 60.64; H, 6.51; N, 11.31. Found: C, 60.38; H, 6.34; N, 11.10.

L-Valyl-L-aspartyl (β-benzyl ester)-L-prolyl-L-isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester Hydrochloride (39) The reaction was carried out using **38** (0.44 g, 0.30 mmol) in a manner similar to the preparation of **12** to give amorphous **39** (0.42 g, quant.), which was used for the subsequent condensation without further purification.

N^{α}-*tert*-Butoxycarbonyl-L-glutamyl(γ-benzyl ester)-L-valyl-L-aspartyl-(β-benzyl ester)-L-prolyl-L-isoleucyl-glycyl-N^{im}-2,4-dinitrophenyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (40) The reaction was carried out using **39** (0.42 g, 0.30 mmol) and *tert*-butoxycarbonyl-L-glutamic acid γ-benzyl ester (0.11 g, 0.33 mmol) in a manner similar to the preparation of **12** to give yellow amorphous **39** (0.42 g, 83%), mp 198—200 °C, $[\alpha]_D$ −24.9° (c=0.53, $CHCl_3$). IR (film): 3268, 1732, 1642, 1537 cm^{-1}. *Anal.* Calcd for $C_{87}H_{105}N_{13}O_{21}\cdot 3/2H_2O$: C, 61.62; H, 6.42; N, 10.74. Found: C, 61.61; H, 6.44; N, 10.46.

N^{α}-*tert*-Butoxycarbonyl-L-glutamyl(γ-benzyl ester)-L-valyl-L-aspartyl-(β-benzyl ester)-L-prolyl-L-isoleucyl-glycyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (41) A solution of compound **40** (0.19 g, 0.11 mmol) in 10% PhSH–DMF (3 ml) was stirred for 4 h at room temperature. After removal of the solvent, the residue was crystallized from ether to give yellow amorphous **41** (0.165 g, 96%).

L-Glutamyl(γ-benzyl ester)-L-valyl-L-aspartyl(β-benzyl ester)-L-prolyl-L-isoleucyl-glycyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Benzyl Ester (42) The reaction was carried out using **41** (0.165 g, 0.11 mmol) in a manner similar to the preparation of **12** to give amorphous **42** (0.16 g, quant.), which was used for the subsequent condensation without further purification.

L-Glutamyl-L-valyl-L-aspartyl-L-prolyl-L-isoleucyl-glycyl-L-histidyl-L-leucyl-*O*-benzyl-L-tyrosine Hydrochloride (9) A solution of **42** (0.16 g, 0.11 mmol) in EtOH (20 ml) was hydrogenated over Pd(OH)$_2$–C (0.20 g) for 6 h at 40 °C, then filtered and concentrated. The residue was purified by RP-HPLC purification (column: Asahipak ODP-50 (0.6×25 cm). Eluent: A, 0.05% TFA; B, CH_3CN, linear gradient from 15% to 35% B in 30 min. Detection: UV at 210 nm. Flow rate: 1.0 ml/min) to give amorphous **6** (0.092 g, 77%), $[\alpha]_D$ −61.6° (c=0.42, 3% AcOH). Positive FAB-MS *m/z*: 1043 $(M+H)^+$.

***N*-Tetradecanoyl-*O*-[2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-β-D-glucopyranosyl]-L-seryl-β-alanine *p*-Nitrophenyl Ester (10)** To a solution of **3** (31 mg, 0.031 mmol) and *p*-nitrophenol (31 mg, 0.22 mmol) in DMF (4 ml) cooled to 0 °C was added EDCl (31 mg, 0.16 mmol), and the mixture was stirred for 16 h at room temperature. The mixture was poured into H_2O (3 ml), the insoluble materials were collected by filtration and dried. This product was purified by silica gel column chromatography using CH_2Cl_2–MeOH (20 : 1) to give **10** (18 mg, 53%) as a yellow solid. $[\alpha]_D$ −18.4° (c=0.22, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (9H, t, J=6.9 Hz, –CH_3), 1.25 (58H, br s, –CH_2–), 1.52—1.60 (6H, m, –CH_2–), 2.07—2.57 (6H, m, –CH_2–), 2.88 (2H, t, NHCH_2C$\underline{H}_2$), 3.19—3.99 (10H, m, H-2, 3, 4, 5, 6, NHC$\underline{H}_2$$CH_2$, OC$\underline{H}_2$CHNH), 4.55 (1H, d, J=7.6 Hz, H-1), 4.57—4.70 (1H, m, OCH_2C$\underline{H}$NH), 5.17—5.23 (1H, m, NHCOCH_2C$\underline{H}$(OCO)), 6.46 (1H, br d, NH), 6.61 (1H, d, J=6.3 Hz, NH), 7.33 (2H, d, J=9.2 Hz, Ph), 8.28 (2H, d, J=8.9 Hz, Ph). Positive FAB-MS *m/z*: 1106 $(M+H)^+$, 1128 $(M+Na)^+$.

***N*-Tetradecanoyl-*O*-[2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-β-D-glucopyranosyl]-L-seryl-β-alanyl-L-isoleucyl-L-prolyl-glycyl-L-leucyl-L-prolyl-L-leucyl-L-seryl-L-leucine (6)** DIEA (10 mg, 0.08 mmol) was added to a solution of **8** (8 mg, 0.01 mmol) and **10** (12 mg, 0.08 mmol) in DMF (3 ml), and cooled to 0 °C. The mixture was stirred at room temperature for 36 h. After evaporation of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH–H_2O 12 : 8 : 1) and Sephadex LH-20 (CH_2Cl_2–MeOH–H_2O 12 : 8 : 1) to give amorphous **4** (12 mg, 69%), after lyophilization from a H_2O suspension, $[\alpha]_D$ −24.3° (c=0.15, MeOH). Positive FAB-MS *m/z*: 1797 $(M+Na)^+$.

***N*-Tetradecanoyl-*O*-[2-deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanoylamino]-β-D-glucopyranosyl]-L-seryl-β-alanyl-L-glutamyl-L-valyl-L-aspartyl-L-prolyl-L-isoleucyl-glycyl-L-histidyl-L-leucyl-L-tyrosine (7)** The reaction was carried out using **9** (6 mg, 0.006 mmol), **10** (8 mg, 0.007 mmol) and DIEA (10 mg, 0.08 mmol) in a manner similar to the preparation of **6** to give amorphous **7** (4 mg, 35%), after purification by silica gel column chromatography (CH_2Cl_2–MeOH–H_2O 12 : 8 : 1) and Sephadex LH-20 (CH_2Cl_2–MeOH–H_2O 12 : 8 : 1), $[\alpha]_D$ −53° (c=0.08, MeOH). Positive FAB-MS *m/z*: 2030 $(M+Na)^+$.

Acknowledgement We thank the MERCIAN CORPORATION (Fujisawa, Japan) for the biological data.

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