

Synthesis of Carboxymethylpullulan–Peptide–Doxorubicin Conjugates and Their Properties

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The amino group of doxorubicin (DXR) was found to be bound to the carboxyl group of carboxymethylpullulan (CMPul) either directly or through tetrapeptide spacers, including Gly–Gly–Phe–Gly, Gly–Phe–Gly–Gly and Gly–Gly–Gly–Gly. These conjugates had DXR contents of 6.1–7.1%, with the degree of substitution of carboxymethyl groups being 0.6 per sugar moiety. These conjugates associate in phosphate-buffered saline (PBS) (pH 7.4), forming micelles with hydrophobic DXR inside and hydrophilic CMPul on the outside. The amounts of DXR released from the conjugates in the presence of rat liver lysosomal enzymes were determined by HPLC. The rate of the drug release differed among the conjugates tested. CMPul–DXR conjugate bound through Gly–Gly–Phe–Gly released 35% of its DXR over 24 h. On the other hand, CMPul–DXR conjugate without spacer released no free DXR. The antitumor effect of each conjugate in rats bearing Walker 256 was studied by monitoring the tumor weights after a single intravenous injection. Compared with DXR, CMPul–DXR conjugates bound through Gly–Gly–Phe–Gly and Gly–Phe–Gly–Gly spacers significantly suppressed the tumor growth, while CMPul–DXR conjugate bound through Gly–Gly–Gly–Gly showed less antitumor effect than DXR. CMPul–DXR conjugate without spacer showed no *in vivo* antitumor effect even at a dose equivalent to as much as 20 mg/kg of DXR.

Key words doxorubicin; peptide spacer; carboxymethylpullulan; polymeric drug

In treating malignant tumors, chemotherapy plays an important role, as well as surgery and radiation therapy. Doxorubicin (DXR), an anthracycline antibiotic, is one of the most widely used drugs in cancer chemotherapy. However, its clinical usefulness is limited by side effects, such as suppression of bone marrow function and cardiotoxicity. In recent years, many attempts have been made to improve the therapeutic index of DXR or daunorubicin (DNR), an analog of DXR, by using various drug delivery systems. One approach has been conjugation of the drug with macromolecular carriers, such as albumin,²⁾ immunoglobulin,³⁾ synthetic polymer,^{4,5)} and poly-amino acid,^{6,7)} as well as polysaccharides,^{8–11)} since macromolecules have a wide variety of characteristics and functions.

Pullulan, an α -1,6-linked linear polymer of maltotriose, has many advantages as a macromolecular drug carrier, including high water solubility, multiple hydroxyl groups that can readily be modified chemically, lack of immunogenicity, and usefulness as a plasma expander.¹²⁾ Takakura *et al.*¹³⁾ reported that carboxymethyl dextran with a molecular size above 70 kDa was retained in blood circulation and accumulated in the tumor. Therefore, pullulan is expected to have similar properties when it is carboxymethylated. Furthermore, drug release can be controlled by selection of a drug-carrier linkage which would be stable in the bloodstream, but degraded in or near the tumor cells. The length and sequence of the peptide spacer can regulate the rate of drug release. For example, Trouet *et al.*²⁾ showed that succinylalbumin–DNR conjugate bound through Ala–Leu–Ala–Leu or Leu–Ala–Leu was much more active against L1210 leukaemia in mice than DNR or the conjugate bound through spacers smaller than dipeptide. Duncan *et al.*⁴⁾ reported that the *N*-(2-hydroxypropyl)methacrylamide (HPMA)–DXR conjugate bound through biodegradable

Gly–Phe–Leu–Gly was more active against mice bearing L1210 than the conjugate bound through non-degradable Gly–Gly. Considerable improvement in the antitumor activity of macromolecule–peptide–drug conjugates has been attained. However, little is known about their efficacy against solid tumors by systemic administration at low dose.

In this paper, we report the synthesis and properties of CMPul–DXR conjugates bound either directly or through tetrapeptide spacers, including Gly–Gly–Phe–Gly, Gly–Phe–Gly–Gly or Gly–Gly–Gly–Gly. We also compared the *in vivo* antitumor effect of these conjugates with that of free DXR.

Results and Discussion

Synthesis The peptide derivatives of DXR (**6a–c**) were prepared according to Masquelier *et al.*¹⁴⁾ with slight modifications. *N*-Triphenylmethyl (trityl) tetrapeptides (**4a–c**), which were prepared by debenzoylation of *N*-trityl *O*-benzyl tetrapeptides (**3a–c**) *via* catalytic transfer hydrogenolysis, were condensed with DXR in the presence of dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HOSu) to obtain *N*-trityl aminoacyl derivatives (**5a–c**). The *N*-trityl groups of **5a–c** were smoothly removed by treatment with 75% AcOH for 1 h at 25 °C. The acetate salts of peptidyl DXR were converted to their hydrochloride salts (**6a–c**).

CMPul (**8**) could be isolated as its sodium salt by treatment of pullulan (**7**) (molecular weight, 150 kDa) with chloroacetic acid in alkaline solution. The degree of substitution of the carboxymethyl group was 0.6 per glucose moiety. Next, **6a–c** or DXR was conjugated with CMPul in the presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in a solution of *N,N*-dimethylformamide (DMF)–H₂O (1:1) to afford the conjugates

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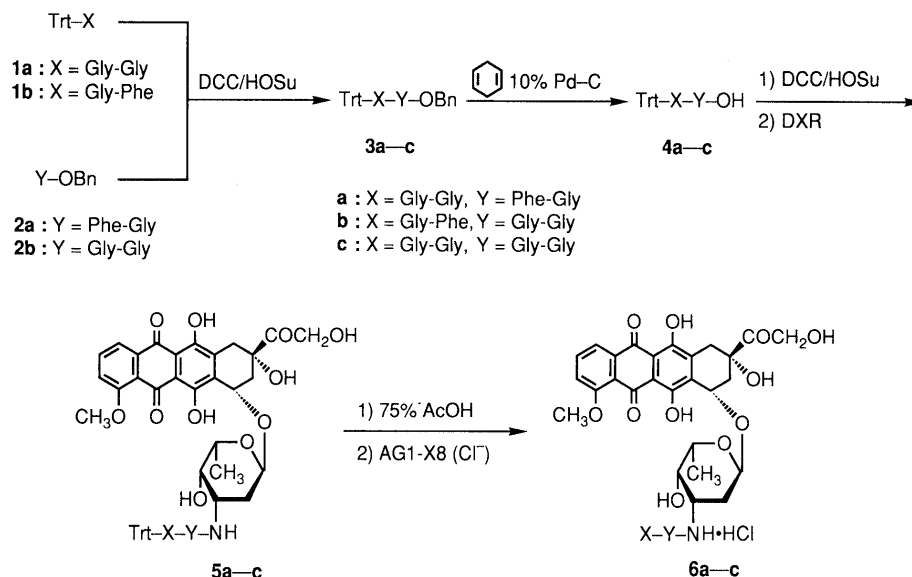


Chart 1

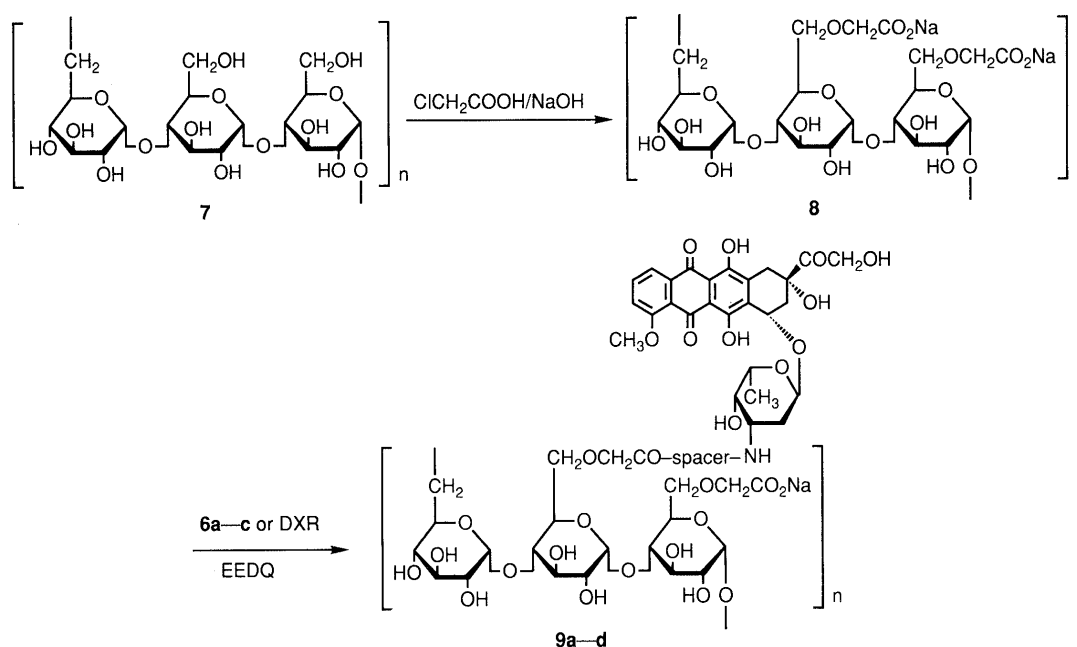


Chart 2

(9a-d). HPLC with detection of **9a** at 480 nm (anthracycline ring), as shown in Fig. 1, confirmed that DXR was bound to the carrier. The possibility of an ester linkage *via* the C-14 hydroxyl group of DXR is considered to be small in this case, because no cross-linking product between the hydroxyl group and the carboxyl group of CMPul was detected, as shown in Fig. 1. However, the exact position of carboxymethylation in pullulan or amide formation between the carboxyl groups of CMPul and amino groups of **6a-c** or DXR could not be determined. Chart 2 shows a typical example. The DXR content of CMPul-DXR conjugates was determined by measuring the absorbance at 480 nm (Fig. 2). CMPul-DXR conjugates had DXR contents of 6.1–7.1%, as shown in Table 1.

In Vitro Stability in Dulbecco's Phosphate-Buffered Saline (PBS) The change in absorbance at 480 nm was monitored for DXR, a mixture of CMPul and DXR, and

9a-d in PBS (pH 7.4) at 37°C, in order to examine the stability of DXR molecules. Absorbance of DXR after incubation for 96 h fell to 70% of the initial level. A mixture of DXR and CMPul showed slightly better stability. On the other hand, **9a-d** retained more than 98% of the initial absorbance after 96 h incubation (data shown only for **9a** and **9d**). HPLC monitoring revealed no DXR release from any of the conjugates (data not shown). The stability of the CMPul-DXR conjugates in PBS was presumably due to the shielding of DXR from hydroxyl ions by CMPul.

Fluorescence Measurements The fluorescence of DXR, **9a** and **9d** in PBS or PBS plus sodium dodecyl sulfate (SDS) was measured for DXR concentrations from 1.2×10^{-7} to 1.0×10^{-3} M. DXR showed 2.2-fold quenching of the fluorescence intensity in PBS at 6.2×10^{-4} M when compared with the intensity in 0.2% (w/v) SDS. This

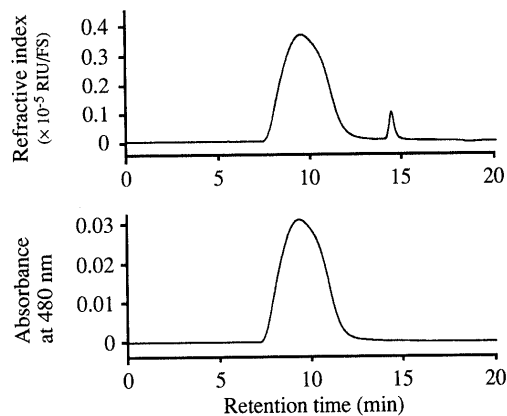


Fig. 1. HPLC Chromatogram of CMPul-Gly-Gly-Phe-Gly-DXR (**9a**)

Chromatographic conditions : column, TSKgel G4000 PW_{XL} (300 mm × 7.8 mm i.d.); eluent, 0.1 M NaCl; flow rate, 0.8 ml/min; column temperature, 40 °C; sample volume, 10 μ l (5 mg/ml).

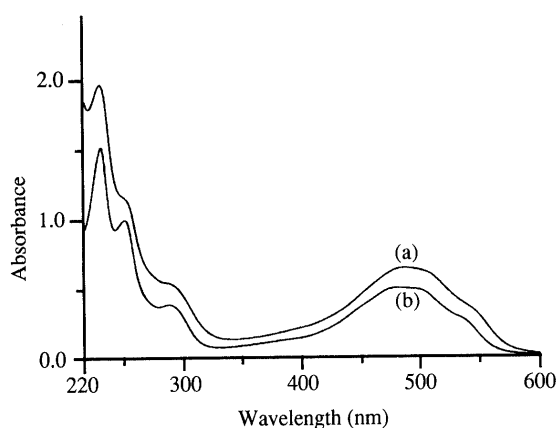


Fig. 2. UV-VIS Spectrum of CMPul-Gly-Gly-Phe-Gly-DXR (**9a**) and DXR in PBS

(a) **9a** (DXR = 5.4×10^{-5} M); (b) DXR (DXR = 4.7×10^{-5} M).

Table 1. DXR Content of CMPul-DXR Conjugates

Run	Spacer	DXR Content ^{a)} (%)
9a	Gly-Gly-Phe-Gly	6.1
9b	Gly-Phe-Gly-Gly	6.1
9c	Gly-Gly-Gly-Gly	6.9
9d	None	7.1

a) The amount of bound DXR was estimated spectrophotometrically at 480 nm in PBS.

quenching resulted from the dimerization of DXR through hydrophobic interactions in aqueous solution,¹⁵⁾ with the interaction being destroyed by addition of SDS. Quenching of the fluorescence intensity of **9a** and **9d** was 7.6- and 6.5-fold, respectively, in PBS at 6.2×10^{-4} M, as compared with that of DXR. Furthermore, quenching of the intensity of **9a** was not cancelled completely by addition of 5% (w/v) SDS. Ulbrich *et al.*¹⁶⁾ showed that the conjugate of HPMA-*p*-nitroaniline bound through oligopeptide spacer forms micelles in water with *p*-nitroaniline inside and HPMA on the outside. CMPul-DXR conjugates are considered to form micelles in PBS with DXR inside and CMPul on the outside, and the DXR molecules are tightly

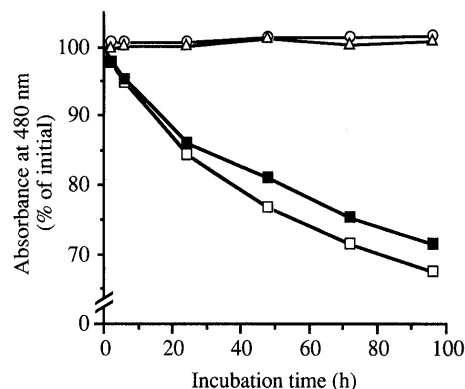


Fig. 3. Stability of DXR, DXR Plus CMPul, CMPul-Gly-Phe-Gly-Gly-DXR (**9a**) and CMPul-DXR (**9d**) in PBS at 37 °C in the Dark, Estimated from the Absorbance at 480 nm

□, DXR (DXR = 2.7×10^{-5} M); ■, DXR (DXR = 2.7×10^{-5} M) plus CMPul (0.3 mg/ml); ○, **9a** (DXR = 3.3×10^{-5} M); ●, **9d** (DXR = 3.1×10^{-5} M).

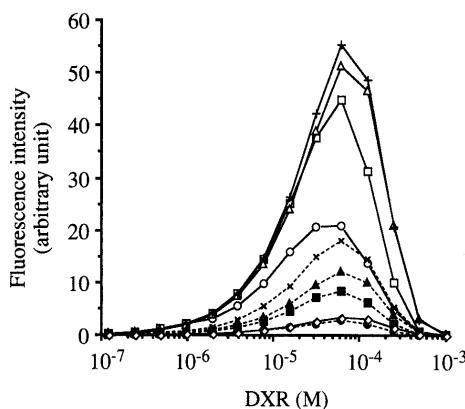


Fig. 4. Plot of Fluorescence Intensity of DXR, CMPul-Gly-Gly-Phe-Gly-DXR (**9a**) and CMPul-DXR (**9d**) in PBS

○, DXR; □, DXR plus 0.2% (w/v) SDS; △, DXR plus 1% (w/v) SDS; +, DXR plus 5% (w/v) SDS; ●, **9a**; ■, **9a** plus 0.2% (w/v) SDS; ▲, **9a** plus 1% (w/v) SDS; ×, **9a** plus 5% (w/v) SDS; ◇, **9d**.

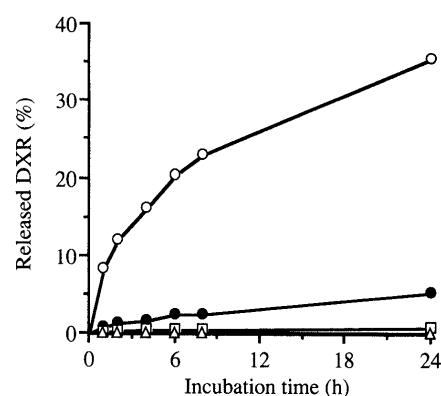


Fig. 5. Digestion of CMPul-DXR Conjugates by Lysosomal Enzymes *In Vitro*

The Conjugates (DXR = 1.8×10^{-5} M) were incubated at 37 °C in 0.2 M citrate buffer (pH 5.5), containing EDTA (1 mM), 0.2% Triton X-100 and reduced glutathione (5 mM) in the presence of lysosomal enzymes from rat liver. After various times, aliquots were taken and DXR released was determined by HPLC. ○, **9a**; ●, **9b**; □, **9c**; △, **9d**.

packed inside a hydrophobic core of the micelles.

In Vitro Digestion by Lysosomal Enzymes In order to examine the sensitivity of **9a**–**d** to hydrolysis by lysosomal enzymes, they were incubated at pH 5.5 in the presence

Table 2. Antitumor Effects of CMPul-DXR Conjugates and DXR on Rats Bearing Walker 256 Tumor

Run	Dose (mg/kg) ^{a)}	T/C (%) ^{b)}
DXR	0.8	55.6 ± 3.6
	5.0	13.7 ± 1.6
9a	0.8	11.0 ± 3.2
9b	0.8	24.1 ± 3.4
9c	0.8	92.1 ± 6.6
9d	5.0	99.7 ± 4.9
	20.0	85.3 ± 7.8

The drugs were given intravenously 3 d after i.m. inoculation of Walker carcinoma 256 into Wistar rats. The tumor weight was measured 7 d after the tumor inoculation. a) The dose is expressed as DXR content. b) T/C (%) = (tumor weight of the treatment group/that of the untreated group) × 100. Means ± S.D. of five rats per group.

of lysosomal enzymes derived from rat liver.¹⁷⁾ The kinetics of hydrolysis of these conjugates into DXR, as determined by HPLC, are illustrated in Fig. 5. The release of DXR by lysosomal enzymes acting on CMPul-DXR conjugates is affected by the presence and composition of the peptide spacer between drug and carrier. After 24 h incubation, **9a**–**c** released 35%, 5.4%, and 0.9% of DXR, respectively, and no free DXR was detected in the case of **9d**.

In Vivo Antitumor Effect The antitumor effects of each conjugate and DXR were tested by monitoring the tumor weights after a single intravenous administration in rats bearing Walker 256. At a dose of 0.8 mg/kg as DXR, T/C values of **9a**–**c** and DXR were about 11%, 24%, 92% and 56%, respectively. Thus, **9a** and **9b** showed a greater antitumor effect than DXR, while **9c** showed a lesser antitumor effect than DXR. Compound **9d** showed no *in vivo* antitumor effect even at the high dose of 20 mg/kg. In addition, the present study showed that the *in vivo* antitumor effect was correlated with the rate of drug release in the presence of lysosomal enzymes *in vitro*.

Conjugates of DXR directly bound to polymer carriers, such as the poly(ethyleneglycol)–poly(aspartic acid) block copolymer¹⁸⁾ or the copolymer of divinyl ether and maleic anhydride (DIVEMA),⁵⁾ were effective *in vivo* against tumor-bearing mice. Hirano *et al.*⁵⁾ showed DXR was released gradually from the conjugate of DIVEMA and DXR under physiological conditions at pH 7.2. DXR showed an antitumor effect on the surface of the cell, when attached to microspheres.¹⁹⁾ In this case, hydrolysis of the attached DXR may not have been necessary, since DXR can exert antitumor activity without entering the cells. However, **9d** showed no *in vivo* antitumor effect at a high dose, and no release of DXR from **9d** in PBS or in the presence of lysosomal enzymes was observed. Therefore, DXR released from CMPul-DXR conjugate may kill tumor cells by intercalation between the base pairs of DNA.²⁰⁾ Although the action mechanism of CMPul-DXR conjugates is still unknown, when they are bound through appropriate tetrapeptide spacers, they showed high *in vivo* antitumor activity against solid tumor even at a low dose, in comparison with DXR.

Experimental

Melting points were measured on a Yanaco melting-point apparatus

without correction. Column chromatography was performed on columns of silica gel (Merck, 230–400 mesh). Optical rotations were measured in CHCl₃ or MeOH with a Perkin-Elmer Model 141 polarimeter. ¹H-NMR spectra were recorded with a Varian VXR-500 FT NMR spectrometer for CDCl₃ or CD₃OD solutions. δ_H values are expressed in ppm downfield from the signal for internal Me₄Si. High-resolution liquid secondary-ion mass spectra (HL-SI-MS) were measured with a Hitachi M-90 mass spectrometer, with Cs⁺ as the primary ion, using *m*-nitrobenzyl alcohol as the matrix and polyethylene glycol 600 with or without KI or NaI as an internal standard. Fluorescence was measured using a Shimadzu RF-5000 fluorescence spectrophotometer, with excitation at 470 nm and emission at 560 nm. UV–VIS spectra were recorded with a Shimadzu UV-3100 spectrophotometer.

Trt-Gly-Gly (1a) Trityl chloride (18.1 g, 64.9 mmol) was added in twelve equal portions within 1 h to a solution of Gly-Gly (6.60 g, 50.0 mmol) in a mixture of H₂O (20 ml), 2-propanol (40 ml) and diethylamine (15 ml). The reaction mixture was stirred for a further 1 h, then H₂O (150 ml) was added. The resulting colorless precipitate was washed with H₂O. Acidification of the combined filtrate and washing solution with AcOH and drying of the precipitate *in vacuo* gave **1a** (15.3 g, 82%). mp 179–180 °C (lit.²¹⁾ 180 °C). ¹H-NMR (CD₃OD) δ: 2.92 (s, 2H, Trt-Gly-αCH₂), 3.97 (s, 2H, Gly-αCH₂), 7.20–7.47 (m, 15H, aromatic H).

Trt-Gly-Phe (1b) Gly-Phe (1.10 g, 5.00 mmol) was dissolved in a mixture of H₂O (2 ml), 2-propanol (4 ml) and diethylamine (1.5 ml), and the reaction mixture was treated with trityl chloride (1.81 g, 6.49 mmol) as described for **1a**, to afford **1b** (1.79 g, 77%). mp 237–238 °C. [α]_D²⁵ + 33.0 (c = 1.0, MeOH). ¹H-NMR (CD₃OD) δ: 2.75 (d, 1H, J = 17.0 Hz, Gly-αCH₂), 2.84 (d, 1H, J = 17.0 Hz, Gly-αCH₂), 3.21 (m, 2H, Phe-βCH₂), 4.71 (t, 1H, J = 5.2 Hz, Phe-αCH), 7.18–7.34 (m, 20H, aromatic H). Anal. Calcd for C₃₀H₂₈N₂O₃ · 0.2H₂O: C, 76.97; H, 6.11; N, 5.98. Found: C, 77.07; H, 6.43; N, 6.09.

Phe-Gly-OBn (2a) A mixture of Phe-Gly-OH · H₂O (25.0 g, 104 mmol), *p*-toluenesulfonic acid monohydrate (20.9 g, 110 mmol) and benzyl alcohol (25 ml) in toluene (200 ml) was heated at reflux for 5 h in a Dean-Stark apparatus. The solution was concentrated, followed by the addition of ethyl ether, giving the *p*-toluenesulfonate of **2a** as colorless crystals (48.1 g, 95%). mp 135–137 °C. [α]_D²⁵ + 17.8° (c = 1.0, MeOH). ¹H-NMR (CD₃OD) δ: 2.36 (s, 3H, Phe-Me), 2.99 (dd, 1H, J = 14.2, 8.5 Hz, Phe-βCH₂), 3.24 (dd, 1H, J = 14.2, 5.9 Hz, Phe-βCH₂), 4.02 (d, 1H, J = 17.8 Hz, Gly-αCH₂), 4.06 (d, 1H, J = 17.8 Hz, Gly-αCH₂), 4.11 (dd, 1H, Phe-αCH, J = 8.5, 5.9 Hz), 5.11 (d, 1H, J = 12.2 Hz, O-CH₂-Phe), 5.17 (d, 1H, J = 12.2 Hz, O-CH₂-Phe), 7.22 (d, 2H, J = 8.1 Hz, aromatic H), 7.28–7.38 (m, 5H, aromatic H), 7.71 (d, 2H, J = 8.1 Hz, aromatic H). Anal. Calcd for C₂₅H₂₈N₂O₆S · 0.2H₂O: C, 61.51; H, 5.82; N, 5.74. Found: C, 61.41; H, 5.79; N, 5.64.

Trt-Gly-Gly-Phe-Gly-OBn (3a) A stirred solution of **1a** (1.54 g, 4.10 mmol) and HOSu (0.52 g, 4.50 mmol) in dry DMF (5 ml) at 4 °C was treated with DCC (0.928 g, 4.50 mmol). After 2 h, a solution of the *p*-toluenesulfonate of **2a** (1.99 g, 4.10 mmol) and *N*-methylmorpholine (NMM) (0.414 g, 4.10 mmol) in dry DMF (10 ml) was added and stirring was continued at 4 °C for 15 h. The precipitate was removed by filtration and the filtrate was concentrated. The residue was partitioned between CHCl₃ and H₂O. The organic phase was successively washed with 5% NaHCO₃ and H₂O, dried (Na₂SO₄), and concentrated. Chromatography of the residue on a column of silica gel with CHCl₃–MeOH (20:1) gave **3a** (1.96 g, 72%). mp 142–146 °C. [α]_D²⁵ – 13.3° (c = 1.0, CHCl₃). ¹H-NMR (CDCl₃) δ: 2.90 (dd, J = 14.0, 8.5 Hz, Phe-βCH₂), 2.91–3.02 (m, 2H, Trt-Gly-αCH₂), 3.22 (dd, 2H, J = 14.0, 5.9 Hz, Phe-βCH₂), 3.88–4.02 (m, 4H, Gly-αCH₂ × 2), 4.50 (m, 1H, Phe-αCH), 5.01 (s, 2H, O-CH₂-Phe), 7.19–7.30 (m, 25H, aromatic H). Anal. Calcd for C₄₁H₄₀N₄O₅: C, 73.63; H, 6.03; N, 8.38. Found: C, 73.53; H, 6.00; N, 8.48.

Trt-Gly-Phe-Gly-Gly-OBn (3b) **1b** (2.32 g, 5.00 mmol) and **2b**, prepared from its *p*-toluenesulfonate²²⁾ (1.97 g, 5.00 mmol) and NMM (0.506 g, 5.00 mmol), were coupled as described for **3a**, and chromatography of the product on a column of silica gel with CHCl₃–MeOH (20:1) gave **3b** (3.00 g, 90%). mp 180–183 °C. [α]_D²⁵ + 28.2° (c = 1.0, CHCl₃). ¹H-NMR (CDCl₃) δ: 2.89 (dd, 1H, J = 17.4, 7.3 Hz, Trt-Gly-αCH₂), 2.90 (dd, 1H, J = 14.0, 8.5 Hz, Phe-βCH₂), 2.98 (dd, 1H, J = 17.4, 9.5 Hz, Trt-Gly-αCH₂), 3.22 (dd, 1H, J = 14.0, 5.9 Hz, Phe-βCH₂), 3.86–4.02 (m, 4H, Gly-αCH₂ × 2), 4.54 (m, 1H, Phe-αCH), 5.01 (s, 2H, O-CH₂-Phe), 7.16–7.35 (m, 25H, aromatic H). Anal. Calcd for C₄₁H₄₀N₄O₅: C, 73.63; H, 6.03; N, 8.38. Found: C, 73.59; H, 5.98;

N, 8.44.

Trt-Gly-Gly-Gly-OBn (3c) 1a (1.87 g, 5.00 mmol) and **2b**, prepared from its *p*-toluenesulfonate²²⁾ (1.97 g, 5.00 mmol) and NMM (0.506 g, 5.00 mmol), were coupled as described for **3a**, and chromatography of the product on a column of silica gel with CHCl₃-MeOH (10:1) gave **3c** (1.75 g, 60%). mp 144–146°C. ¹H-NMR (CDCl₃) δ: 2.93 (s, 2H, Trt-Gly-αCH₂), 3.94 (s, 2H, Gly-αCH₂), 3.96 (s, 2H, Gly-αCH₂), 4.00 (s, 2H, Gly-αCH₂), 5.14 (s, 2H, O-CH₂-Phe), 7.19–7.47 (m, 20H, aromatic H). *Anal.* Calcd for C₃₄H₃₄N₄O₅·0.2H₂O: C, 70.13; H, 5.95; N, 9.62. Found: C, 70.15; H, 5.99; N, 9.86.

Trt-Gly-Gly-Phe-Gly-OH (4a) A mixture of a solution of **3a** (1.34 g, 2.00 mmol) in DMF (10 ml), 10% Pd-C (0.150 g) and 1,4-cyclohexadiene (0.300 g) was stirred for 30 min at room temperature. After removal of the catalyst by filtration, the filtrate was evaporated. The resulting mixture was applied to a column of silica gel with CHCl₃-MeOH (7:1) to afford **4a** (1.13 g, 98%). mp 219–224°C (dec.). [α]_D²⁵ -14.3° (c=1.0, MeOH). ¹H-NMR (CD₃OD) δ: 2.90 (s, 2H, Trt-Gly-αCH₂), 2.90 (dd, 1H, J=14.2, 8.8 Hz, Phe-βCH_a), 3.23 (dd, 1H, J=14.2, 5.6 Hz, Phe-βCH_b), 3.64 (d, 1H, J=17.5 Hz, Gly-αCH_a), 3.78 (d, 1H, J=17.5 Hz, Gly-αCH_b), 3.80 (d, 1H, J=16.5 Hz, Gly-αCH_a), 3.94 (d, 1H, J=16.5 Hz, Gly-αCH_b), 4.64 (dd, 1H, J=8.8, 5.6 Hz, Phe-αCH), 7.18–7.45 (m, 20H, aromatic H). *Anal.* Calcd for C₃₄H₃₄N₄O₅·2H₂O: C, 66.43; H, 6.23; N, 9.11. Found: C, 66.58; H, 5.93; N, 9.21.

Trt-Gly-Phe-Gly-Gly-OH (4b) A solution of **3b** (2.01 g, 3.00 mmol) in DMF was hydrogenated according to the method described for **4a**, and chromatography of the product on a column of silica gel with CHCl₃-MeOH (7:1) gave **4b** (1.65 g, 95%). mp 213–215°C (dec.). [α]_D²⁵ +18.2° (c=1.0, MeOH). ¹H-NMR (CD₃OD) δ: 2.75 (d, 1H, J=16.8 Hz, Trt-Gly-αCH_a), 2.81 (d, 1H, J=16.8 Hz, Trt-Gly-αCH_b), 2.99 (dd, 1H, J=14.2, 8.8 Hz, Phe-βCH_a), 3.25 (dd, 1H, J=14.2, 5.6 Hz, Phe-βCH_b), 3.84 (d, 1H, J=17.2 Hz, Gly-αCH_a), 3.90 (s, 2H, Gly-αCH₂), 3.96 (d, 1H, J=17.2 Hz, Gly-αCH_b), 4.58 (dd, 1H, J=8.8, 5.6 Hz, Phe-αCH), 7.20–7.33 (m, 20H, aromatic H). C₃₄H₃₄N₄O₅·2H₂O: C, 66.43; H, 6.23; N, 9.11. Found: C, 66.28; H, 6.26; N, 8.93.

Trt-Gly-Gly-Gly-Gly-OH (4c) A solution of **3c** (1.34 g, 2.00 mmol) in DMF was hydrogenated according to the method described for **4a**, and chromatography of the product on a column of silica gel with CHCl₃-MeOH (5:1) gave **4c** (1.12 g, 97%). mp 190–191°C (dec.). ¹H-NMR (CD₃OD) δ: 2.94 (s, 2H, Trt-Gly-αCH₂), 3.93 (s, 2H, Gly-αCH₂), 3.94 (s, 2H, Gly-αCH₂), 3.97 (s, 2H, Gly-αCH₂), 7.19–7.48 (m, 15H, aromatic H). *Anal.* Calcd for C₂₇H₂₈N₄O₅·0.6H₂O: C, 64.94; H, 5.89; N, 11.22. Found: C, 64.90; H, 5.65; N, 11.42.

3'-N-(Gly-Gly-Phe-Gly)-DXR·HCl (6a) A stirred solution of **4a** (0.475 g, 0.820 mmol) and HOSu (0.104 g, 0.900 mmol) in dry DMF (4 ml) at 4°C was treated with DCC (0.186 g, 0.900 mmol). The reaction mixture was stirred for 2 h. A solution of DXR, prepared from its hydrochloride (0.476 g, 0.820 mmol) and NMM (0.083 g, 0.820 mmol), in dry DMF (10 ml) was poured into the solution and the whole was stirred at 4°C for 10 h. The precipitate was removed by filtration and the filtrate was concentrated. The residue was partitioned between CHCl₃ and H₂O. The organic phase was dried (Na₂SO₄) and concentrated, and chromatography of the residue on a column of silica gel with CHCl₃-MeOH (20:1) gave **5a** (0.766 g). A solution of **5a** in 3 ml of 75% AcOH was stirred for 1 h at room temperature, then H₂O (50 ml) was poured into the solution, the precipitate was removed by filtration, and the filtrate was lyophilized. The product obtained was dissolved in H₂O (2 ml) and passed through an anion exchange resin column (AG 1-X8, Cl⁻ form, BIO-RAD). The aqueous layer was extracted with CHCl₃ and lyophilized to afford **6a** (0.462 g, 63%). [α]_D²⁵ +219.1° (c=0.1, MeOH). ¹H-NMR (CD₃OD) δ: 1.28 (d, 3H, J=6.6 Hz, H-6'), 1.71 (dd, 1H, J=12.7, 4.6 Hz, H-2'), 1.98 (ddd, 1H, J=12.7, 12.7, 3.9 Hz, H-2'), 2.19 (dd, 1H, J=14.7, 5.1 Hz, H-8_b), 2.38 (d, 1H, J=14.7 Hz, H-8_a), 2.94 (dd, 1H, J=13.9, 8.4 Hz, Phe-βCH_a), 3.00 (d, 1H, J=18.6 Hz, H-10_a), 3.10 (d, 1H, J=18.6 Hz, H-10_b), 3.13 (dd, 1H, J=13.9, 6.6 Hz, Phe-βCH_b), 3.59 (d, 1H, J=16.9 Hz, Gly-αCH_a), 3.62 (d, 1H, J=1.5 Hz, H-4'), 3.73 (d, 1H, J=15.9 Hz, Gly-αCH_a), 3.77 (d, 1H, J=15.9 Hz, Gly-αCH_b), 3.83 (d, 1H, J=17.0 Hz, Gly-αCH_a), 3.86 (d, 1H, J=16.9 Hz, Gly-αCH_b), 4.02 (s, 3 H, 4-OCH₃), 4.03 (d, 1H, J=17.0 Hz, Gly-αCH_b), 4.16 (m, 1H, H-3'), 4.30 (q, 1H, J=6.6 Hz, H-5'), 4.43 (dd, 1H, J=8.4, 6.6 Hz, Phe-αCH), 4.73 (s, 2H, H-14), 5.13 (brs, 1H, H-7), 5.43 (d, 1H, J=3.9 Hz, H-1'), 7.16–7.26 (m, 5H, Phe-aromatic), 7.54 (d, 1H, J=8.3 Hz, H-3), 7.80 (t, 1H, H-2), 7.91 (d, 1H, J=7.6 Hz, H-1). HL-SI-MS *m/z*: Calcd for C₄₂H₄₈N₅O₁₅ (M+H⁺): 862.3147. Found:

862.3147.

3'-N-(Gly-Phe-Gly-Gly)-DXR·HCl (6b) Compound **4b** (0.579 g, 1.00 mmol) was treated with DXR prepared from its hydrochloride (0.580 g, 1.00 mmol) and NMM (0.101 g, 1.00 mmol), according to the method described for **6a**, to afford **6b** (0.366 g, 40%). [α]_D²⁴ +206.2° (c=0.1, MeOH). ¹H-NMR (CD₃OD) δ: 1.28 (d, 3H, J=6.6 Hz, H-6'), 1.71 (dd, 1H, J=12.7, 4.6 Hz, H-2'), 2.05 (ddd, 1H, J=12.7, 12.7, 3.4 Hz, H-2'), 2.19 (dd, 1H, J=14.7, 5.1 Hz, H-8_a), 2.38 (d, 1H, J=14.7 Hz, H-8_b), 2.94 (dd, 1H, J=14.0, 8.4 Hz, Phe-βCH_a), 3.04 (d, 1H, J=18.5 Hz, H-10_a), 3.12 (d, 1H, J=18.5 Hz, H-10_b), 3.12 (dd, 1H, J=14.0, 6.0 Hz, Phe-βCH_b), 3.56 (d, 1H, J=16.1 Hz, Gly-αCH_a), 3.62 (d, 1H, J=1.5 Hz, H-4'), 3.68 (d, 1H, J=16.1 Hz, Gly-αCH_a), 3.69 (d, 1H, J=16.1 Hz, Gly-αCH_b), 3.79 (d, 1H, J=16.6 Hz, Gly-αCH_a), 3.84 (d, 1H, J=16.1 Hz, Gly-αCH_b), 3.85 (d, 1H, J=16.6 Hz, 1H, Gly-αCH_a), 4.03 (s, 3 H, 4-OCH₃), 4.14 (m, 1H, H-3'), 4.28 (q, 1H, J=6.6 Hz, H-5'), 4.59 (dd, 1H, J=8.4, 6.0 Hz, Phe-αCH), 4.70 (d, 1H, J=20.8 Hz, H-14_a), 4.75 (d, 1H, J=20.8 Hz, H-14_b), 5.17 (brs, 1H, H-7), 5.44 (d, 1H, J=3.4 Hz, H-1'), 7.18–7.28 (m, 5H, Phe-aromatic), 7.57 (d, 1H, J=8.3 Hz, H-3), 7.84 (t, 1H, H-2), 7.97 (d, 1H, J=7.3 Hz, H-1). HL-SI-MS *m/z*: C₄₂H₄₈N₅O₁₅ (M+H⁺): 862.3147. Found: 862.3144.

3'-N-(Gly-Gly-Gly-Gly)-DXR·HCl (6c) Compound **4c** (0.488 g, 1.00 mmol) was treated with DXR prepared from its hydrochloride (0.580 g, 1.00 mmol) and NMM (0.101 g, 1.00 mmol), according to the method described for **6a**, to afford **6c** (0.497 g, 62%). [α]_D²⁴ +239.2° (c=0.1, MeOH). ¹H-NMR (CD₃OD) δ: 1.29 (d, 3H, J=6.4 Hz, H-6'), 1.75 (dd, 1H, J=12.7, 3.9 Hz, H-2'), 2.03 (ddd, 1H, J=12.7, 12.7, 3.4 Hz, H-2'), 2.18 (dd, 1H, J=14.7, 3.4 Hz, H-8_a), 2.36 (d, 1H, J=14.7 Hz, H-8_b), 3.04 (d, 1H, J=18.6 Hz, H-10_a), 3.07 (d, 1H, J=18.6 Hz, H-10_b), 3.65 (d, 1H, J=1.5 Hz, H-4'), 3.81 (s, 2H, Gly-αCH₂), 3.86 (s, 2H, Gly-αCH₂), 3.90 (s, 2H, Gly-αCH₂), 3.98 (d, 1H, J=16.6 Hz, Gly-αCH_a), 4.03 (d, 1H, J=16.6 Hz, Gly-αCH_b), 4.04 (s, 3 H, 4-OCH₃), 4.16 (m, 1H, H-3'), 4.28 (q, 1H, J=6.4 Hz, H-5'), 4.74 (d, 1H, J=21.0 Hz, H-14_a), 4.79 (d, 1H, J=21.0 Hz, H-14_b), 5.09 (brs, 1H, H-7), 5.43 (d, 1H, J=3.4 Hz, H-1'), 7.55 (d, 1H, J=8.3 Hz, H-3), 7.83 (t, 1H, H-2), 7.87 (d, 1H, J=7.3 Hz, H-1). HL-SI-MS *m/z*: Calcd for C₃₅H₄₂N₅O₁₅ (M+H⁺): 772.2678. Found: 772.2674.

Synthesis of CMPul (8) Chloroacetic acid (30.0 g) was added to a solution of **7** (10.0 g) (molecular weight, 150 kDa, Hayashibara) in 6N NaOH (100 ml) at 70°C, and the mixture was stirred for 2 h. When the reaction was complete, the solution was adjusted to pH 7.0 with AcOH at 20°C. The solution was poured into MeOH (1000 ml) and the whole was centrifuged. The precipitate, dissolved in deionized H₂O (100 ml), was dialyzed using membrane tubing (molecular weight cut-off, 12–14 kDa, Spectrum) against deionized H₂O for 2 d and lyophilized to give **8** as the sodium salt (8.90 g).

Measurement of Degree of Substitution of CM Groups Compound **8** was dissolved in H₂O, and chromatography of the solution on a column of cation exchange resin (AG 50W-X8, H⁺ form, BIO-RAD) followed by lyophilization gave **8** as a free form. The compound (*s* mg) was dissolved in 0.1 N NaOH (10 ml) and titrated with 0.1 N HCl (*t* ml) using phenolphthalein solution as an indicator. The degree of substitution (*DS*) of CM groups was determined from the following equation.

$$DS = 16.2 \times (10 - t) / [s - 5.8 \times (10 - t)]$$

Synthesis of CMPul-Gly-Gly-Phe-Gly-DXR (9a) A solution of **6a** (220 mg) in DMF-H₂O (1:1) (10 ml) was added to a solution of the sodium salt of **8** (1.00 g) in DMF-H₂O (1:1) (30 ml). EEDQ (1.00 g) was added to the mixture and the whole was stirred for 2 h. Undissolved compound was removed by centrifugation, and the supernatant was poured into ethanol (100 ml). The precipitate was dissolved in deionized H₂O (30 ml) and dialyzed using membrane tubing (molecular weight cut-off 12–14 kDa, Spectrum) against deionized H₂O for 2 d. The solution was passed through a cation exchange resin column (AG 50W-X8, Na⁺ form), dialyzed against deionized H₂O for 2 d, and lyophilized to afford **9a** (0.93 g). Other conjugates (**9b–d**) were prepared via the same procedure.

HPLC of 9a Compound **9a** in 0.1 M NaCl was applied to a column of TSKgel G4000PW_{XL} (300 mm × 7.8 mm i.d., Tosoh) and developed with 0.1 M NaCl at a flow rate of 0.8 ml/min. The system was operated at 40°C. Detection was performed with a Hitachi L-3300 RI monitor and with a Hitachi L-4200 UV-VIS detector at 480 nm.

In Vitro Drug Release in PBS One ml of the sample was mixed with 1 ml of 1 M Na₂CO₃/NaHCO₃ (pH 9.8) and extracted with 2.5 ml of CHCl₃-MeOH (3:1). The organic layer was evaporated and the residue

was dissolved in the following eluent (200 μ l). A 20 μ l aliquot was injected onto a column of Inertsil octadecyl silica (ODS) (150 mm \times 4.6 mm i.d., GL Sciences Inc.) and eluted with 0.035 M HCOOH/HCOONH₄ (pH 3.0)-MeOH (45:55) at a flow rate of 1.0 ml/min. The system was operated at 40 °C. Detection was performed with a Shimadzu RF-535 fluorescence monitor with excitation at 480 nm and emission at 590 nm.

Enzymes A mixture of rat liver lysosomal enzymes was prepared according to the method of Trouet.¹⁷⁾ The activity of the thiol enzyme in lysosomal enzymes was determined by using Z-Arg-Arg-MCA as the substrate according to Barrett and Kirschke.²³⁾ Specific activity was 11.8 U/min per ml of enzyme stock solution.

In Vitro Digestion by Lysosomal Enzymes Each of 9a-d (DXR = 1.8×10^{-5} M) was incubated at 37 °C in 0.2 M citrate buffer (pH 5.5), containing EDTA (1 mM), 0.2% Triton X-100 and reduced glutathione (5 mM), with the enzyme solution (0.6 ml ml⁻¹). At various time intervals, 0.1 ml of the incubation mixture was placed in a propylene tube and MeOH solution (0.1 ml) containing DNR (1.9×10^{-6} M) was added as an internal standard. One ml of H₂O, 1 M Na₂CO₃/NaHCO₃ (pH 9.8) (0.1 ml) and CHCl₃-MeOH (3:1) (2.5 ml) were added and mixed. The organic layer was evaporated and free DXR was determined by HPLC using the method described above.

In Vivo Antitumor Effect Walker carcinosarcoma 256 was inoculated i.m. into female Wistar rats, 6 weeks old, at 1×10^7 cells per rat. CMPul-DXR conjugates and DXR were dissolved in isotonic saline and administered once i.v. at 3 d after inoculation of the tumor. Four days after the drug injection, the excised tumor weight of each treated group was compared with that of the control group.

References and Notes

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