Synthesis and Antitumor Activity of Duocarmycin Derivatives

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A series of duocarmycin B2 derivatives, modified at the phenolic hydroxyl group to ester, carbonate and carbamate, was synthesized. Antitumor activity of these analogs was preliminarily evaluated by assays of growth inhibition of HeLa S3 cells (*in vitro*) and antitumor activity against murine sarcoma 180 (*in vivo*). The stability of the compounds under aqueous conditions was examined, and we found a correlation between antitumor activity *in vivo* and stability in aqueous solution, that is, the more stable derivatives exhibited higher antitumor activity. Among these derivatives, the *N,N*-dialkylcarbamoyl analogs exhibited both improved antitumor activity and higher stability compared with duocarmycin B2. These analogs were subjected to further biological evaluation and they expressed broad-spectrum activity toward murine solid tumors M5076, Colon 26 and Colon 38, and human xenografted carcinoma MX-1.

Key words duocarmycin; carbamoyl derivative; antitumor activity; prodrug; stability

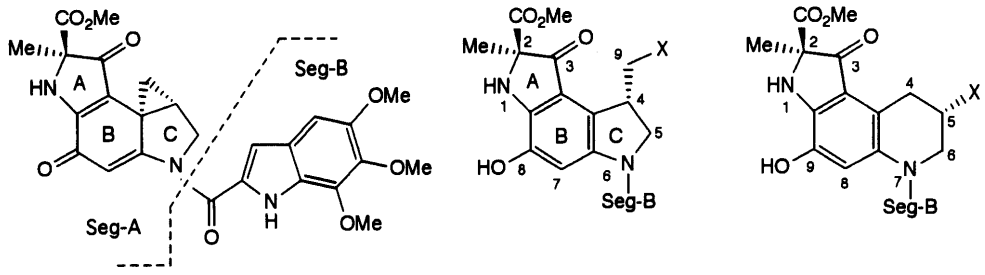
Duocarmycin(DUM)s (A, B1, B2, C1, C2) are novel antitumor antibiotics isolated from the culture broth of *Streptomyces* sp.[2)] The structures of DUMs were confirmed by spectroscopic and chemical analysis.[3)] DUMA (**1a**),[2a)] which is considered as an active form among DUMs, possesses a unique cyclopropane ring and has the ability to alkylate DNA irreversibly.[4)] It alkylates DNA by a mechanism similar to that of CC-1065.[5)] We are interested in synthesizing analogs in order to enhance the antitumor activity, and to broaden the spectrum of DUMs.[6)] Since DUMs are unstable both in aqueous solution and in serum, and decompose to inactive forms through **1a**,[7)] we sought to prevent this decomposition by modifying the phenolic hydroxyl group of DUMB2 (**1c**), that is, by using a prodrug strategy. It is, of course, essential for the success of this strategy that the compounds modified at the phenolic hydroxyl group of **1c** are capable of delivering **1a**. In addition, to improve the solubility of these derivatives, we synthesized a variety of analogs with a hydrophilic moiety on the phenolic hydroxyl group.

In this paper, we describe the synthesis of derivatives modified at the phenolic hydroxyl group of **1c** to ester, carbonate and carbamate, and the evaluation of their antitumor activity and stability under various conditions.

Chemistry

The carboxylic acid esters (**2a—c**, **3a—c**, **4a, b**) with a hydrophilic moiety and the *N*-benzyloxycarbonyl (*Z*)-protected amino acid esters (**5a, b**) of **1c** were prepared in moderate yields by reaction with the corresponding carboxylic acid in methylene chloride in the presence of dicyclohexylcarbodiimide, as depicted in Chart 2. The carboxylic acids with a hydrophilic residue were synthesized by adding the appropriate acid anhydride or acyl chloride to the amine.[8)] In order to obtain the amino acid esters of **1c**, we tried the deprotection reaction of **5a** and **5b**. However, we could not acquire the corresponding amino acid esters of **1c** by hydrogenolysis in various solvents; **1c** was exclusively recovered. It was considered that the amino acid esters of **1c** were unstable, and might be readily hydrolyzed to **1c**. The *N,N*-dialkylcarbamoyl derivatives (**6a—e**) were prepared in good yields by reaction with *p*-nitrophenyl chloroformate in methylene chloride in the presence of triethylamine to afford the carbonate as an intermediate, followed by the addition of various secondary amines. The hydrochloride of **6b** was obtained upon treatment with hydrogen chloride in ethanol. The solubility of this salt (**7**) in water was found to be 10 mg/ml. The reaction of **1c** with isocyanates in methylene chloride in the presence of triethylamine gave the *N*-monoalkyl and *N*-monoarylcarbamoyl derivatives (**8a, b**).

Stability of DUMs and DUMB2 Derivatives under Various Conditions The stability of DUMB2 and DUMB2



DuocarmycinA(DUMA;**1a**) X=Br;DUMB2(**1c**) =Cl;DUMC2(**1e**) =OH;**1f** X=Br;DUMB1(**1b**) =Cl;DUMC1(**1d**) =OH;**1g**

Chart 1

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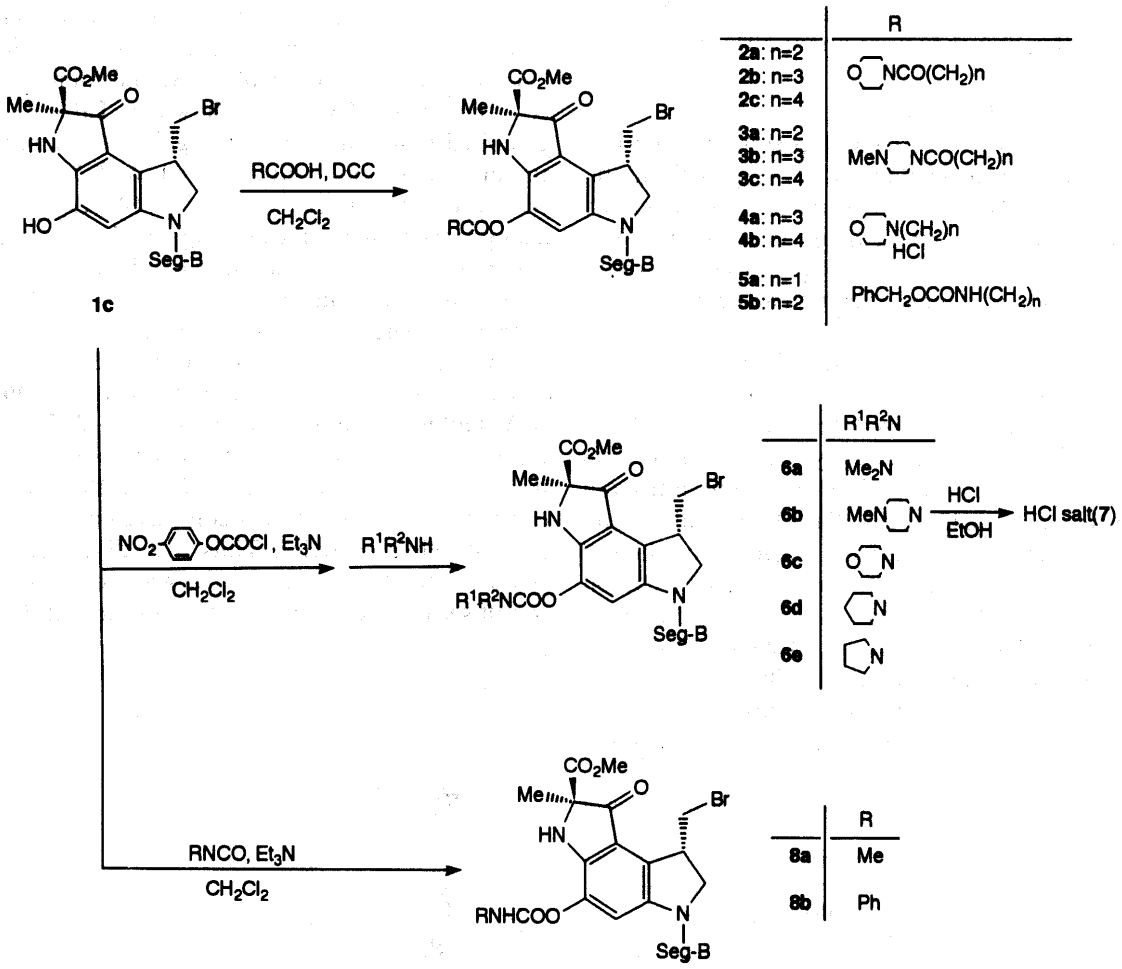

Chart 2

derivatives was measured in a solution containing 20% CH_3CN by HPLC analysis under the following three conditions: in aqueous buffer solution (pH 7), in culture medium that was used for measuring anticellular activity against HeLa S3 and in calf serum. Compound **1c** was readily hydrolyzed to inactive forms (**1f** and **1g**) through **1a** (Fig. 1).[9] With regard to the chemical stability of the DUMB2 derivatives (Table 1), the ester analogs with an amido substituent in the alkyl chain (**2a—c**, **3a—c**) were stable in aqueous solution ($T_{1/2}$; 45—143 h). In contrast, the morpholinylalkyl ester derivatives (**4a, b**) were rather unstable in aqueous solution ($T_{1/2}$; <1 h), being readily hydrolyzed to **1a** through **1c**. This result is consistent with the fact that we could not prepare the amino acid esters of **1c** by deprotection of the *Z*-protecting amino acid esters (**5a, b**). The *N*-monoalkyl and *N*-monoarylcarbamoyl derivatives (**8a, b**) were also very unstable ($T_{1/2}$; <1 h), and they were readily converted to **1a** through **1c**.[10] On the other hand, the *N,N*-dialkylcarbamoyl derivatives (**6a—d**, **7**) were as stable as the ester derivatives (**2a—c**, **3a—c**) in aqueous solution ($T_{1/2}$; 49—102 h).

As mentioned above, the ester derivatives (**2a—c**, **3a—c**) and the *N,N*-dialkylcarbamoyl derivatives (**6a—d**, **7**) were fairly stable in aqueous solution. To examine the stability under the conditions used for measuring biological activity, we measured the stability of the compounds in culture medium and calf serum. As shown in Table 1, the ester derivatives (**2a—c**, **3a—c**) were unstable

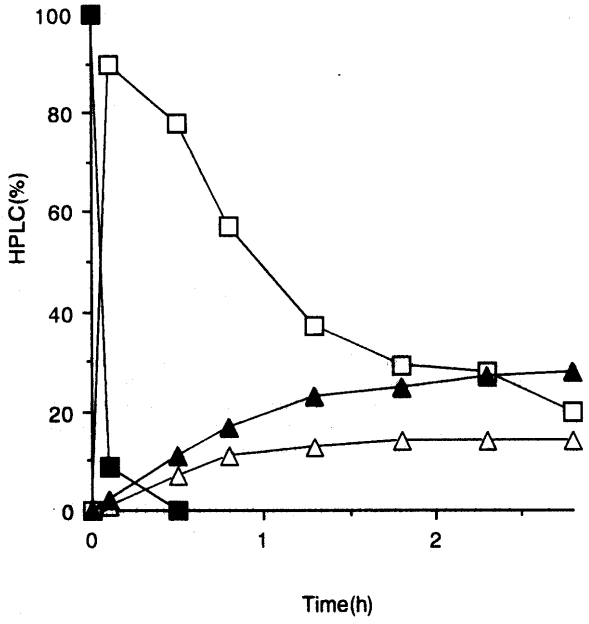

Fig. 1. Stability of DUMB2 in Aqueous Solution

Stability was measured in aqueous buffer solution. DUMB2 (**1c**; ■) was dissolved in 20% acetonitrile–0.01 M phosphate buffer (pH 7), and the solution obtained was kept at 35°C. DUMB2 immediately decomposed to DUMA (**1a**; □), which subsequently gave innactive diols (**1f**; ▲ and **1g**; △). The half life was below 1 h.

and were readily converted under these conditions to **1a**, followed by decomposition. The *N,N*-dialkylcarbamoyl derivatives (**6a—d**, **7**) were about 10 times more stable

than the ester derivatives (**2a—c**, **3a—c**); even after 15 h exposure to calf serum they were about 50% unchanged.

Biological Results and Discussion

The antitumor activity of some representative derivatives was evaluated primarily by assays of growth inhibition against HeLa S3 cells (*in vitro*), and antitumor activity against murine sarcoma 180 (*in vivo*). As shown in Table 1, the efficacy *in vivo* is expressed as T/C, where T and C represent mean tumor volume of treated and control mice, respectively.

The anticellular activity of the ester derivatives (**2a—c**, **3b**) was as potent as that of **1c**. It is considered that these ester derivatives readily released **1c** in culture medium, and indeed their anticellular activity was similar to that of **1c**. *In vivo* they did not exhibit superior antitumor activity to **1c** (T/C; 0.42—0.6). The *N*-monoalkyl and *N*-monoarylcarbamoyl derivatives (**8a, b**) also showed very potent anticellular activity *in vitro*, but did not exhibit superior efficacy to **1c** *in vivo*. In contrast, the *N,N*-dialkylcarbamoyl derivatives (**6a—d, 7**) showed decreased anticellular activity, being about 2×10^2—2×10^3 times inferior to **1c**. They exhibited higher activity than **1c** *in vivo* (T/C; 0.087—0.13). Consequently, the *N,N*-dialkylcarbamoyl derivatives (**6a** and **7**) were selected for further evaluation against several murine solid tumors (M5076, Colon 26 and Colon 38) and human xenografted carcinoma (MX-1). As shown in Table 2, **6a** and **7** exhibited remarkable activity against other murine solid tumors in addition to sarcoma 180. With M5076 sarcoma, their T/C values were 0.088 and 0.064, respectively. They also possessed high efficiency against human xenografted carcinoma MX-1. On the other hand, **1c** was not effective against murine solid tumors, Colon 26 and Colon 38 or human xenografted carcinoma MX-1.

It was considered that difference of activity *in vitro* and *in vivo* between the ester derivatives (**2a—c**, **3b**) and the *N,N*-dialkylcarbamoyl derivatives (**6a—d**, **7**) would correspond to the stability of those compounds. In culture medium, the ester derivatives (**2a—c**, **3b**) were gradually converted to **1a**, which was considered as the active form for anticellular activity. Thus they displayed high anticellular activity *in vitro*. As suggested by the stability in calf serum, they could produce **1a** through **1c**, and then

Table 1. Results of Stability Tests, and Anticellular and Antitumor Activities

Compd.	Stability $T_{1/2}$ (h)[a]			HeLa S_3 IC_{50}[b] (nM)	Sarcoma 180 (s.c.-i.v.)[c]	
	In aqueous solution	In culture medium	In calf serum		mg/kg	T/C[d]
2a	55	6	2	0.54	0.25	0.42
2b	67	3	<1	0.18	0.25	0.49
2c	102	3	1	0.092	0.25	0.6
3a	45	4	1			
3b	143	9	<1	0.25		
3c	119	8	<1			
4a	<1					
6a	75	39	20	56	1.0	0.087
6b	49	23	15	71	1.0	0.12
6c	75	26	18	30	0.5	0.13
6d	102	30	14	6.6	0.5	0.13
7	61	23	15	15	1.0	0.13
8a	1	<1	<1	0.074		
8b	<1	<1	<1	0.016	0.25	0.35
1a	1	<1	<1	0.0058	0.075	0.26
1c	<1	<1	<1	0.028	0.25	0.24

a) Drug concentration was 0.02 mg/ml. See the experimental section. *b*) Drug concentration required to inhibit the growth of HeLa S_3 cells by 50%. *c*) Mice (five mice/group) were implanted subcutaneously (s.c.) with tumor cells, and the drug was dosed (mg/kg) intravenously (i.v.). *d*) T and C are the values of mean tumor volume of treated and control mice, respectively.

Table 2. Antitumor Activities against Murine and Human Xenografted Solid Tumors

Compd.	M5076 (s.c.-i.v.)[a]		Colon 26 (s.c.-i.v.)		Colon 38 (s.c.-i.v.)		MX-1 (s.c.-i.v.)	
	mg/kg	T/C[b]	mg/kg	T/C	mg/kg	T/C	mg/kg	T/C
6a	1.0	0.088	0.5	0.18	0.5	0.15	1.0	0.06
7	1.0	0.064	0.5	0.12	N.T.		1.0	0.10
1c	N.T.		0.13	0.54	0.13	0.59	0.16	0.46
MMC	6.0	0.065	6.0	0.18	6.0	0.062	4.25	0.004

a) Mice (five mice/group) were implanted subcutaneously (s.c.) with tumor cells, and the drug was dosed (mg/kg) intravenously (i.v.). *b*) T and C are the values of mean tumor volume of treated and control mice, respectively. N.T., not tested.

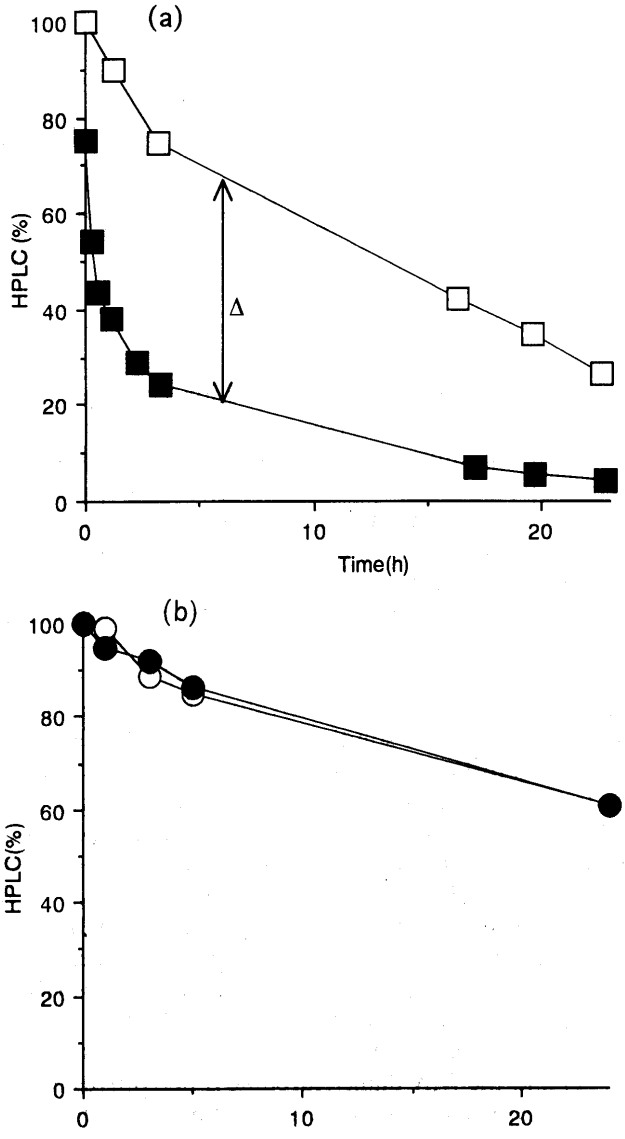

Fig. 2. Interaction of **1a** and **6a** with Calf Thymus DNA

(a) Time course of disappearance of compound **1a** in the presence of DNA (■) and in the absence of DNA (□). They were measured in 0.01 M phosphate buffer (pH 7) containing 10% DMF at 35°C. (b) Interaction with DNA of compound **6a** (●) and its control (○).

readily decomposed to inactive forms. As a result, they could not maintain a sufficient concentration of the active form *in vivo*. They did not exhibit superior antitumor activity to **1c**.

On the other hand, the *N,N*-dialkylcarbamoyl derivatives (**6a—d**, **7**) showed decreased anticellular activity *in vitro*, because it could not produce **1a** through **1c** in culture medium or calf serum. We also established a simple method of investigating by HPLC whether the drug interacted directly with calf thymus DNA.[11] As shown in Fig. 2, **1a** disappeared faster in the presence of calf thymus DNA than in its absence, while the rate of disappearance of **6a** was not influenced by DNA. Accordingly, **6a** had no direct interaction with calf thymus DNA, and showed very weak anticellular activity. However the compounds exhibited promising antitumor activity *in vivo*. It seemed that the carbamoyl residue would be hydrolyzed by an enzyme (esterase or protease) *in vivo* and the derivatives would gradually produce **1a**. More detailed research is necessary to clarify whether the *N,N*-dialkylcarbamoyl derivatives are hydrolyzed enzymatically *in vivo*.

As described above, the stability and anticellular and antitumor activity of DUMB2 derivatives were examined under several conditions. These results indicated a correlation between stability and antitumor activity, and suggested that DUMB2 derivatives would require suitable stability in serum in order to exhibit excellent antitumor activity *in vivo*. The *N,N*-dialkylcarbamoyl derivatives showed both promising antitumor activity and pronounced stability.

Experimental

All melting points were measured on a Yanagimoto micro melting point apparatus without correction. Infrared spectra (IR) were recorded on a JASCO IR-810; ^{1}H- and ^{13}C-NMR spectra were measured on Varian EM-390, JEOL FX-100, and Bruker AM-400 spectrometers. Chemical shifts were reported in parts per million (ppm) downfield from tetramethylsilane. Mass spectra were measured with a Hitachi B-80. For column chromatography, silica gel (SiO_2, Wako C-200) was used. Analytical thin-layer chromatography (TLC) was performed on Silica gel $60F_{254}$ plates (Merck). All organic solvent extracts were dried over anhydrous sodium sulfate. Substituted carboxylic acids were prepared by literature procedures.[8] (3-(4-Morpholinylcarbonyl)propionic acid, 4-(4-morpholinylcarbonyl)butyric acid, 5-(4-morpholinylcarbonyl)valeric acid, 3-(4-methyl-1-piperazinylcarbonyl)propionic acid, 4-(4-methyl-1-piperazinylcarbonyl)butyric acid, 5-(4-methyl-1-piperazinylcarbonyl)-valeric acid, 4-(4-morpholinyl)butyric acid hydrochloride and 5-(4-morpholinyl)valeric acid hydrochloride).

Typical Procedures for the Preparation of the Carboxylic Acid Esters of DUMB2 (2a—c, 3a—c, 5a, b). 8-*O*-[3-(4-Morpholinylcarbonyl)propionyl]DUMB2 (2a) Dicyclohexylcarbodiimide (79 mg, 0.38 mmol) was added to a solution of DUMB2 (150 mg, 0.25 mmol) in methylene chloride (10 ml), and the mixture was stirred at 0 °C for 0.5 h. Then a solution of 3-(4-morpholinylcarbonyl)propionic acid (0.5 g, 2.67 mmol) in methylene chloride (5 ml) was added, and stirring was continued at room temperature for 18 h. The reaction mixture was filtered, then water was added to the filtrate, and the mixture was extracted with methylene chloride twice. The combined extracts were washed with 1 N HCl, aqueous $NaHCO_3$ and brine. The organic layer was dried and concentrated under reduced pressure. AcOEt was added to the residue, and the mixture was stirred at 0 °C for 1 h. A small amount of precipitate (dicyclohexylurea) was removed by filtration, and then the filtrate was concentrated under reduced pressure. The residue was subjected to column chromatography ($CHCl_3$–MeOH, 50 : 1) to give 80 mg (42%) of **2a** as a pale yellow solid. mp 123—126 °C (AcOEt/*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.63 (3H, s, CH_3), 2.86 (4H, m, CH_2–CO × 2), 3.51 (2H, m, N–CH_2), 3.61 (3H, m, N–CH_2, 9-H), 3.69 (5H, m, O–CH_2 × 2, 9-H), 3.71 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.08 (3H, s, OCH_3), 4.22 (1H, m, 4-H), 4.56 (1H, dd, J=10.8, 4.5 Hz, 5-H), 4.62 (1H, br d, J=9.4 Hz, 5-H), 6.70 (1H, br s, NH), 6.87 (1H, s, 4′-H), 6.94 (1H, d, J=2.3 Hz, 3′-H), 8.39 (1H, s, 7-H), 9.32 (1H, br s, NH). IR (KBr): 2934, 1745, 1696, 1627, 1495, 1435, 1307, 1219, 1115, 1033 cm^{-1}. SIMS *m/z*: 759 757 $(M+1)^+$, 590 588, 234. *Anal.* Calcd for $C_{34}H_{37}BrN_4O_{11}\cdot 2H_2O$: C, 51.46; H, 5.21; N, 7.06. Found: C, 51.48; H, 5.5; N, 6.74.

8-*O*-[4-(4-Morpholinylcarbonyl)butyryl]DUMB2 (2b) Yield: 50% (a pale yellow solid). mp 118—119 °C (AcOEt/*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.67 (3H, s, CH_3), 2.13 (2H, m, CH_2), 2.52 (2H, t, J=6.4 Hz, CH_2–CO), 2.75 (2H, t, J=6.4 Hz, CH_2–CO), 3.49 (2H, m, N–CH_2), 3.62 (3H, m, N–CH_2, 9-H), 3.67 (5H, m, O–CH_2 × 2, 9-H), 3.75 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.08 (3H, s, OCH_3), 4.26 (1H, m, 4-H), 4.57 (1H, dd, J=10.8, 4.6 Hz, 5-H), 4.63 (1H, br d, J=9.3 Hz, 5-H), 6.10 (1H, br s, NH), 6.87 (1H, s, 4′-H), 6.95 (1H, d, J=2.3 Hz, 3′-H), 8.43 (1H, s, 7-H), 9.33 (1H, br s, NH). IR (KBr): 2938, 1746, 1700, 1618, 1558, 1495, 1429, 1388, 1308, 1114, 1034 (cm^{-1}). SIMS *m/z*: 773 771 $(M+1)^+$, 590 588, 234. *Anal.* Calcd for $C_{35}H_{39}BrN_4O_{11}$: C, 54.48; H, 5.09; N, 7.26. Found: C, 54.37; H, 5.48; N, 7.41.

8-*O*-[5-(4-Morpholinylcarbonyl)valeryl]DUMB2 (2c) Yield: 36% (a pale yellow solid). mp 105—110 °C (AcOEt/*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.66 (3H, s, CH_3), 1.84 (4H, m, CH_2 × 2), 2.39 (2H, t, J=6.4 Hz, CH_2–CO), 2.68 (2H, t, J=6.5 Hz, CH_2–CO), 3.49 (2H, m, N–CH_2), 3.62 (3H, m, N–CH_2, 9-H), 3.69 (5H, m, O–CH_2, 9-H), 3.73 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.08 (3H, s, OCH_3), 4.23 (1H, m, 4-H), 4.57 (1H, dd, J=10.8, 4.6 Hz, 5-H), 4.63 (1H, br d, J=9.3 Hz, 5-H), 6.18 (1H, br s, NH), 6.87 (1H, s, 4′-H), 6.95 (1H, d, J=2.3 Hz, 3′-H), 8.46 (1H, s, 7-H), 9.32 (1H, br s, NH). IR (KBr): 2936, 1743, 1617, 1520, 1493, 1387, 1306, 1234, 1114 cm^{-1}. SIMS *m/z*: 787 785 $(M+1)^+$, 590 588, 234. *Anal.* Calcd for $C_{36}H_{41}BrN_4O_{11}$: C, 55.04; H, 5.26; N, 7.13. Found: C, 54.93; H, 5.47; N, 6.98.

8-*O*-[3-(4-Methyl-1-piperazinylcarbonyl)propionyl]DUMB2 (3a) Yield: 64% (a pale yellow powder). mp 135—138 °C (dec.). ^{1}H-NMR (CD_3OD) δ: 1.57 (3H, s, CH_3), 2.37 (3H, s, N–CH_3), 2.59 (4H, m, N–CH_2 × 2), 2.66 (4H, m, CH_2–CO × 2), 3.62 (4H, m, N–CH_2 × 2), 3.68 (3H, s, OCH_3), 3.83 (1H, dd, J=10.1, 7.1 Hz, 9-H), 3.87 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.94 (1H, dd, J=10.1, 3.2 Hz, 9-H), 4.04 (3H, s, OCH_3), 4.19 (1H, m, 4-H), 4.51 (1H, dd, J=11.0, 4.1 Hz, 5-H), 4.68 (1H, dd, J=11.0, 9.6 Hz, 5-H), 5.47 (1H, br s, NH), 6.96 (1H, s, 4′-H), 7.04 (1H, d, J=2.3 Hz, 3′-H), 8.25 (1H, s, 7-H), 8.49 (1H, br s, NH). IR (KBr): 2936, 1738, 1617, 1539, 1458, 1411, 1378, 1305, 1221, 1117, 1023 cm^{-1}. SIMS *m/z*: 772 770 $(M+1)^+$, 690, 234. *Anal.* Calcd for $C_{35}H_{40}BrN_5O_{10}\cdot H_2O$: C, 53.3; H, 5.37; N, 8.88. Found: C, 53.35; H, 5.43; N, 8.67.

8-*O*-[4-(4-Methyl-1-piperazinylcarbonyl)butyryl]DUMB2 (3b) Yield: 91% (a light-tan powder). mp 110—112 °C (AcOEt/*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.67 (3H, s, CH_3), 2.13 (2H, m, CH_2), 2.41 (2H, m, CO–CH_2), 2.42 (3H, s, N–CH_3), 2.54 (4H, m, N–CH_2, CO–CH_2), 2.75 (2H, m, N–CH_2), 3.71 (3H, m, N–CH_2, 9-H), 3.75 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.08 (3H, s, OCH_3), 4.23 (1H, m, 4-H), 4.57 (1H, dd, J=10.8, 4.6 Hz, 5-H), 4.62 (1H, br d, J=9.1 Hz, 5-H), 6.27 (1H, br s, NH), 6.87 (1H, s, 4′-H), 6.95 (1H, d, J=2.3 Hz, 3′-H), 8.42 (1H, s, 7-H), 9.33 (1H, br s, NH). IR (KBr): 2938, 1734, 1614, 1526, 1460, 1440, 1369, 1304, 1264, 1115 cm^{-1}. SIMS *m/z*: 786 784 $(M+1)^+$, 234. *Anal.* Calcd for $C_{36}H_{42}BrN_5O_{10}\cdot 0.5H_2O$: C, 54.48; H, 5.46; N, 8.82. Found: C, 54.44; H, 5.78; N, 8.43.

8-*O*-[5-(4-Methyl-1-piperazinylcarbonyl)valeryl]DUMB2 (3c) Yield: 47% (a light-tan powder). mp 115—116 °C. ^{1}H-NMR (CD_3OD) δ: 1.57 (3H, s, CH_3), 1.82 (4H, m, CH_2 × 2), 2.38 (3H, s, N–CH_3), 2.58 (4H, m, CO–CH_2), 2.68 (4H, br s, N–CH_2), 3.62 (5H, m, N–CH_2, 9-H), 3.68 (3H, s, OCH_3), 3.70 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 3.95 (1H, br d, J=7.5 Hz, 9-H), 4.05 (3H, s, OCH_3), 4.23 (1H, m, 4-H), 4.52 (1H, dd, J=10.8, 4.6 Hz, 5-H), 4.68(1H, br d, J=9.1 Hz, 5-H), 5.47 (1H, br s, NH), 6.98 (1H, d, J=2.7 Hz, 4′-H), 7.04 (1H, s, 3′-H), 8.25 (1H, s, 7-H), 8.49 (1H, br s, NH). IR (KBr): 2938, 1734, 1614, 1526, 1460, 1440, 1369, 1304, 1264, 1115, 995 cm^{-1}. SIMS *m/z*: 800 798 $(M+1)^+$, 234. *Anal.* Calcd for $C_{37}H_{44}BrN_5O_{10}$: C, 55.64; H, 5.55; N, 8.77. Found: C, 55.56; H, 5.26; N, 8.43.

8-*O*-(N-Benzyloxycarbonyl glycyl)DUMB2 (5a) Yield: 98% (a pale yellow powder). mp 122—123 °C (AcOEt/*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.68 (3H, s, CH_3), 3.62 (1H, dd, J=9.3, 9.3 Hz, 9-H), 3.75 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.04 (1H, dd, J=9.3, 3.2 Hz, 9-H), 4.08 (3H, s, OCH_3), 4.26 (2H, m, 5-H, 4-H), 4.58 (1H, dd, J=10.7, 4.6 Hz, 5-H), 4.64 (2H, m, CO–CH_2), 5.15 (1H, d, J=16.1 Hz,

Ar–$\underline{CH_2}$), 5.19 (1H, d, J=16.1 Hz, Ar–$\underline{CH_2}$), 5.68 (1H, br s, NH), 6.87 (1H, s, 4'-H), 6.95 (1H, d, J=2.3 Hz, 3'-H), 7.35 (5H, m, Ar-H), 8.47 (1H, s, 7-H), 9.32 (1H, br s, NH). IR (KBr): 2938, 1734, 1614, 1526, 1460, 1440, 1369, 1304, 1264, 1115, 995 cm^{-1}. SIMS m/z: 781 779 $(M+1)^+$, 590 588, 234. *Anal.* Calcd for $C_{36}H_{35}BrN_4O_{11}\cdot H_2O$: C, 54.21; H, 4.68; N, 7.02. Found: C, 54.42; H, 4.64; N, 6.74.

8-*O*-(*N*-Benzyloxycarbonyl-β-alanyl)DUMB2 (5b) Yield: 89% (a pale yellow powder). mp 112—114 °C (AcOEt/*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.67 (3H, s, CH_3), 2.87 (2H, t, J=5.8 Hz, CO–CH_2), 3.62 (1H, dd, J=10.0, 9.0 Hz, 9-H), 3.63 (2H, m, NH–$\underline{CH_2}$), 3.75 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.04 (1H, dd, J=10.0, 3.5 Hz, 9-H), 4.08 (3H, s, OCH_3), 4.25 (1H, m, 4-H), 4.58 (1H, dd, J=10.9, 4.5 Hz, 5-H), 4.65 (1H, dd, J=10.9, 10.9 Hz, 5-H), 5.12 (2H, d, J=13.6 Hz, Ar–$\underline{CH_2}$), 5.87 (1H, br s, NH), 6.87 (1H, s, 4'-H), 6.95 (1H, d, J=2.3 Hz, 3'-H), 7.34 (5H, m, Ar-H), 8.48 (1H, s, 7-H), 9.30 (1H, br s, NH). IR (KBr): 2938, 1734, 1614, 1526, 1460, 1440, 1369, 1304, 1264, 1115, 995 cm^{-1}. SIMS m/z: 795 793 $(M+1)^+$, 590 588, 234. *Anal.* Calcd for $C_{37}H_{37}BrN_4O_{11}\cdot H_2O$: C, 54.75; H, 4.84; N, 6.90. Found: C, 54.71; H, 4.78; N, 6.57.

Typical Procedures for the Preparation of the Amino Acid Esters of DUMB2 (4a, b). 8-*O*-[4-(4-Morpholinyl)butyryl]DUMB2 hydrochloride (4a) Dicyclohexylcarbodiimide (16 mg, 0.078 mmol) was added to a solution of DUMB2 (30 mg, 0.051 mmol) in methylene chloride (3 ml), and the mixture was stirred at 0 °C for 0.5 h. Then 4-(4-morphilinyl)-butyric acid hydrochloride (16 mg, 0.076 mmol) was added, and stirring was continued at room temperature for 18 h. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. To the residue was added AcOEt, and the mixture was stirred at 0 °C for 1 h. A small amount of precipitate (dicyclohexylurea) was removed by filtration, then the filtrate was concentrated under reduced pressure. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (5 : 1) to give 27.9 mg (74%) of **4a** as a white crystalline compound. mp 140—146 °C (2-PrOH). ^{1}H-NMR (DMSO-d_6) δ: 1.49 (3H, s, CH_3), 1.78 (2H, m, CH_2), 2.79 (2H, br s, CO–CH_2), 2.95 (6H, br s, N–$CH_2\times3$), 3.09 (1H, br s, 9-H), 3.63 (3H, s, OCH_3), 3.79 (5H, m, $OCH_2\times2$, 9-H), 3.80 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.01 (1H, br s, NH), 4.19 (1H, m, 4-H), 4.34 (1H, dd, J=10.4, 5.7 Hz, 5-H), 4.68 (1H, dd, J=10.4, 10.4 Hz, 5-H), 6.98 (1H, s, 4'-H), 7.00 (1H, d, J=2.0 Hz, 3'-H), 7.88 (1H, s, 7-H), 11.32 (1H, br s, NH). IR (KBr): 2941, 1745, 1652, 1620, 1529, 1490, 1386, 1306, 1110 cm^{-1}. SIMS m/z: 745 743 $(M+1)^+$, 590 588, 234. *Anal.* Calcd for $C_{34}H_{39}BrN_4O_{10}\cdot HCl\cdot H_2O$: C, 51.17; H, 5.30; N, 7.02. Found: C, 50.98; H, 5.7; N, 6.96.

8-*O*-[5-(4-Morpholinyl)valeryl]DUMB2 Hydrochloride (4b) Yield: 35% (white crystals). mp 149—150 °C (2-PrOH). ^{1}H-NMR (DMSO-d_6) δ: 1.50 (3H, s, CH_3), 1.71 (4H, br s, $CH_2\times2$), 2.34 (2H, br s, CO–CH_2), 2.73 (6H, br s, N–$CH_2\times3$), 3.30 (1H, br s, 9-H), 3.64 (3H, s, OCH_3), 3.65 (5H, m, $OCH_2\times2$, 9-H), 3.80 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 4.03 (1H, br s, NH), 4.20 (1H, m, 4-H), 4.35 (1H, dd, J=10.3, 4.3 Hz, 5-H), 4.69 (1H, dd, J=10.3, 10.3 Hz, 5-H), 6.97 (1H, s, 4'-H), 7.01 (1H, d, J=1.9 Hz, 3'-H), 7.85 (1H, s, 7-H), 11.35 (1H, br s, NH). IR (KBr): 2930, 1743, 1652, 1612, 1520, 1489, 1387, 1306, 1112 cm^{-1}. SIMS m/z: 759 757 $(M+1)^+$, 677, 590 588, 234. *Anal.* Calcd for $C_{35}H_{41}BrN_4O_{10}\cdot HCl\cdot 0.2H_2O$: C, 52.70; H, 5.36; N, 7.02. Found: C, 52.75; H, 5.74; N, 6.89.

Typical Procedures for the Preparation of the *N*,*N*-Dialkylcarbamoyl Derivatives of DUMB2 (6a—e and 7). I. 8-*O*-(4-Methyl-1-piperazinyl-carbamoyl)DUMB2 (6b) *p*-Nitrophenyl chloroformate (26 mg, 0.13 mmol) and triethylamine (0.014 ml, 0.1 mmol) were added to a solution of DUMB2 (30 mg, 0.051 mmol) in dry methylene chloride (5 ml) under cooling at 0 °C. The mixture was stirred at 0 °C for 1 h. Then 4-methylpiperazine (0.017 ml, 0.15 mmol) was added, and stirring was continued at 0 °C for 2 h. The mixture was diluted with $CHCl_3$ and washed with aqueous $NaHCO_3$ and brine. The organic layer was dried and concentrated under reduced pressure. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (50 : 1) to give 35 mg (96%) of **6b** as a light-tan powder. mp 185—190 °C (dec., AcOEt/*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.69 (3H, s, CH_3), 2.37 (3H, s, N–CH_3), 2.52 (4H, br s, N–$CH_2\times2$), 3.61 (1H, dd, J=10.1, 8.9 Hz, 9-H), 3.64 (2H, br s, N–CH_2), 3.76 (2H, br s, N–CH_2), 3.78 (3H, s, OCH3), 3.92 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.04 (1H, dd, J=10.1, 3.3 Hz, 9-H), 4.08 (3H, s, OCH_3), 4.25 (1H, m, 4-H), 4.59 (1H, dd, J=10.8, 4.5 Hz, 5-H), 4.63 (1H, br d, J=9.3 Hz, 5-H), 5.48 (1H, br s, NH), 6.87 (1H, s, 4'-H), 6.94 (1H, d, J=2.3 Hz, 3'-H), 8.45 (1H, s, 7-H), 9.32 (1H, br s, NH). IR (KBr): 2940, 1710, 1621, 1521, 1493, 1430, 1385, 1289, 1231, 1049, 1002 cm^{-1}. SIMS m/z: 716 714 $(M+1)^+$, 482 480, 234. *Anal.* Calcd for $C_{32}H_{36}BrN_5O_9\cdot H_2O$: C, 52.47; H, 5.23; N, 9.56. Found: C, 52.42; H, 5.37; N, 9.17.

8-*O*-(Dimethylcarbamoyl)DUMB2 (6a) Yield: 87% (a pale yellow powder). mp 125—129 °C (dec.). ^{1}H-NMR ($CDCl_3$) δ: 1.68 (3H, s, CH_3), 3.06 (3H, s, N–CH_3), 3.16 (3H, s, N–CH_3), 3.60 (1H, dd, J=10.1, 8.9 Hz, 9-H), 3.78 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.05 (1H, dd, J=10.1, 3.4 Hz, 9-H), 4.08 (3H, s, OCH_3), 4.25 (1H, m, 4-H), 4.59 (1H, dd, J=10.8, 4.7 Hz, 5-H), 4.64 (1H, dd, J=10.8, 9.1 Hz, 5-H), 5.53 (1H, br s, NH), 6.87 (1H, s, 4'-H), 6.96 (1H, d, J=2.5 Hz, 3'-H), 8.44 (1H, s, 7-H), 9.35 (1H, br s, NH). IR (KBr): 1717, 1619, 1495, 1308, 1164 cm^{-1}. SIMS m/z: 661 659 $(M+1)^+$, 234. *Anal.* Calcd for $C_{29}H_{31}BrN_4O_9$: C, 52.82; H, 4.74; N, 8.50. Found: C, 52.98; H, 5.07; N, 8.50.

8-*O*-(Morpholinylcarbamoyl)DUMB2 (6c) Yield: 84% (a light-tan powder). mp 145—146 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.69 (3H, s, CH_3), 3.61 (2H, br s, N–CH_2), 3.62 (1H, dd, J=10.1, 4.4 Hz, 9-H), 3.73 (2H, br s, N–CH_2), 3.77 (4H, m, OCH_2), 3.78 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.04 (1H, dd, J=10.1, 3.3 Hz, 9-H), 4.08 (3H, s, OCH_3), 4.26 (1H, m, 4-H), 4.59 (1H, dd, J=10.7, 4.4 Hz, 5-H), 4.63 (1H, br d, J=9.3 Hz, 5-H), 5.47 (1H, br s, NH), 6.87 (1H, s, 4'-H), 6.96 (1H, d, J=2.3 Hz, 3'-H), 8.46 (1H, s, 7-H), 9.31 (1H, br s, NH). IR (KBr): 2940, 1714, 1616, 1520, 1494, 1429, 1386, 1306, 1231, 1115, 1051 cm^{-1}. SIMS m/z: 703 701 $(M+1)^+$, 469 467, 234. *Anal.* Calcd for $C_{31}H_{33}BrN_4O_{10}$: C, 53.08; H, 4.74; N, 7.99. Found: C, 52.9; H, 5.05; N, 7.7.

8-*O*-(Piperidinylcarbamoyl)DUMB2 (6d) Yield: 75% (a pale yellow powder). mp 137—138 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.68 (3H, s, CH_3), 2.27 (6H, m, $CH_2\times3$), 3.54 (2H, br s, N–CH_2), 3.60 (1H, dd, J=10.1, 9.0 Hz, 9-H), 3.66 (2H, br s, N–CH_2), 3.78 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.04 (1H, dd, J=10.1, 3.4 Hz, 9-H), 4.08 (3H, s, OCH_3), 4.26 (1H, m, 4-H), 4.58 (1H, dd, J=10.8, 4.5 Hz, 5-H), 4.62 (1H, br d, J=9.2 Hz, 5-H), 5.52 (1H, br s, NH), 6.87 (1H, s, 4'-H), 6.96 (1H, d, J=2.3 Hz, 3'-H), 8.44 (1H, s, 7-H), 9.33 (1H, br s, NH). IR (KBr): 2940, 1702, 1621, 1523, 1494, 1429, 1387, 1306, 1228, 1141, 1049 cm^{-1}. SIMS m/z: 701 699 $(M+1)^+$, 234. *Anal.* Calcd for $C_{32}H_{35}BrN_4O_9$: C, 54.94; H, 5.04; N, 8.01. Found: C, 54.8; H, 5.26; N, 7.85.

8-*O*-(Pyrrolidinylcarbamoyl)DUMB2 (6e) Yield: 87% (a pale yellow powder). mp 148—149 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.68 (3H, s, CH_3), 1.98 (4H, m, $CH_2\times2$), 3.52 (2H, t, J=6.7 Hz, N–CH_2), 3.60 (1H, dd, J=10.1, 9.9 Hz, 9-H), 3.63 (2H, t, J=6.7 Hz, N–CH_2), 3.77 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.05 (1H, dd, J=10.1, 3.4 Hz, 9-H), 4.08 (3H, s, OCH_3), 4.25 (1H, m, 4-H), 4.58 (1H, dd, J=10.4, 4.5 Hz, 5-H), 4.64 (1H, br d, J=9.4 Hz, 5-H), 5.59 (1H, br s, NH), 6.87 (1H, s, 4'-H), 6.95 (1H, d, J=2.4 Hz, 3'-H), 8.45 (1H, s, 7-H), 9.32 (1H, br s, NH). IR (KBr): 2941, 1705, 1616, 1539, 1521, 1492, 1386, 1306, 1166, 1109, 1051 cm^{-1}. SIMS m/z: 687 685 $(M+1)^+$, 453 451, 234. *Anal.* Calcd for $C_{31}H_{33}BrN_4O_9\cdot 0.5H_2O$: C, 53.61; H, 4.93; N, 8.07. Found: C, 54.00; H, 5.11; N, 7.73.

II. 8-*O*-(4-Methyl-1-piperazinylcarbamoyl)DUMB2 Hydrochloride (7) A solution of **6b** (29 mg, 0.041 mmol) in ethanol (1.5 ml) was treated with anhydrous 5.8 N HCl in ethanol (0.01 ml) at 0 °C for 1 h. The resulting mixture was poured into diethyl ether (30 ml) and the whole was vigorously stirred at 0 °C for 1 h. The resulting precipitate was collected by filtration and dried under reduced pressure to give 16.3 mg (53%) of **7** as a bright yellow crystalline compound. mp 183—185 °C (EtOH). ^{1}H-NMR (DMSO-d_6) δ: 1.50 (3H, s, CH_3), 2.83 (3H, s, N–CH_3), 3.20 (2H, br s, N–CH_2), 3.48 (2H, br s, N–CH_2), 3.79 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.90 (1H, dd, J=10.0, 6.7 Hz, 9-H), 3.93 (6H, s, OCH_3), 3.97 (1H, dd, J=10.0, 3.1 Hz, 9-H), 4.18 (2H, br s, N–CH_2), 4.20 (1H, m, 4-H), 4.34 (1H, dd, J=10.4, 4.4 Hz, 5-H), 4.38 (2H, br s, N–CH_2), 4.68 (1H, dd, J=10.4, 10.4 Hz, 5-H), 6.96 (1H, s, 4'-H), 7.00 (1H, d, J=2.2 Hz, 3'-H), 7.83 (1H, s, NH), 8.30 (1H, br s, HCl), 10.40 (1H, br s, 7-H), 11.29 (1H, br s, NH). IR (KBr): 2930, 1718, 1617, 1522, 1492, 1457, 1430, 1387, 1308, 1234, 1170, 1109 cm^{-1}. *Anal.* Calcd for $C_{32}H_{36}BrN_5O_9\cdot HCl\cdot 0.5H_2O$: C, 50.57; H, 5.04 N, 9.21. Found: C, 50.28; H, 4.71; N, 9.30.

Typical Procedures for the Preparation of the *N*-Monoalkyl and *N*-Monoarylcarbamoyl Derivatives of DUMB2 (8a, b). 8-*O*-(*N*-Phenylcarbamoyl)DUMB2 (8b) Phenyl isocyanate (0.009 ml, 0.083 mmol) and triethylamine (0.012 ml, 0.086 mmol) were added to a solution of DUMB2 (20 mg, 0.034 mmol) in dry methylene chloride (4 ml) at 0 °C. The reaction mixture was stirred for 1 h, then poured into 1 N HCl and

extracted with $CHCl_3$. The extract was washed with aqueous $NaHCO_3$ and brine. The organic layer was dried and concentrated under reduced pressure. The residue was chromatographed on silica gel with *n*-hexane–AcOEt (1 : 1) to give 19.7 mg (82%) of **8b** as a pale yellow powder. mp 147—150 °C (AcOEt/*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.69 (3H, s, CH_3), 3.61 (1H, dd, J=10.0, 9.0 Hz, 9-H), 3.78 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.04 (1H, dd, J=10.0, 3.5 Hz, 9-H), 4.07 (3H, s, OCH_3), 4.24 (1H, m, 4-H), 4.58 (1H, dd, J=10.7, 4.5 Hz, 5-H), 4.62 (1H, br d, J=9.4 Hz, 5-H), 5.44 (1H, s, NH), 6.87 (1H, s, 4′-H), 6.95 (1H, d, J=2.3 Hz, 3′-H), 7.15 (2H, m, Ph–H), 7.36 (2H, m, Ph-H), 7.45 (1H, m, Ph-H), 8.56 (1H, s, 7-H), 9.37 (1H, br s, NH). IR (KBr): 2941, 1734, 1602, 1524, 1442, 1388, 1308, 1193, 1040 cm^{-1}. SIMS *m/z*: 709 707 $(M+1)^+$, 234. *Anal.* Calcd for $C_{33}H_{31}BrN_4O_9 \cdot 0.5H_2O$: C, 55.32; H, 4.50; N, 7.82. Found: C, 55.13; H, 4.63; N, 7.58.

8-*O*-(*N*-Methylcarbamoyl)DUMB2 (8a) Yield: 62% (a pale yellow powder). mp 125—129 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.68 (3H, s, CH_3), 2.86 (1H, s, NH), 2.96 (3H, s, N–CH_3), 3.59 (1H, dd, J=10.1, 9.1 Hz, 9-H), 3.78 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.05 (1H, dd, J=10.1, 3.4 Hz, 9-H), 4.08 (3H, s, OCH_3), 4.24 (1H, m, 4-H), 4.58 (1H, dd, J=10.7, 4.4 Hz, 5-H), 4.62 (1H, br d, J=9.4 Hz, 5-H), 5.44 (1H, s, NH), 6.86 (1H, s, 4′-H), 6.95 (1H, d, J=2.3 Hz, 3′-H), 8.46 (1H, s, 7-H), 9.32 (1H, br s, NH). IR (KBr): 2936, 1735, 1615, 1589, 1490, 1418, 1386, 1306, 1232, 1110 cm^{-1}. SIMS *m/z*: 647 645 $(M+1)^+$, 590 588, 234. *Anal.* Calcd for $C_{28}H_{29}BrN_4O_9$: C, 52.1; H, 4.53; N, 8.68. Found: C, 52; H, 4.47; N, 8.55.

Stability of Drug in Aqueous Solution, Culture Medium and Calf Serum The stability of the DUMB2 derivatives under various conditions was examined by chromatography on a UNISIL pack 5C18 reversed-phase HPLC column (GL Science, Co., Ltd., Tokyo, Japan). The compound (1 mg) was dissolved in acetonitrile (10 ml). This solution (2 ml) was diluted with aqueous solution or culture medium or calf serum (each 8 ml). Aqueous solution and culture medium were composed of 0.01 M phosphate buffer (pH 7) and Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Grand Island Biological Co.), respectively. The resulting solution was incubated at 35 °C. Samples were removed at intervals and injected directly into the HPLC injection port. The compound was eluted with 0.05 M phosphate buffer (pH 5.9)–acetonitrile (30 : 70) and detected by measuring absorbance at 330 nm.

Interaction of 1a and 6a with Calf Thymus DNA Calf thymus DNA from Sigma (16.1 mg) was dissolved in 0.01 M phosphate buffer (pH 7, 50 ml). The examined compounds were dissolved in *N*,*N*-dimethylformamide (DMF) to give 6.10×10^{-4} M drug concentration. To this drug solution (9 ml) was added the buffer solution (1 ml) containing calf thymus DNA (or no DNA as the control), and the mixture was kept at 35 °C. Samples were removed at intervals and injected directly into the HPLC injection port. Analytical conditions were as for the stability studies.

Biological Studies HeLa S3 cells (5×10^4) were seeded in Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Grand Island Biological Co.) and 0.06 mg/ml of kanamycin. Graded concentrations of drugs, appropriately diluted with growth medium, were added 24 h after the cells were seeded. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2. After 72 h of drug exposure, the monolayer cells were washed with phosphate-buffered salts solution (Flow Laboratories) and incubated with 0.05% trypsin (Difco Laboratories, Detroit, MI) and 0.02% EDTA (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan). The cells were counted with a Toa Micro-Cell counter (Toa Medical Electronics Co., Ltd., Kobe, Japan) and the IC_{50} value (drug concentration required for 50% inhibition of the cell growth) was determined. Sarcoma 180 (5×10^6 cells/mouse) was inoculated subcutaneously (s.c.) at the axillary region in ddY mice in groups of five. Drugs were administered intravenously (i.v.) beginning 1 d after tumor inoculation. Antitumor efficacy was expressed as *T*/*C*, where *T* and *C* are the values of mean tumor volume of treated and control mice. Tumor volume was calculated by using the formula for a prolate ellipsoid

$$\text{tumor volume} = L\,(\text{mm}) \times W^2\,(\text{mm})/2$$

in which L is the length of the major axis and W is the length of the minor axis.[12] BALB/c-nu/nu mice were given a tumor fragment equivalent to 8 mm^3 MX-1 (human mammary carcinoma) tumor passed in nude mouse. When the tumor volume reached 100—300 mm^3, the mice were pair-matched in groups of five each and the drug was administered intravenously. Antitumor efficacy was expressed as *T*/*C*, as described for sarcoma 180.

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References and Notes

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