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Preparation of antibodies against a novel immunosuppressant, FTY720, and development of an enzyme immunoassay for FTY720

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Abstract—FTY720 (**1**) is a novel immunosuppressant (immunomodulator), derived from ISP-I (**2**: myriocin and thermozymocidin). To clarify the pharmacokinetic properties of **1**, antibodies against **1** were prepared and a competitive enzyme immunoassay (EIA) was developed. Two kinds of haptens, **3** and **4**, for **1** were synthesized and coupled to ovalbumin (OVA). Rabbits were immunized with **3**-OVA or **4**-OVA, and corresponding antibodies were obtained. Both antibodies recognized the 2-amino-2-(2-phenylethyl)propane-1,3-diol moiety in **1**. Using the anti-**3**-OVA antibody, a competitive EIA for **1** was developed and evaluated. The range of quantification by the EIA was 0.06–10 ng/mL. The application of the EIA has enabled us to measure the FTY720 concentration in serum after oral administration of **1** (1 mg/kg) to rats.

1. Introduction

Immunosuppressants have important clinical roles in organ transplantation and the treatment of autoimmune diseases. At present, the established immunosuppressants, cyclosporin A (CsA),[1] and tacrolimus (FK506),[2–4] are widely used in organ transplantation. However, both drugs, which have similar mechanisms of action,[5] have severe side effects, notably renal toxicity.[6,7] Therefore, it would be desirable to develop a novel immunosuppressant with less toxicity and an entirely different mechanism of action.

In the late 1980s, Fujita et al. of our group isolated a unique immunosuppressant, ISP-I[8] (**2**: myriocin[9,10] and thermozymocidin[11,12]), from the fermentation broth of *Isaria sinclairii* (ATCC24400), which is the imperfect stage of *Cordyceps sinclairii* and is parasitic on cicada larvae. It has been reported that ISP-I inhibits serine palmitoyltransferase of an interleukin-2 (IL-2)-dependent mouse cytotoxic T cell line, CTLL-2, at picomolar concentrations.[13] His group simplified the structure of ISP-I in order to reduce its toxicity, to improve its physicochemical properties, and to identify the essential structure for immunosuppressive activity[14] and obtained a candidate compound, FTY720 (**1**: 2-amino-2-(2-(4-octylphenyl)ethyl)propane-1,3-diol hydrochloride),[15,16] which does not inhibit serine palmitoyltransferase. Compound **1** has an entirely different action mechanism from established drugs such as CsA and FK506. It does not affect IL-2 production from mitogen-stimulated rat spleen cells[17] and suppresses the immune response by sequestering lymphocytes from peripheral blood and spleen to the secondary lymphoid tissues (lymph nodes, Peyer's patches, and so on).[18–20] Recently, it was reported by Mandala et al.[21] and Brinkmann et al.[22] that the phosphorylated form of **1** acts as an agonist of sphingosine-1-phosphate (S1P). Like sphingosine, **1** is converted by sphingosine kinase to FTY720 monophosphate, which is the active form of the drug. FTY720 monophosphate signals through four of the five known S1P receptors, which belong to the endothelial-differentiation-gene (EDG) family of G-protein-coupled receptors.

Keywords: FTY720; Immunosuppressant; Hapten; Enzyme immunoassay.
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Very recently, Matloubian et al.[23] established, by using **1**, that S1P receptor 1 is essential for lymphocyte recirculation and regulates egress from both thymus and peripheral lymphoid organs. It has been shown that **1** has a more potent immunosuppressive activity than CsA and FK506 in rat skin and cardiac allograft models.[17,18,24] Furthermore, combination therapy with the other drugs, CsA and FK506, showed a remarkable synergistic effect in prolonging skin, cardiac, and renal allograft survival.[17,18,25–27] Compound **1** is also expected to be useful for the treatment of other diseases, including multiple sclerosis,[22] rheumatoid arthritis,[28] spontaneous dermatitis,[29] and myasthenia gravis,[30] based on findings in animal models.

Recently, a clinical trial of **1** in renal transplantation patients has been performed.[31] Quantitative analysis of **1** in the blood was accomplished by HPLC/MS/MS.[31] The instrumentation is expensive and requires skill to operate. On the other hand, immunoassay is a common laboratory method, which is suitable to screen large numbers of samples. Therefore, the development of immunoassay technology for **1** would be of great value.

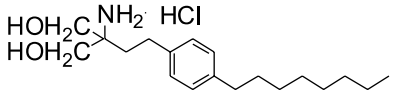
We have succeeded in preparing the first specific antibodies against **1**. A part of this work was reported in our preliminary communication.[32] Here, we present full details of the synthesis of two FTY720 analogues, 2-amino-2-(2-(4-(4-mercaptobutyl)phenyl)ethyl)propane-1,3-diol hydrochloride (**3**) and 2-amino-2-(2-(4-(8-mercaptooctyl)phenyl)ethyl)propane-1,3-diol hydrochloride (**4**), as haptens, the preparation of hapten-carrier proteins and their use to raise antibodies, the specificity of the antibodies, and the development of an EIA for **1**.

2. Results

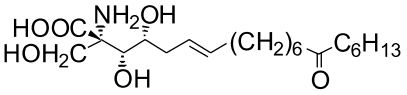
2.1. Synthesis of haptens and preparation of hapten-carrier proteins

To prepare antibodies in animals against a low-molecular compound such as **1**, conjugation to a carrier protein such as ovalbumin (OVA) is generally required. We designed compounds **3** and **4** as haptens containing the characteristic structure, 2-aminopropane-1,3-diol and a side chain including a phenyl group, of **1**. The thiol group of the haptens was expected to undergo Michael-type addition to maleimide groups of a carrier protein bearing suitable groups, such as ε-maleimidocaproic acid *N*-hydroxysuccinimide ester (EMCS).[33]

The haptens **3** and **4** were synthesized as follows. Ethyl 4-phenylbutyrate **5**[34] was bromoacetylated to give **6**, which was reduced with triethylsilane in trifluoroacetic acid to afford **7**.[35] The bromide **7** was condensed with diethyl acetamidomalonate to give the triethylester **8**. Reduction of **8** with sodium borohydride, followed by treatment with 2,2-dimethoxypropane, afforded a protected monoester **9** as a major product and an alcohol **10** as a minor product. The ester **9** was reduced with lithium aluminum hydride to give the alcohol **10**, which was oxidized with oxalyl chloride to afford an aldehyde **11**. The Wittig condensation product obtained from the aldehyde **11** with (3-(ethoxycarbonyl)-2-propenyl)triphenylphosphonium bromide was hydrogenated to afford an ester **12**. The ester **12** was reduced with lithium aluminum hydride to give an alcohol **13**. The alcohols, **10** and **13**, were treated with tosyl chloride to give tosylates, **14** and **15**, respectively. Compounds **14** and **15** were treated with potassium thioacetate to give thioacetates **16** and **17**, and then acid hydrolysis yielded the desired haptens, **3** and **4**, respectively (Chart 1).

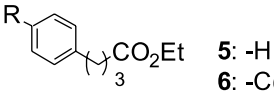


FTY720 (**1**)

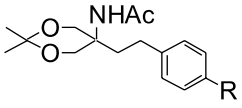


ISP-I (**2**: myriocin, thermozymocidin)

3: n = 4
4: n = 8



R
5: -H
6: -C(=O)CH_2Br
7: -$(CH_2)_2$Br
8: -$(CH_2)_2C(CO_2Et)_2(NHAc)$



R
9: $(CH_2)_3CO_2Et$
10: $(CH_2)_4OH$
11: $(CH_2)_3CHO$
12: $(CH_2)_7CO_2Et$
13: $(CH_2)_8OH$
14: $(CH_2)_4OTs$
15: $(CH_2)_8OTs$
16: $(CH_2)_4SAc$
17: $(CH_2)_8SAc$

Chart 1. Structures of FTY720 and related compounds.

Antigens were prepared as shown in Scheme 1. OVA was selected as a carrier protein for the immunogen. Maleimide groups were introduced into OVA by using EMCS to give maleimide-OVA. **3**-OVA conjugate and **4**-OVA conjugate were prepared by Michael-type addition to the maleimide-OVA of thiols **3** and **4**, respectively. The average numbers of **3** and **4** molecules introduced per OVA molecule were 6.8 and 2.4, respectively, as calculated from the decrease in the number of maleimide groups. The residual maleimide moieties were protected with 2-mercaptoethylamine hydrochloride and purified by gel filtration on a column to give the **3**- and **4**-OVA conjugates, respectively.

To develop an enzyme immunoassay (EIA) for **1** and an enzyme-linked immunosorbent assay (ELISA) for anti-**3** and anti-**4** IgGs, we selected bovine serum albumin (BSA) as a carrier protein for the antigen. Horseradish peroxidase (HRP) was selected as a label. BSA conjugates and HRP conjugates of **3** or **4** were prepared in a similar manner to that used for the preparation of **3**-OVA.

2.2. Preparation and specificity of antibodies

Three rabbits were intracutaneously immunized three times with **3**- or **4**-OVA conjugate in Freund's complete adjuvant at three-week intervals. Antibody IgG against **3** or **4** could be detected in sera from the corresponding rabbits by ELISA (data not shown), in which a **3**-BSA- or **4**-BSA-coated solid phase was incubated with test sera and then, after washing, with HRP-labeled goat (anti-rabbit IgG) Fab′.

To examine the specificity of anti-**3**-OVA IgG and anti-**4**-OVA IgG, their epitopes were analyzed by a competitive EIA, using FTY720 (**1**), the derivatives (**18**–**27**),[14,16] and the metabolites (**28**–**31**)[36] in Chart 2 with HRP-labeled **3** or HRP-labeled **4** (Fig. 1, method A). The results clearly indicate that the antibodies bind not only with **3** and **4**, but also with **1**, as shown in Table 1. Compounds **21**–**27**, which have a phenyl ring at a variety of positions within the side chain of **1**, but with a constant chain length ($m + n = 10$), were slightly cross-reactive with anti-**3**-OVA IgG, but did not cross-react at all with anti-**4**-OVA IgG. The non-aromatic **20** was not cross-reactive at all with either of the antibodies. The 2-aminoalcohol **18** and *N*-acetate **19** were slightly cross-reactive with anti-**3**-OVA IgG, but were not

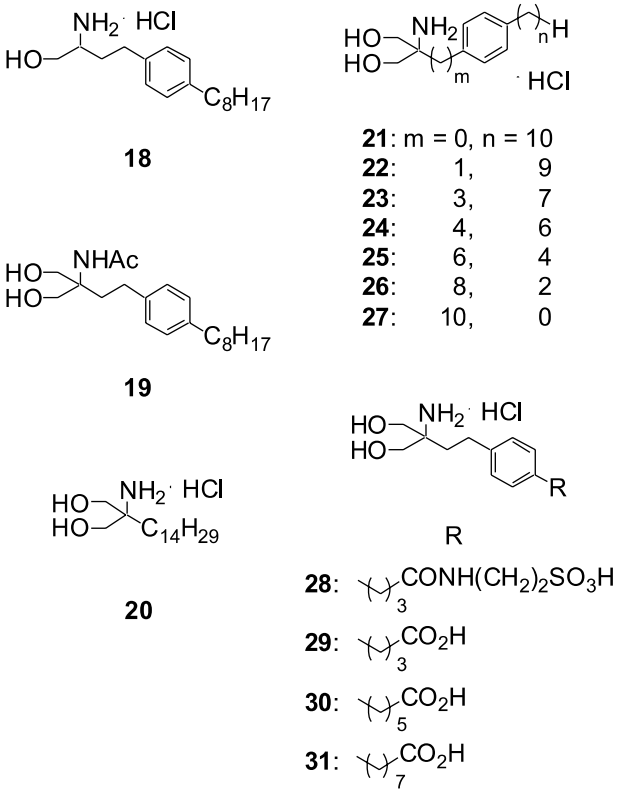
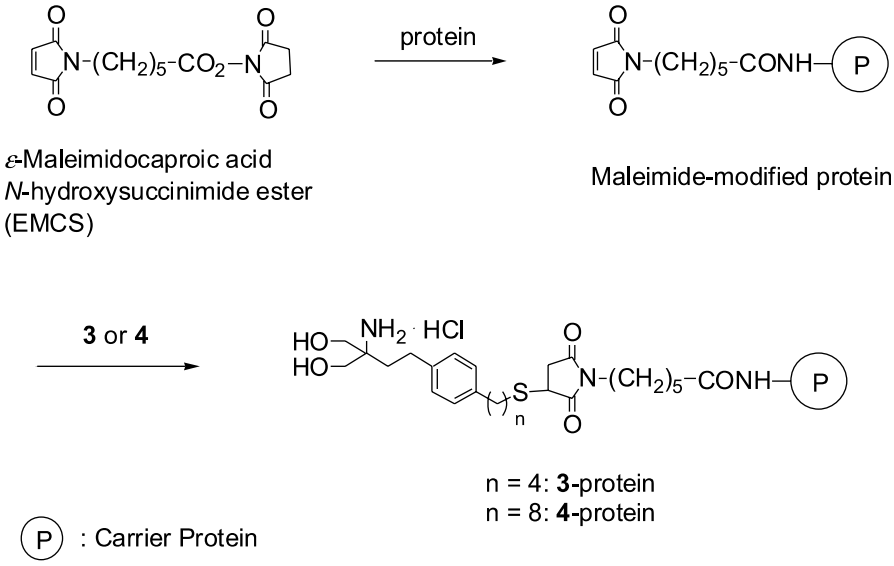


Chart 2. Structures of derivatives (**18**–**27**) and metabolites (**28**–**31**) of **1**.



Scheme 1. Preparation of hapten-carrier protein.

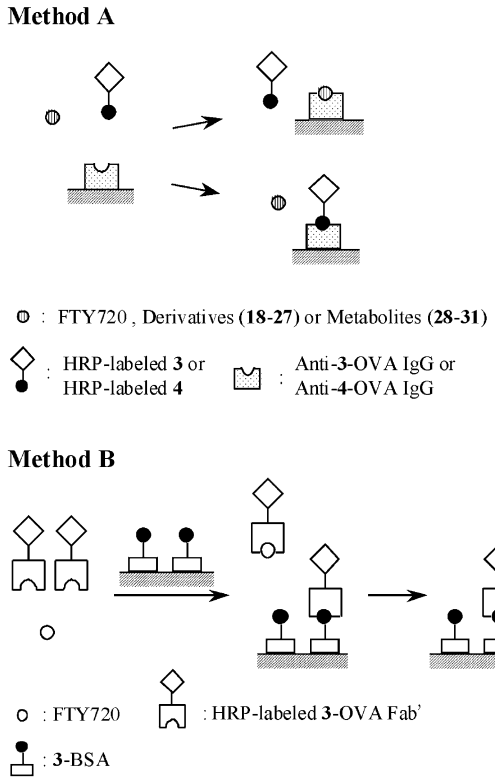


Figure 1. Procedure of epitope analysis of anti-**3**-OVA IgG and anti-**4**-OVA IgG by method A, and a competitive EIA for **1** by method B. Method A: FTY720 (or related compounds **18**–**31**) and HRP-labeled **3** (or HRP-labeled **4**) are mixed and trapped on an anti-**3**-OVA IgG-coated solid phase (or anti-**4**-OVA IgG-coated solid phase). The solid phase is washed, and the bound HRP activity is detected. Method B: compound **1** is incubated with HRP-labeled anti-**3**-OVA Fab′, and then free HRP-labeled anti-**3**-OVA Fab′ is trapped on a **3**-BSA-coated solid phase. The solid phase is washed, and the bound HRP activity is detected.

Table 1. Epitope analysis and cross-reactivity of derivatives (**18**–**27**) and metabolites (**28**–**31**) of **1**

Compound	Reactivity (%)[a]	
	Anti-**3**-OVA IgG	Anti-**4**-OVA IgG
1	100	100
3	345	300
4	178	231
18	0.6	0
19	14	0
20	0	0
21	5.9	0
22	0.4	0
23	3.7	0
24	8.9	0
25	13	0
26	15	0
27	19	0
28	178	173
29	141	79
30	326	300
31	668	363

Epitope and cross-reactivity were examined by using a competitive EIA (method A). The concentration (C) of the compounds was calculated by curve fitting using **1** as a standard. The reactivity (%) = $C \times 100$/actual concentration.

[a] Binding reactivity of the antibodies for **1** is designated as 100.

cross-reactive with anti-**4**-OVA IgG. These results indicate that both antibodies recognize the 2-amino-2-(2-phenylethyl)propane-1,3-diol moiety.

The metabolites **28**–**31**, having 2-amino-2-(2-phenylethyl)propane-1,3-diol and carboxylic acid groups, were reactive with anti-**3**-OVA IgG and anti-**4**-OVA IgG, because the antibodies recognized the 2-amino-2-(2-phenylethyl)propane-1,3-diol moiety.

2.3. Development of an EIA for FTY720

An EIA for FTY720 (**1**) based on a competitive format (Fig. 1, method B) was conducted to examine the applicability of anti-**3**-OVA IgG for the assay of **1**. A calibration curve for **1** obtained by this method is shown in Figure 2 (open circles). We developed a specific EIA for **1** using this method, since the sensitivity by this method was higher than that by the method A.

To develop a specific EIA for **1**, it was necessary to remove the acidic metabolites **28**–**31** from samples which contain the metabolites, that is, serum and plasma. For this purpose, alkaline-chloroform extraction was performed before measuring **1** by this method. The acidic metabolites **28**–**31**, which cross-reacted with anti-**3**-OVA antibody, were efficiently eliminated (Fig. 3). The recovery rate of **1** in the presence of serum was extremely low, probably due to the matrix effect of the serum proteins. Alkaline-chloroform extraction also improved the recovery rate of **1** in serum. The calibration curve for **1** in the presence of serum was almost identical with that in the absence of serum (Fig. 2). The range of quantification was 0.06–10 ng/mL in the presence of serum.

To examine the applicability of the specific EIA, the concentration of **1** in serum after oral administration

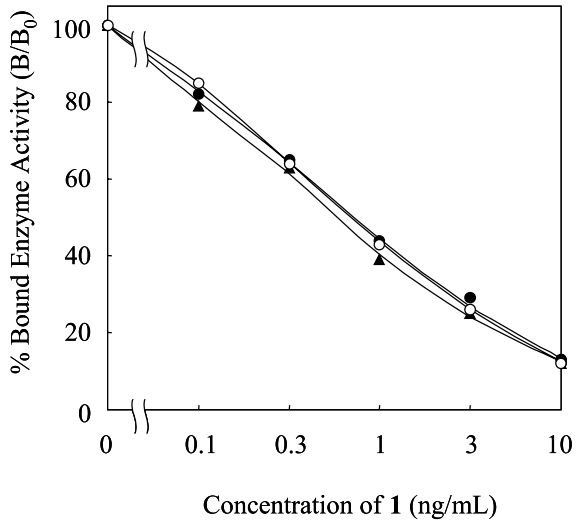


Figure 2. Calibration curves for **1**. Compound **1** was diluted with buffer and subjected to competitive EIA (open circle). Compound **1** was diluted with buffer (closed circle) and with rat control serum (closed triangle), then subjected to alkaline-chloroform extraction, followed by competitive EIA.

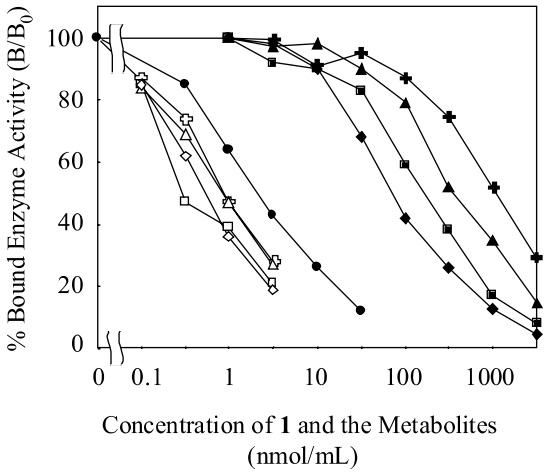


Figure 3. Dose–response curves for **1** and the metabolites (**28**–**31**) before and after alkaline-chloroform extraction. Open symbols, before extraction; closed symbols, after extraction. Compound **1** (circle), **28** (cross), **29** (triangle), **30** (square), and **31** (diamond) were subjected to EIA for **1** before and after alkaline-chloroform extraction.

of **1** to rats (1 mg/kg) was measured. A time–course curve of **1** is shown in Figure 4. The concentration of **1** reached a maximum at 12 h (C_{max}: 33 ng/mL) and decreased with a half life of approximately 60 h ($t_{1/2}$). AUC_{0-96h} was 3100 ng h/mL.

3. Discussion

Very recently, it was clarified that the active form of FTY720 (**1**) is the monophosphate.[21,22] Therefore, it will be important to examine the pharmacokinetics of intracellular FTY720 monophosphate. The pharmacokinetics of intracellular triphosphorylated compounds,[37] such as the active forms of the antiviral agents lamivudine and zidovudine, has been investigated with HPLC/MS/MS, and this approach is also likely to be applicable to FTY720 monophosphate.

Compound **1** is expected to be widely applied in clinical treatment not only for organ transplantation patients, but also for patients with immunological disorders, such as multiple sclerosis,[22] rheumatoid arthritis,[28] dermatitis,[29] and myasthenia gravis.[30] The present immunoassay will be useful to assess the safety and efficacy of **1** in the clinical setting.

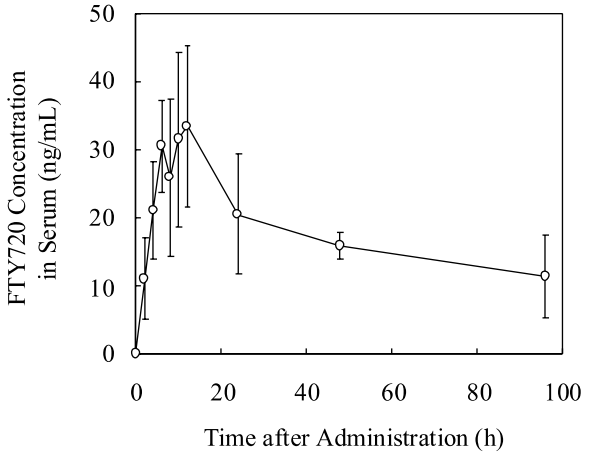


Figure 4. Concentration of **1** in serum after oral administration of **1** (1 mg/kg) to rats (n = 4–6). Each value indicates the mean, and vertical bars indicate ±SD.

4. Conclusion

Initially, the hapten **3** was synthesized and coupled with OVA, and antibody against the **3**-OVA was prepared in rabbits. The obtained anti-**3**-OVA antibody binds with FTY720 (**1**), and recognizes the 2-amino-2-(2-phenylethyl)propane-1,3-diol moiety, but is highly cross-reactive with the metabolites (**28**–**31**) of **1**. To obtain an antibody specific for the alkyl side chain moiety of **1**, the hapten **4**, which has the same length of alkyl side chain as **1**, was synthesized and coupled with OVA, and antibody against the **4**-OVA was prepared. The specificity of the anti-**4**-OVA antibody for the 2-amino-2-(2-phenylethyl)propane-1,3-diol moiety is slightly higher than that of the anti-**3**-OVA antibody, but the specificity for the alkyl side chain moiety is similar to that of the anti-**3**-OVA. The solubility of **3** in buffer is better than that of **4**, and the conjugate of **3** could be easily prepared. Therefore, we selected anti-**3**-OVA antibody to develop a competitive EIA for **1**. Consequently, the acidic metabolites of **1** had to be removed by alkaline-chloroform extraction prior to quantification of **1**. The range of quantification of the EIA was 0.06–10 ng/mL. The developed EIA should be suitable to measure blood levels of **1** in large numbers of samples from patients in the clinical setting.

5. Experimental

5.1. Chemistry

5.1.1. General information. Melting points were determined on a Yanagimoto micro melting point apparatus (MP-S3) without correction. IR spectra were taken on a Nihon Bunkou A-102 infrared spectrophotometer. UV spectra were taken on a Hitachi U-2000 spectrometer. Mass spectra (EI-MS and FAB-MS) were taken on a JEOL JMS-DX300 spectrometer. ^{1}H NMR (400 MHz, $CDCl_3$) spectra were taken on a JEOL JNM-GX400 spectrometer with TMS as an internal standard; chemical shifts are reported in parts per million. TLC was performed on aluminum seated silica gel 60 F_{254} (Merck). For column chromatography, silica gel (70–230 mesh, Merck) was used. Organic solvent extracts were dried over anhydrous magnesium sulfate, and evaporation of solvents was performed under reduced pressure.

5.1.2. Ethyl 4-(4-(bromoacetyl)phenyl)butyrate (6). To a stirred solution of **5** (20.0 g, 1.0×10^{-1} mol) and bromoacetyl chloride (10.4 mL, 1.3×10^{-1} mol) in dried $CHCl_3$ (250 mL) was added portionwise $AlCl_3$ (30.6 g, 2.3×10^{-1} mol) over a period of 15 min at −10 °C, and the mixture was stirred at room temperature for 2 h. The reaction mixture was poured into ice water (200 mL) and extracted with $CHCl_3$ (100 mL). The extract was washed with saturated aqueous $NaHCO_3$

(200 mL) and then brine (50 mL), dried, and evaporated. The residue was chromatographed on silica gel with *n*-hexane–AcOEt (9:1) to give **6** (28.8 g, 88%) as a colorless oil: bp 188–192 °C (0.1 mmHg); IR ν_{max} (CCl_4) cm^{-1}: 1740 (*CO*OEt, $BrCH_2CO$); ^1H NMR δ: 1.24 (3H, t, $J = 7.0$ Hz, $CO_2CH_2CH_3$), 1.98 (2H, quintet, $J = 7.5$ Hz, $CH_2CH_2CH_2CO_2Et$), 2.33 (2H, t, $J = 7.5$ Hz, CH_2CO_2Et), 2.73 (2H, t, $J = 7.5$ Hz, $PhCH_2$), 4.15 (2H, q, $J = 7.0$ Hz, $CO_2CH_2CH_3$), 4.43 (2H, s, $BrCH_2CO$), 7.30, 7.91 (each 2H, d, $J = 8.0$ Hz, Ar*H*); EI-MS *m*/*z*: 313 ($[M]^+$).

5.1.3. Ethyl 4-(4-(2-bromoethyl)phenyl)butyrate (7). To a stirred solution of **6** (7.9 g, 2.5×10^{-2} mol) in trifluoroacetic acid (25 mL) was added triethylsilane (8.9 mL, 5.6×10^{-2} mol) under ice cooling and the mixture was stirred at room temperature for 4 h. The reaction mixture was poured into a mixture of ice water (150 mL) and AcOEt (200 mL), and $NaHCO_3$ (27 g) was added. The extracted organic solution was washed with saturated aqueous $NaHCO_3$ (20 mL) and brine (2× 15 mL), then dried, and evaporated. The residue was chromatographed on silica gel with *n*-hexane–AcOEt (40:1) to give **7** (5.1 g, 68%) as a colorless oil: IR ν_{max} (CCl_4) cm^{-1}: 1740 (*CO*OEt); ^1H NMR δ: 1.25 (3H, t, $J = 7.0$ Hz, $CO_2CH_2CH_3$), 1.94 (2H, quintet, $J = 7.5$ Hz, $CH_2CH_2CH_2CO_2Et$), 2.31 (2H, t, $J = 7.5$ Hz, CH_2CO_2Et), 2.63 (2H, t, $J = 7.5$ Hz, $PhCH_2(CH_2)_2CO_2Et$), 3.13 (2H, t, $J = 7.5$ Hz, $BrCH_2CH_2$), 3.54 (2H, t, $J = 7.5$ Hz, $BrCH_2CH_2$), 4.12 (2H, q, $J = 7.0$ Hz, $CO_2CH_2CH_3$), 7.13 (4H, br s, Ar*H*); EI-MS *m*/*z*: 298 ($[M]^+$).

5.1.4. Diethyl 2-(acetylamino)-2-(2-(4-(3-(ethoxycarbonyl)propyl)phenyl)ethyl)propane-1,3-dioate (8). To a solution of diethyl (acetylamino)malonate (4.8 g, 2.2×10^{-2} mol) in dried DMF (10 mL) was added 60% sodium hydride (0.74 g, 1.9×10^{-2} mol) under an argon atmosphere, and the mixture was stirred at 90 °C. To the mixture were added dried THF (10 mL) and a solution of **7** (2.2 g, 7.4×10^{-3} mol) in dried THF (5 mL). The mixture was refluxed for 2 h and concentrated. To the residue was added ice water (20 mL), and the whole mixture was extracted with Et_2O (80 mL). The extract was washed with brine (2× 20 mL), dried, and evaporated. The residue was chromatographed on silica gel with *n*-hexane–AcOEt (2:1) to give **8** (2.6 g, 81%) as a colorless oil: bp 200–230 °C (oil bath temp, 0.1 mmHg); IR ν_{max} (CCl_4) cm^{-1}: 1740 (*CO*OEt), 1690 (*CO*NH); ^1H NMR δ: 1.12–1.27 (9H, $CO_2CH_2CH_3$ 3×), 1.92 (2H, quintet, $J = 7.5$ Hz, $CH_2CH_2CH_2CO_2Et$), 1.97 (3H, s, $NHCOCH_3$), 2.30 (2H, t, $J = 7.5$ Hz, $Ph(CH_2)_2CH_2CO_2Et$), 2.43–2.47 (2H, m, $PhCH_2CH_2C$), 2.60 (2H, t, $J = 7.5$ Hz, $PhCH_2(CH_2)_2CO_2Et$), 2.65–2.69 (2H, m, $PhCH_2CH_2C$), 4.12 (2H, q, $J = 7.0$ Hz, $Ph(CH_2)_3CO_2CH_2CH_3$), 4.18–4.22 (4H, m, $C(CO_2CH_2CH_3)_2$), 6.79 (1H, br s, N*H*), 7.07 (4H, br s, Ar*H*); EI-MS *m*/*z*: 435 ($[M]^+$); Anal. Calcd for $C_{23}H_{33}NO_7$: C, 63.43; H, 7.64; N, 3.22. Found: C, 63.43; H, 7.41; N, 3.07.

5.1.5. Ethyl 4-(4-(2-(5-(acetylamino)-2,2-dimethyl-1,3-dioxan-5-yl)ethyl)phenyl)butanoate (9) and *N*-(5-(2-(4-(4-hydroxybutyl)phenyl)ethyl)-2,2-dimethyl-1,3-dioxan-5-yl)ethanamide (10). To a stirred solution of **8** (1.9 g, 4.4×10^{-3} mol) in EtOH (7 mL) was added sodium borohydride (0.80 g, 2.1×10^{-2} mol) under ice cooling and the mixture was stirred at room temperature for 7 h. The reaction mixture was poured into a mixture of ice water (10 mL) and AcOEt (50 mL), and the aqueous layer was acidified to pH 3 with 1 N HCl. The extracted organic solution was washed with brine (10 mL), dried, and evaporated. The residue (1.6 g) was dissolved in 2,2-dimethoxypropane (9.0 mL, 7.3×10^{-2} mol) and to this stirred solution was added *p*-toluenesulfonic acid monohydrate (82 mg, 4.3×10^{-4} mol). The mixture was stirred at room temperature for 18 h and concentrated. To the residue was added ice water (10 mL), followed by extraction with AcOEt (30 mL). The extract was washed with brine (5 mL), dried, and evaporated. The residue was chromatographed on silica gel with *n*-hexane–AcOEt (2:1) to give **9** (0.84 g, 49%) and with *n*-hexane–AcOEt (1:2) to give **10** (0.23 g, 15%).

Compound **9**: colorless needles (recrystallized from *n*-hexane–Et_2O); mp 52–53 °C; IR ν_{max} (CCl_4) cm^{-1}: 1735 (*CO*OEt), 1680 (*CO*NH); ^1H NMR δ: 1.25 (3H, t, $J = 7.0$ Hz, $CO_2CH_2CH_3$), 1.42 (6H, s, $C(CH_3)_2$), 1.92 (2H, quintet, $J = 7.5$ Hz, $PhCH_2CH_2CH_2CO_2Et$), 2.01 (3H, s, $NHCOCH_3$), 2.03–2.07 (2H, m, $PhCH_2CH_2C$), 2.30 (2H, t, $J = 7.5$ Hz, $Ph(CH_2)_2CH_2CO_2Et$), 2.49–2.53 (2H, m, $PhCH_2CH_2C$), 2.60 (2H, t, $J = 7.5$ Hz, $PhCH_2(CH_2)_2CO_2Et$), 3.67, 3.97 (each 2H, d, $J = 12.0$ Hz, $(CH_2O)_2C(CH_3)_2$), 4.12 (2H, q, $J = 7.0$ Hz, $CO_2CH_2CH_3$), 5.71 (1H, br s, N*H*), 7.08 (4H, br s, Ar*H*); EI-MS *m*/*z*: 391 ($[M]^+$); Anal. Calcd for $C_{22}H_{33}NO_5$: C, 67.49; H, 8.50; N, 3.58. Found: C, 67.45; H, 8.40; N, 3.63.

Compound **10**: colorless plates (recrystallized from benzene); mp 107–108 °C; IR ν_{max} (KBr) cm^{-1}: 3440 (*OH*), 1675 (*CO*NH); ^1H NMR δ: 1.42 (6H, s, $C(CH_3)_2$), 1.57–1.63 (2H, m, $PhCH_2CH_2(CH_2)_2OH$), 1.63–1.69 (2H, m, $Ph(CH_2)_2CH_2CH_2OH$), 2.01 (3H, s, $NHCOCH_3$), 2.02–2.07 (2H, m, $PhCH_2CH_2C$), 2.49–2.53 (2H, m, $PhCH_2CH_2C$), 2.60 (2H, t, $J = 7.5$ Hz, $PhCH_2(CH_2)_3OH$), 3.65 (2H, t, $J = 7.4$ Hz, $Ph(CH_2)_3CH_2OH$), 3.66, 3.97 (each 2H, d, $J = 12.0$ Hz, $(CH_2O)_2C(CH_3)_2$), 5.74 (1H, br s, N*H*), 7.08 (4H, br s, Ar*H*); EI-MS *m*/*z*: 349 ($[M]^+$); Anal. Calcd for $C_{20}H_{31}NO_4$: C, 68.74; H, 8.94; N, 4.01. Found: C, 68.66; H, 8.66; N, 3.83.

5.1.6. *N*-(5-(2-(4-(4-Hydroxybutyl)phenyl)ethyl)-2,2-dimethyl-1,3-dioxan-5-yl)ethanamide (10). To a stirred solution of **9** (1.3 g, 3.3×10^{-3} mol) in dried Et_2O (80 mL) was added lithium aluminum hydride (0.38 g, 1.0×10^{-2} mol) under ice cooling, and the reaction mixture was stirred at room temperature for 2 h. Saturated aqueous Rochelle salt ($KNaC_4H_4O_6{\cdot}4H_2O$) was added to the mixture under ice cooling, and the organic solution was decanted, dried, and evaporated. The residue was recrystallized from benzene to give **10** (0.78 g, 67%).

5.1.7. *N*-(2,2-Dimethyl-5-(2-(4-(4-oxobutyl)phenyl)ethyl)-1,3-dioxan-5-yl)ethanamide (11). To a stirred solution of oxalyl chloride (88.3 μL, 1.0×10^{-3} mol) in dried CH_2Cl_2

(7 mL) was added dried DMSO (158 μL, 2.2×10^{-3} mol) in CH_2Cl_2 (7 mL) at −70 °C under an argon atmosphere, and the solution was stirred for 10 min. To the solution was added a solution of **10** (300 mg, 8.6×10^{-4} mol) in CH_2Cl_2 (7 mL). The mixture was stirred for 10 min and then dried Et_3N (0.60 mL, 4.3×10^{-3} mol) was added. The reaction mixture was poured into ice water (10 mL). The organic layer was washed with brine (2× 5 mL), dried, and evaporated. The residue was chromatographed on silica gel with *n*-hexane–AcOEt (3:2) to give **11** (0.28 g, 93%): colorless needles (recrystallized from Et_2O); mp 102–104 °C; IR ν_{max} (KBr) cm^{-1}: 1720 (*CHO*), 1680 (*CO*NH); ^1H NMR δ: 1.42 (6H, s, $C(CH_3)_2$), 1.94 (2H, quintet, J = 7.4 Hz, $PhCH_2CH_2CH_2CHO$), 2.02 (3H, s, $NHCOCH_3$), 2.02 (2H, m, $PhCH_2CH_2C$), 2.44 (2H, dt, J = 7.4 Hz and 2.0 Hz, $Ph(CH_2)_2CH_2CHO$), 2.49–2.53 (2H, m, $PhCH_2CH_2C$), 2.63 (2H, t, J = 7.4 Hz, $PhCH_2(CH_2)_2CHO$), 3.69, 3.97 (each 2H, d, J = 12.0 Hz, $(CH_2O)_2C(CH_3)_2$), 5.69 (1H, br s, N*H*), 7.07, 7.10 (each 2H, d, J = 8.3 Hz, Ar*H*), 9.74 (1H, t, J = 2.0 Hz, C*H*O); EI-MS *m*/*z*: 347 ($[M]^+$); Anal. Calcd for $C_{20}H_{29}NO_4$: C, 69.14; H, 8.41; N, 4.03. Found: C, 68.88; H, 8.22; N, 3.81.

5.1.8. Ethyl 8-(4-(2-(5-(acetylamino)-2,2-dimethyl-1,3-dioxan-5-yl)ethyl)phenyl)octanoate (12). To a stirred solution of triphenylphosphine (5.0 g, 1.9×10^{-2} mol) in dried benzene (5 mL) was added ethyl 4-bromocrotonate (4.83 mL, 2.6×10^{-2} mol) under an argon atmosphere, and the mixture was stirred at room temperature for 4 h. The precipitate was washed with dried benzene (2× 10 mL) followed with $CHCl_3$ (2× 10 mL) and recrystallized from CH_3CN to give (3-(ethoxycarbonyl)-2-propenyl)triphenylphosphonium bromide (7.2 g, 83%) as a white powder. To a suspension of 60% sodium hydride (24 mg, 5.8×10^{-4} mol) in dried DMF (6 mL) was added (3-ethoxycarbonylallyl-propyl)triphenylphosphonium bromide (0.28 g, 6.3×10^{-4} mol) at −25 °C under an argon atmosphere and the mixture was stirred for 10 min. To the mixture was added a solution of **11** (0.20 g, 5.8×10^{-4} mol) in dried DMF (5 mL) and the mixture was stirred at the same temperature for 0.5 h. The reaction mixture was poured into ice water (20 mL) and extracted with Et_2O (30 mL). The extract was washed with brine (3 mL), dried, and evaporated. The residue was chromatographed on silica gel with Et_2O to give ethyl 8-(4-(2-(5-(acetylamino)-2,2-dimethyl-1,3-dioxan-5-yl)ethyl)-phenyl)-2,4-octadienoate (0.13 g, 52%) as a yellow oil. A solution of ethyl 8-(4-(2-(5-(acetylamino)-2,2-dimethyl-1,3-dioxan-5-yl)ethyl)phenyl)-2,4-octadienoate (0.51 g, 1.1×10^{-3} mol) in dried AcOEt (4 mL) was stirred under a hydrogen atmosphere in the presence of 10% Pd–C (0.10 g) for 2 h. The catalyst was removed by filtration and the filtrate was evaporated to give **12** (0.49 g, 99%) as a pale yellow oil: IR ν_{max} (CCl_4) cm^{-1}: 1720 (*CO*OEt), 1675 (*CO*NH); ^1H NMR δ: 1.25 (3H, t, J = 7.1 Hz, $CO_2CH_2CH_3$), 1.24–1.31 (8H, m, $Ph(CH_2)_2(CH_2)_4CH_2$–), 1.42 (6H, s, $C(CH_3)_2$), 1.94 (2H, quintet, J = 7.4 Hz, $PhCH_2CH_2(CH_2)_5$–), 2.01 (3H, s, $NHCOCH_3$), 2.03–2.07 (2H, m, $PhCH_2CH_2C$), 2.20 (2H, t, J = 7.4 Hz, CH_2CO_2Et), 2.48–2.53 (2H, m, $PhCH_2CH_2C$), 2.60 (2H, t, J = 7.4 Hz, $PhCH_2(CH_2)_6$–), 3.66, 3.96 (each 2H, d, J = 12.0 Hz, $(CH_2O)_2C(CH_3)_2$), 4.12 (2H, q, J = 7.1 Hz, $CO_2CH_2CH_3$), 5.75 (1H, br s, N*H*), 7.07 (4H, br s, Ar*H*); EI-MS *m*/*z*: 447 ($[M]^+$); HR-MS: Anal. Calcd for $C_{26}H_{41}NO_5$: 447.2985. Found: 447.2984.

5.1.9. *N*-(5-(2-(4-(8-Hydroxyoctyl)phenyl)ethyl)-2,2-dimethyl-1,3-dioxan-5-yl)ethanamide (13). To a stirred solution of **12** (0.26 g, 5.8×10^{-4} mol) in dried Et_2O (80 mL) was added lithium aluminum hydride (72 mg, 1.9×10^{-3} mol) at room temperature for 2 h. The reaction mixture was treated in a similar manner to that used for the preparation of **10** to give **13** (0.17 g, 72%): colorless needles (recrystallized from *n*-hexene–Et_2O); mp 86–88 °C; IR ν_{max} ($CHCl_3$) cm^{-1}: 1680 (*CO*NH). ^1H NMR δ: 1.29–1.35 (8H, m, $Ph(CH_2)_2(CH_2)_4(CH_2)_2OH$), 1.42 (6H, s, $C(CH_3)_2$), 1.53–1.60 (4H, m, $PhCH_2CH_2(CH_2)_4CH_2CH_2$–), 2.01 (3H, s, $NHCOCH_3$), 2.03–2.07 (2H, m, $PhCH_2CH_2C$), 2.49–2.53 (2H, m, $PhCH_2CH_2C$), 2.54 (2H, t, J = 7.4 Hz, $PhCH_2(CH_2)_7$–), 3.62–3.65 (2H, m, $Ph(CH_2)_7CH_2O$ H), 3.67, 3.98 (each 2H, d, J = 12.0 Hz, $(CH_2O)_2C(CH_3)_2$), 5.71 (1H, br s, N*H*), 7.07 (4H, br s, Ar*H*); EI-MS *m*/*z*: 405 ($[M]^+$); Anal. Calcd for $C_{20}H_{31}NO_4$: C, 71.07; H, 9.69; N, 3.45. Found: C, 70.89; H, 9.40; N, 3.26.

5.1.10. 4-(4-(2-(5-(Acetylamino)-2,2-dimethyl-1,3-dioxan-5-yl)ethyl)phenyl)butyl 4-methylbenzenesulfonate (14). To a stirred solution of **10** (0.45 g, 1.3×10^{-3} mol) in dried CH_2Cl_2 (5 mL) and dried Et_3N (0.36 mL, 2.6×10^{-3} mol) was added *p*-toluenesulfonyl chloride (0.30 g, 1.6×10^{-3} mol) under ice cooling. The reaction mixture was stirred at room temperature for 13 h and concentrated. To the residue was added ice water (5 mL), and the whole mixture was extracted with AcOEt (20 mL). The extract was washed with brine (1 mL), dried, and evaporated. The residue was chromatographed on silica gel with *n*-hexane–AcOEt (1:3) to give **14** (0.49 g, 75%) as a white powder: mp 123–124 °C; IR ν_{max} ($CHCl_3$) cm^{-1}: 1675 (*CO*NH); ^1H NMR δ: 1.42 (6H, s, $C(CH_3)_2$), 1.60–1.65 (4H, m, $PhCH_2(CH_2)_2CH_2$–), 2.02 (3H, s, $NHCOCH_3$), 2.02–2.07 (2H, m, $PhCH_2CH_2C$), 2.44 (3H, s, $PhCH_3$), 2.48–2.53 (2H, m, $PhCH_2CH_2C$), 2.60 (2H, t, J = 7.5 Hz, $PhCH_2(CH_2)_3$–), 3.67, 3.97 (each 2H, d, J = 12.0 Hz, $(CH_2O)_2C(CH_3)_2$), 4.02 (2H, t, J = 6.0 Hz, CH_2OTs), 5.73 (1H, br s, N*H*), 7.00, 7.07 (each 2H, d, J = 8.0 Hz, Ar*H*), 7.33, 7.78 (each 2H, d, J = 8.0 Hz, Ar*H*); EI-MS *m*/*z*: 503 ($[M]^+$).

5.1.11. 8-(4-(2-(5-(Acetylamino)-2,2-dimethyl-1,3-dioxan-5-yl)ethyl)phenyl)octyl 4-methylbenzenesulfonate (15). To a stirred solution of **13** (0.16 g, 3.9×10^{-4} mol) in dried CH_2Cl_2 (2.5 mL) and dried Et_3N (0.11 mL, 1.9×10^{-4} mol) was added *p*-toluenesulfonyl chloride (89 mg, 4.5×10^{-4} mol) under ice cooling, and the reaction mixture was stirred at room temperature for 15 h. The reaction mixture was treated in a similar manner to that used for the preparation of **14** to give **15** (0.18 g, 81%): mp 61–63 °C; IR ν_{max} ($CHCl_3$) cm^{-1}: 1180, 1190 (SO_2), 1680 (*CO*NH); ^1H NMR δ: 1.20–1.32 (8H, m, $Ph(CH_2)_2(CH_2)_4(CH_2)_2OTs$),

1.42 (6H, s, $C(CH_3)_2$), 1.52–1.66 (4H, m, $PhCH_2CH_2(CH_2)_4CH_2CH_2$–), 2.01 (3H, s, $NHCOCH_3$), 2.03–2.07 (2H, m, $PhCH_2CH_2C$), 2.44 (3H, s, $PhCH_3$), 2.49–2.53 (2H, m, $PhCH_2CH_2C$), 2.53 (2H, t, J = 7.4 Hz, $PhCH_2(CH_2)_7$–), 3.67, 3.98 (each 2H, d, J = 12.0 Hz, $(CH_2O)_2C(CH_3)_2$), 4.01 (2H, t, J = 6.4 Hz, CH_2OTs), 5.71 (1H, br s, NH), 7.06, 7.08 (each 2H, d, J = 8.0 Hz, ArH), 7.34, 7.79 (each 2H, d, J = 8.0 Hz, ArH); EI-MS m/z: 559 ($[M]^+$); HR-MS: Anal. Calcd for $C_{31}H_{45}NO_6S$: 559.2967. Found: 559.2967.

5.1.12. *S*-(4-(4-(2-(5-(Acetylamino)-2,2-dimethyl-1,3-dioxan-5-yl)ethyl)phenyl)butyl)ethanethioate (16). To a solution of **14** (0.45 g, 8.9×10^{-4} mol) in dried EtOH (10 mL) was added 90% potassium thioacetate (0.13 g, 1.0×10^{-3} mol) under an argon atmosphere. The mixture was refluxed for 1 h and concentrated. To the residue was added ice water (5 mL) and the whole mixture was extracted with AcOEt (20 mL). The extract was washed with brine (1 mL), dried, and evaporated. The residue was chromatographed on silica gel with *n*-hexane–AcOEt (1:1) to give **16** (0.29 g, 80%) as a white powder: mp 77.5–79 °C; ^{1}H NMR δ: 1.42 (6H, s, $C(CH_3)_2$), 1.58–1.66 (4H, m, $PhCH_2(CH_2)_2CH_2$–), 2.01 (3H, s, $NHCOCH_3$), 2.03–2.07 (2H, m, $PhCH_2CH_2C$), 2.32 (3H, s, $SCOCH_3$), 2.49–2.53 (2H, m, $PhCH_2CH_2C$), 2.57 (2H, t, J = 7.5 Hz, $PhCH_2(CH_2)_3$–), 2.88 (2H, t, J = 7.0 Hz, CH_2SAc), 3.67, 3.98 (each 2H, d, J = 11.0 Hz, $(CH_2O)_2C(CH_3)_2$), 5.71 (1H, br s, NH), 7.05, 7.08 (each 2H, d, J = 8.5 Hz, ArH); EI-MS m/z: 407 ($[M]^+$).

5.1.13. *S*-(8-(4-(2-(5-(Acetylamino)-2,2-dimethyl-1,3-dioxan-5-yl)ethyl)phenyl)octyl)ethanethioate (17). To a solution of **15** (0.18 g, 3.2×10^{-4} mol) in dried EtOH (10 mL) was added 90% potassium thioacetate (44 mg, 9.5×10^{-5} mol) under an argon atmosphere. The reaction mixture was treated in a similar manner to that used for the preparation of **16** to give **17** (0.14 g, 94%): mp 33–35 °C; IR ν_{max} ($CHCl_3$) cm^{-1}: 1690, 1680 (*CO*NH, *CO*S). ^{1}H NMR δ: 1.23–1.35 (8H, m, $Ph(CH_2)_2(CH_2)_4(CH_2)_2SAc$), 1.42 (6H, s, $C(CH_3)_2$), 1.52–1.59 (4H, m, $PhCH_2CH_2(CH_2)_4CH_2CH_2$–), 2.01 (3H, s, $NHCOCH_3$), 2.03–2.07 (2H, m, $PhCH_2CH_2C$), 2.32 (3H, s, $SCOCH_3$), 2.49–2.53 (2H, m, $PhCH_2CH_2C$), 2.54 (2H, t, J = 7.4 Hz, $PhCH_2(CH_2)_7$–), 2.86 (2H, t, J = 7.4 Hz, CH_2SAc), 3.67, 3.98 (each 2H, d, J = 12.0 Hz, $(CH_2O)_2C(CH_3)_2$), 5.71 (1H, br s, NH), 7.08 (4H, br s, ArH); EI-MS m/z: 463 ($[M]^+$); HR-MS: Anal. Calcd for $C_{26}H_{41}NO_4S$: 463.2756. Found: 463.2762.

5.1.14. 2-Amino-2-(2-(4-(4-mercaptobutyl)phenyl)ethyl)propane-1,3-diol hydrochloride (3). To a solution of **16** (77 mg, 1.9×10^{-4} mol) in EtOH (2 mL) was added 10% HCl (1.5 mL) under an argon atmosphere and the mixture was refluxed for 8 h. The reaction mixture was concentrated to give **3** (60 mg, 99%): colorless plates (recrystallized from MeOH–Et_2O); mp 105–113 °C (dec); IR ν_{max} (KBr) cm^{-1}: 3550–3150 (*OH*), 3150–2400 (NH_3^+); ^{1}H NMR (DMSO-d_6) δ: 1.51–1.55 (2H, m, $Ph(CH_2)_2CH_2CH_2$–), 1.61–1.63 (2H, m, $PhCH_2CH_2(CH_2)_2$–), 1.76–1.79 (2H, m, $PhCH_2CH_2C$), 2.23 (1H, t, J = 8.0 Hz, SH), 2.46–2.51 (4H, m, $PhCH_2(CH_2)_2CH_2SH$), 2.53–2.59 (2H, m, $PhCH_2CH_2C$), 3.52 (4H, d, J = 5.0 Hz, CH_2OH 2×), 5.36 (2H, t, J = 5.0 Hz, CH_2OH 2×), 7.11 (4H, br s, ArH), 7.82 (3H, br s, NH_3^+); FAB-MS (positive) m/z: 284 ($[M+H-HCl]^+$); Anal. Calcd for $C_{15}H_{26}NO_2SCl$: C, 56.32; H, 8.19; N, 4.38. Found: C, 56.37; H, 8.09; N, 4.49.

5.1.15. 2-Amino-2-(2-(4-(8-mercaptooctyl)phenyl)ethyl)propane-1,3-diol hydrochloride (4). To a solution of **17** (60 mg, 1.3×10^{-4} mol) in EtOH (2 mL) was added 10% HCl (1.0 mL) under an argon atmosphere and the mixture was refluxed for 5 h. The reaction mixture was concentrated to give **4** (31 mg, 62%) as a powder: white powder (recrystallized from MeOH–Et_2O); mp 97–104 °C (dec); ^{1}H NMR (DMSO-d_6) δ: 1.23–1.35 (8H, m, $Ph(CH_2)_2(CH_2)_4(CH_2)_2SH$), 1.48–1.56 (4H, m, $PhCH_2CH_2(CH_2)_4CH_2CH_2$–), 1.75–1.80 (2H, m, $PhCH_2CH_2C$), 2.20 (1H, t, J = 7.4 Hz, SH), 2.45 (2H, q, J = 7.4 Hz, $Ph(CH_2)_7CH_2SH$), 2.50–2.54 (2H, m, $PhCH_2(CH_2)_7$–), 2.55–2.60 (2H, m, $PhCH_2CH_2C$), 3.52 (4H, d, J = 5.0 Hz, CH_2OH 2×), 5.37 (2H, t, J = 5.0 Hz, CH_2OH 2×), 7.10 (4H, br s, ArH), 7.83 (3H, br s, NH_3^+); FAB-MS (positive) m/z: 340 ($[M+H-HCl]^+$).

5.2. Biological procedures

5.2.1. Buffers. The regularly used buffers were 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mmol/L NaCl (buffer A); buffer A containing 1 g/L bovine serum albumin (BSA, Nacalai Tesque, Kyoto, Japan) (buffer B); 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5 mmol/L EDTA (buffer C).

5.2.2. Measurement of thiol groups[38]. The concentration of thiol groups was calculated from the absorbance at 324 nm (ε: 19,800) of pyridine-4-thione, which is the reduction product of 4,4′-dithiodipyridine.

5.2.3. Measurement of maleimide groups[38]. Samples including maleimide group were incubated with a known amount of 2-mercaptoethylamine hydrochloride and the remaining thiol groups was measured as above. The amount of maleimide groups was calculated from the difference between the former and the latter.

5.2.4. 3-Protein conjugate and 4-protein conjugate

5.2.4.1. Maleimide-protein. Maleimide groups were introduced into ovalbumin (OVA, Nacalai Tesque, Kyoto, Japan), BSA, or horseradish peroxidase (HRP, Boehringer Mannheim GmbH, Mannheim, Germany) using ε-maleimidocaproic acid *N*-hydroxysuccinimide ester (EMCS, Dojindo Laboratories, Kumamoto, Japan). The protein solution (10 mg/mL, 0.6 mL) in 0.1 mol/L sodium phosphate buffer, pH 7.0, was incubated with EMCS (30–80 mmol/L, 0.06 mL) in DMF at 30 °C for 30 min. The reaction mixture was subjected to gel filtration on a column of Sephadex G-25 (Pharmacia LKB Biotechnology, Uppsala, Sweden) with buffer C or centrifuged column procedure using Sephadex G-50 (Pharmacia LKB Biotechnology) with buffer C to give

maleimide-protein. The concentration of maleimide-protein was calculated from the absorbance at 280 nm (OVA: extinction coefficient, 0.74 g^{-1} L cm^{-1}, M_r (relative molecular mass), 45,000, BSA: extinction coefficient, 0.63 g^{-1} L cm^{-1}, M_r, 66,200) or 403 nm (HRP: extinction coefficient, 2.275 g^{-1} L cm^{-1}, M_r, 40,000).

5.2.4.2. 3-OVA conjugate. Compound **3** (1.2 mg) was dissolved in buffer C (3.0 mL) and passed through a Millipore filter (0.45 μm). The maleimide-OVA (0.484 mL, 66.8 nmol), in which the average number of maleimide groups introduced per OVA molecule had been 8.4, was incubated with an aliquot (1.48 mL) of the **3** solution (666 nmol as thiol group) at 4 °C for 20 h. The reaction mixture was further incubated with 0.1 mol/L 2-mercaptoethylamine hydrochloride in buffer C (18.6 μL) at 30 °C for 15 min and subjected to gel filtration on a column (1× 30 cm) of Sephadex G-25 with buffer A to give **3**-OVA conjugate. The average number of **3** moieties conjugated per OVA molecule was 6.8, as calculated from the decrease in the number of maleimide groups. The amount of the **3**-OVA conjugate obtained was 2.27 mg.

5.2.4.3. 4-OVA conjugate. Compound **4** (1.6 mg) was dissolved in MeOH (0.43 mL). The maleimide-OVA (3.3 mL, 347 nmol), in which the average number of maleimide groups introduced per OVA molecule had been 4.8, was diluted with a mixture of ice MeOH (10.0 mL) and buffer C (20.5 mL). The diluted maleimide-OVA was incubated with an aliquot (0.23 mL) of the **4** solution (690 nmol as thiol group) at 4 °C overnight. The reaction mixture was centrifuged at 7000 rpm for 10 min. The supernatant (30.0 mL) was incubated with 0.25 mmol/L 2-mercaptoethylamine hydrochloride in buffer C (80.0 μL) at 30 °C for 15 min and then with 0.5 mmol/L *N*-ethylmaleimide in buffer C (0.16 mL) at 30 °C for 15 min. The reaction mixture was dialyzed against buffer A (1 L) overnight and subjected to gel filtration on a column (1× 30 cm) of Sephadex G-25 with buffer A to give **4**-OVA conjugate. The **4**-OVA conjugate was concentrated in an ultrafiltration apparatus (Amicon 8010) using a Diaflo Ultrafiltration Membrane PM-10 (Amicon, Inc., Bervery, MA, USA). The average number of **4** moieties conjugated per OVA molecule was 2.4, as calculated from the decrease in the number of maleimide groups. The amount of the **4**-OVA conjugate obtained was 3.20 mg.

5.2.4.4. 3-BSA conjugate. The maleimide-BSA (0.45 mL, 45.5 nmol), in which the average number of maleimide groups introduced per BSA molecule had been 10.1, was incubated with an aliquot (1.01 mL) of the **3** solution (666 nmol as thiol group) used in Section 5.2.4.2 at 4 °C for 20 h. The reaction mixture was further incubated with 0.1 mol/L 2-mercaptoethylamine hydrochloride in buffer C (14.6 μL) at 30 °C for 15 min and subjected to gel filtration on a column (1× 30 cm) of Sephadex G-25 using buffer A. The average number of **3** moieties conjugated per BSA molecule was 9.6, as calculated from the decrease in the number of maleimide groups. The amount of the **3**-BSA conjugate obtained was 2.09 mg.

5.2.4.5. 4-BSA conjugate. Compound **4** (1.4 mg) was dissolved in MeOH (0.37 mL). The maleimide-BSA (0.25 mL, 26.5 nmol), in which the average number of maleimide groups introduced per BSA molecule had been 2.0, was diluted with a mixture of ice MeOH (0.32 mL) and buffer C (0.58 mL). The diluted maleimide-BSA was incubated with an aliquot (32.6 μL) of the **4** solution (134 nmol as thiol group) at 4 °C overnight. The reaction mixture was centrifuged at 3000 rpm for 10 min. The supernatant (0.89 mL) was incubated with 0.01 mol/L 2-mercaptoethylamine hydrochloride in buffer C (87.4 μL) at 30 °C for 15 min and then with 0.01 mol/L *N*-ethylmaleimide in buffer C (184.1 μL) at 30 °C for 15 min. The mixture was dialyzed against buffer A (1 L) overnight to give **4**-BSA conjugate. The average number of **4** moieties conjugated per BSA molecule was 2.0, as calculated from the decrease in the number of maleimide groups. The amount of the **4**-BSA conjugate obtained was 0.54 mg.

5.2.4.6. HRP-labeled 3. Compound **3** (0.5 mg) was dissolved in water (0.5 mL). The maleimide-HRP (0.566 mL, 79.8 nmol), in which the average number of maleimide groups introduced per HRP molecule had been 0.93, was incubated with an aliquot (1.47 mL) of the **3** solution (516 nmol as thiol group) at room temperature for 1 h. After incubation, the reaction mixture (0.6 mL) was subjected to the centrifuged column procedure using Sephadex G-50 and buffer C. The filtrate (0.784 mL) was incubated with 0.1 mol/L 2-mercaptoethylamine hydrochloride in buffer C (7.84 μL) at room temperature for 15 min and dialyzed against buffer A (500 mL) for two days. The average number of **3** moieties conjugated per HRP molecule was 1.0, as calculated from the decrease in the number of maleimide groups. The amount of the HRP-labeled **3** obtained was 1.93 mg.

5.2.4.7. HRP-labeled 4. Compound **4** (0.4 mg) was dissolved in 4 mmol/L β-cyclodextrin (Wako Pure Chemical Industries, Osaka, Japan) in buffer C (0.5 mL). The maleimide-HRP (0.20 mL, 48 nmol), in which the average number of maleimide groups introduced per HRP molecule had been 0.66, was incubated with an aliquot (0.718 mL) of the **4** solution (240 nmol as thiol group) at 4 °C for two days. The reaction mixture was incubated with 0.1 mol/L 2-mercaptoethylamine hydrochloride in buffer C (19 μL) at 30 °C for 15 min and then with 0.1 mol/L *N*-ethylmaleimide in buffer C (38 μL) at 30 °C for 15 min. The mixture was subjected to gel filtration on a column (1× 30 cm) of Sephadex G-25 with buffer A to give HRP-labeled **4**. The average number of **4** moieties conjugated per HRP molecule was 0.66, as calculated from the decrease in the number of maleimide groups. The amount of the HRP-labeled **4** obtained was 1.40 mg.

5.2.5. Antisera. Three rabbits (Japanese White, 2.7–2.8 kg, female) were intracutaneously injected 3 times with the **3**-OVA conjugate (0.1 mg) or the **4**-OVA conjugate (0.1 mg) in Freund's complete adjuvant at three-week intervals. Blood was collected a week after the last injection, and the antisera were stored at −20 °C.

5.2.6. Rabbit (anti-3-OVA) IgG, rabbit (anti-4-OVA) IgG, and rabbit (anti-3-OVA) Fab′. IgG, $F(ab')_2$ and Fab′ were prepared by Ishikawa's method.[38] The amounts of rabbit (anti-**3**-OVA) IgG and rabbit (anti-**4**-OVA) IgG obtained were 23.1 and 25.9 mg, respectively, from 10 mL of the corresponding serum. The amount of rabbit (anti-**3**-OVA) Fab′ obtained was 1.5 mg from 5.0 mg of the corresponding IgG.

5.2.7. HRP-labeled rabbit (anti-3-OVA) Fab′. The maleimide-HRP (0.398 mL, 32.6 nmol), in which the average number of maleimide groups introduced per HRP molecule had been 1.85, was incubated with rabbit (anti-**3**-OVA) Fab′ (32.9 nmol) in buffer C (0.54 mL) at 4 °C overnight. The reaction mixture was subjected to gel filtration on a column (1.5× 45 cm) of Ultrogel AcA 44 (Bio Sepra S.A. Villeneuve la Garenne Cedex, France) using 0.1 mol/L sodium phosphate buffer, pH 7.0, to give HRP-labeled rabbit (anti-3-OVA) Fab′. The average number of rabbit (anti-**3**-OVA) Fab′ molecules conjugated per HRP molecule was 0.82, as calculated from the absorbance at 280 and at 403 nm.[38] The amount of the HRP-labeled rabbit (anti-**3**-OVA) Fab′ obtained was 1.8 mg.

5.2.8. Antibody response

5.2.8.1. Anti-3 IgG. Before and after (7, 10, 22, 26, 31, and 41 days) administration of **3**-OVA conjugate, blood samples were collected from an ear into plastic tubes, and the sera (approx. 0.1 mL) were stored at −20 °C.

The **3**-BSA conjugate (0.01 mg/mL) in 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 1 g/L NaN_3 (0.15 mL) was added to a microtiter plate (Sanko Junyaku, Tokyo, Japan) and incubated at 4 °C overnight. The **3**-BSA conjugate was removed by aspiration and the plate was washed four times with buffer A (0.2 mL). The washed plate was incubated with buffer B containing 1 mmol/L $MgCl_2$ and 1 g/L NaN_3 (0.2 mL) at 37 °C for 2 h, and the buffer was removed. The serum samples (0.15 mL), which had been diluted 10^5-fold with buffer B, were added to the plate and incubated at 37 °C for 2 h. The serum samples were removed and the plate was washed four times with buffer A (0.2 mL). To the plate was added HRP-labeled goat (anti-rabbit IgG) Fab′ (5 ng) in buffer B (0.15 mL), and incubation was continued at 37 °C for 1 h. After incubation, HRP-labeled goat (anti-rabbit IgG) Fab′ was removed and the plate was washed four times with buffer A (0.25 mL). HRP activity bound to the plate was measured by incubating the plate with 7.5 mmol/L *o*-phenylenediamine (Nacalai Tesque, Kyoto, Japan) in 50 mmol/L sodium acetate buffer, pH 5.0, containing 0.25 mg/mL BSA, 5 mmol/L EDTA, and 0.025% hydrogen peroxide (0.1 mL) at 37 °C for 10 min. The enzyme reaction was terminated by addition of 1.2 mol/L H_2SO_4 containing 2.4 g/L Na_2SO_3 (0.05 mL), and the absorbance at 490 nm was measured using a microplate reader.

5.2.8.2. Anti-4 IgG. Before and after (7, 14, 22, 35, and 43 days) administration of **4**-OVA conjugate, blood samples were collected, and anti-**4** IgG in serum was measured in a similar manner to that used for anti-**3** IgG.

5.2.9. Epitope analysis and test for cross-reactivity using a competitive EIA (method A). Rabbit (anti-**3**-OVA) IgG (0.1 mg/mL) in 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 1 g/L NaN_3 was diluted 100-fold with normal rabbit IgG (0.1 mg/mL) in the same buffer. The diluted rabbit (anti-**3**-OVA) IgG (0.15 mL) was added to a microtiter plate and incubated at room temperature for 2 h. After incubation, the diluted rabbit (anti-**3**-OVA) IgG was removed and the plate was washed four times with buffer A (0.2 mL). The washed plate was incubated with buffer B (0.2 mL) at room temperature for 2 h. An aliquot (0.075 mL) of HRP-labeled **3** (0.2 pmol) in buffer B was mixed with compound **1** (0.1, 0.3, 1, 3, and 10 pmol), **3**, **4** or **18**–**31** (0.3, 3, or 30 pmol) in buffer B (0.075 mL), and the mixture was added to the plate and incubated at room temperature for 3 h. After incubation, the mixture was removed and the plate was washed four times with ice-cold buffer A (0.25 mL). The HRP activity bound to the plate was assayed as described in Section 5.2.8.1. Epitope analysis and test for cross-reactivity of rabbit (anti-**4**-OVA) IgG were performed in a similar manner to that used for rabbit (anti-**3**-OVA) IgG.

5.2.10. Competitive EIA for 1

5.2.10.1. Alkaline-chloroform extraction of 1 from serum. The sample (0.4 mL) containing **1** was mixed with 1 N NaOH (0.1 mL) and water (0.5 mL). $CHCl_3$ (5 mL) was added to the mixture and the whole mixture was shaken for 10 min. After shaking, the mixture was centrifuged at 3000 rpm for 10 min. The $CHCl_3$ layer was transferred to two glass test tubes (1.5 mL each). The extract was evaporated to dryness at 40 °C using a centrifugal concentrator (Taitec Co., Saitama, Japan). The residue was subjected to a competitive EIA as described in Section 5.2.10.2.

5.2.10.2. Competitive EIA (method B). **3**-BSA conjugate (0.01 mg/mL) in 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 1 g/L NaN_3 was diluted 300-fold with BSA (0.01 mg/mL) in the same buffer. The diluted **3**-BSA conjugate (0.15 mL) was added to a microtiter plate and incubated at 4 °C overnight. The diluted **3**-BSA conjugate was removed and the plate was washed four times with buffer A (0.2 mL). The washed plate was incubated with buffer B (0.2 mL) at room temperature for 2 h. Buffer B was removed from the plate prior to use. The residue containing **1** was dissolved in 10 mmol/L sodium phosphate buffer, pH 8.5, containing 0.1 mol/L NaCl and 1 g/L BSA (0.12 mL), and incubated with an aliquot (0.06 mL) of the HRP-labeled rabbit (anti-**3**-OVA) Fab′ (60 fmol) in the same buffer at 4 °C overnight. After incubation, an aliquot (0.15 mL) of the reaction mixture was added to the plate and was incubated at 4 °C for 6 h. The reaction mixture was removed and the plate was washed four times with ice-cold 10 mmol/L sodium phosphate buffer, pH 8.5, containing 0.1 mol/L NaCl (0.25 mL). The HRP activity bound to the plate was assayed as described in Section 5.2.8.1.

5.2.11. Measurement of bioavailability. Wistar rats (10-weeks-old, male, Japan SLC, Inc., Shizuoka, Japan) were fasted, but allowed free access to water overnight

before all experiments. Compound **1** (1 mg/mL) in water was administered orally at a dose of 1 mg/kg without anesthesia. Before and after (2, 4, 6, 8, 10, 12, 24, 48, and 96 h) administration, blood samples (approx. 0.3 mL) were collected from a tail vein through a heparinized needle into plastic tubes (1.5 mL), and the sera (approx. 0.1 mL) were stored at −20 °C. The sera were diluted 8-fold with water and subjected to the competitive EIA for **1**.

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