Synthesis and Antitumor Activity of Duocarmycin Derivatives: Modification of Segment A of Duocarmycin B2

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Several A-ring pyrrole derivatives of duocarmycin B2 were synthesized effectively from the 3-hydroxy compounds by utilizing an interesting acid-catalyzed rearrangement, their anticellular activity was preliminarily evaluated by assays of growth inhibition of HeLa S_3 cells (*in vitro*) and antitumor activity against murine sarcoma 180 (*in vivo*). The 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives of the A-ring pyrrole compound showed remarkably potent *in vivo* antitumor activity, superior to that of duocarmycin B2. These derivatives were subjected to further biological evaluation. They exhibited potent antitumor activity toward murine solid tumors including M5076 sarcoma, B-16 melanoma and Colon 26 adenocarcinoma. Their most noteworthy feature was their efficacy against various human xenografts including LC-6 (lung), St-4 (stomach), and Co-3 (colon).

Key words duocarmycin; antitumor activity; Wagner–Meerwein rearrangement; KW-2189

Duocarmycin (DUM)s (A; **1a**, SA; **1c**, B2; **1d**, C2; **1e**, B1; **1f**, C1; **1g**) are novel antitumor antibiotics isolated from the culture broth of *Streptomyces* sp. (Fig. 1).[2)] The structures of DUMs have been confirmed by spectroscopic and chemical analysis.[3)] DUMA (**1a**),[2a)] which is considered to be an active form among these DUMs, possesses a unique cyclopropane ring and has the ability to alkylate DNA. The mechanism of this alkylation appears to be similar to that of CC-1065 (**1b**),[4-6)] which binds to the DNA minor groove and alkylates the N-3 position of adenine.[7)] DUMs are known to exhibit potent growth-inhibitory activity against human uterine cervix carcinoma HeLa S_3 cells *in vitro*, and have a fairly broad antitumor spectrum against murine transplantable solid tumors.[8)] However, their marginal activity against human solid tumors and their instability and insolubility dissuaded us from further evaluation. Instead, we have synthesized analogs with the aim of enhancing and broadening the spectrum of the antitumor activity, and improving the stability and solubility. We previously found that the 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**1h**, **1i**) of **1d** possessed potent antitumor activity *in vivo* and improved stability, superior to that of **1d**.[9)] In parallel with the modification of the C_8 hydroxyl group of **1d**, we modified the A-ring part of **1d**, and found that the A-ring pyrrole compound could be produced in good yield by acid-catalyzed rearrangement of the 3-hydroxy derivatives of **1d**. This A-ring pyrrole compound was converted to 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives, in view of the above information. As anticipated, the 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives demonstrated potent *in vivo* antitumor activity.

In this paper, we describe the synthesis of the A-ring pyrrole derivatives, and the evaluation of their antitumor activity and stability under various conditions.

Chemistry

The A-ring pyrrole analogs of DUMB2 (**1d**) were synthesized according to Chart 1. The hydroxyl group of **1d** was protected with *tert*-butyldimethylsilyl chloride in *N*,*N*-dimethylformamide (DMF) to give **2** quantitatively. The protection facilitated further chemical modification since **1d** is easily transformed to **1a**, followed by decomposition, under basic conditions.[9)] Compound **2** was reduced with sodium borohydride in methyl alcohol to afford the 3α- or 3β-hydroxy compounds (**3a**, **3b**), the 2-decarbomethoxy-3-hydroxy compound (**3c**) and the diols (**3d**, **3e**).[10)] The production ratio of **3a**—**3e** was greatly affected by the reaction solvent and temperature. We found that the reduction was best carried out in allyl alcohol as a reaction solvent at 0 °C to give **3a** as the main compound in 74% yield.[10)] The configuration at the C_3 center was confirmed by NMR. Obtained **3a** or **3b** was treated with camphorsulfonic acid (CSA) in toluene. In this reaction an interesting rearrangement of the methoxycarbonyl group occurred to afford the A-ring pyrrole analog **4a** in reasonable yield. The structure of **4a** was elucidated on the basis of NMR and mass spectrometry. Nuclear Overhauser effect (NOE) and long-range coupling were observed between the C_2-methyl group and NH proton in the NMR spectra, but NOEs from the C_2-methyl group to 4-H or 9-H_2 were not observed.[11)] The mechanism was considered to be a Wagner–Meerwein type rearrangement, as reported by Berner *et al.*[12)] The 2-decarbomethoxy-3-hydroxy compound **3c** was also treated with CSA to afford the dehydration product **4b** in 77% yield. In contrast, the diols (**3d**, **3e**) gave no rearrangement products under the same conditions.

The desilylation of **4a** and **4b** was carried out with *n*-Bu_4NF in tetrahydrofuran (THF) to give **5a** and **5b**, respectively. Compound **5a** was treated with 48% HBr, followed by addition of 4-nitrophenyl chloroformate in the presence of triethylamine in methylene chloride at −78 °C to give a carbonate **6a** as an intermediate. Various secondary amines were added to **6a** to give the 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**7a**—**d**) in good yields. The HBr adducts of **5a** and **5b** could not be isolated due to chemical instability. They immediately reverted to the cyclopropane compounds during purification. The hydrobromide of **7b** was obtained upon treatment with 48%

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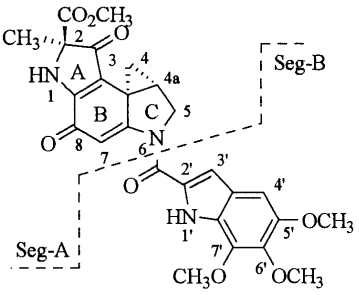
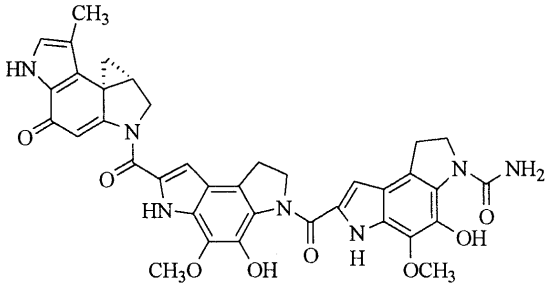
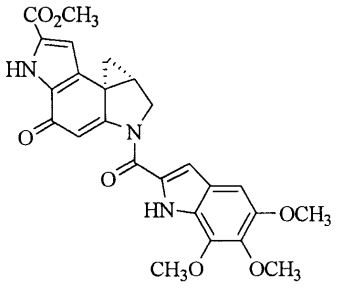


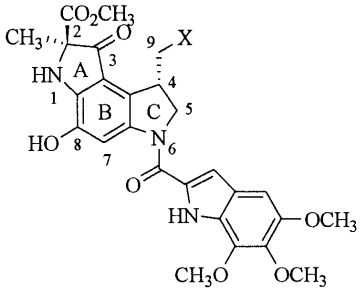
duocarmycin A (**1a**)



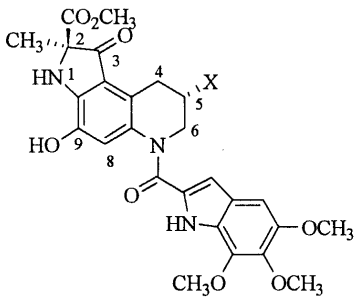
CC-1065 (**1b**)



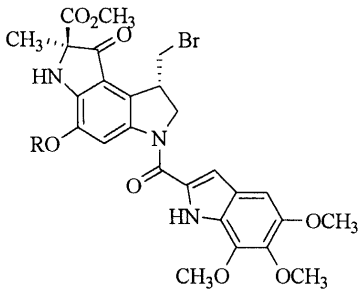
duocarmycin SA (**1c**)



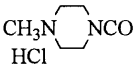
X=Br; duocarmycin B2 (**1d**)
X=Cl; duocarmycin C2 (**1e**)



X=Br; duocarmycin B1 (**1f**)
X=Cl; duocarmycin C1 (**1g**)



1h ; R=$(CH_3)_2NCO$

1i ; R= 

HCl

Fig. 1. Structures of Duocarmycins, CC-1065 and Duocarmycin Derivatives

hydrobromic acid in acetone–ethanol. The solubility of this salt (**7e**) in water was found to be about 10 mg/ml. Therefore **5a** was treated with HCl in place of HBr in the above reaction scheme to afford **7f** and **7g** in good yields. Compound **7g** was converted to the hydrochloride (**7h**) upon treatment with hydrogen chloride in ethanol.

In order to investigate the antitumor mechanism of the 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**7a**—**h**), we prepared the A-ring pyrrole analog (**8**) of DUMB1 (**1f**) in 43% yield based on **1f** in 4 steps (Fig. 2).[11b]

Stability of A-Ring Pyrrole Analogs in Aqueous Solution and in Calf Serum The stability of A-ring pyrrole analogs was measured in aqueous solution and in calf serum by HPLC analysis. As shown in Table 1, the synthetic intermediate **4a** of the A-ring pyrrole compound was unstable under these conditions. It was readily decomposed to **5a**. On the other hand, compounds **5a** and **5b**, which are considered as active forms of A-ring pyrrole analogs, were very much more stable than **1a**. It is speculated that this unusual stability is a consequence of overlap of the π-system in the A-ring part with the cyclohexadienone π-system.[13]

In a previous study, the 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**1h**, **1i**) of **1d** showed good stability.[9] The 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**7a**—**e**), however, were not as stable as the A-ring pyrrolidone analogs (**1h**,

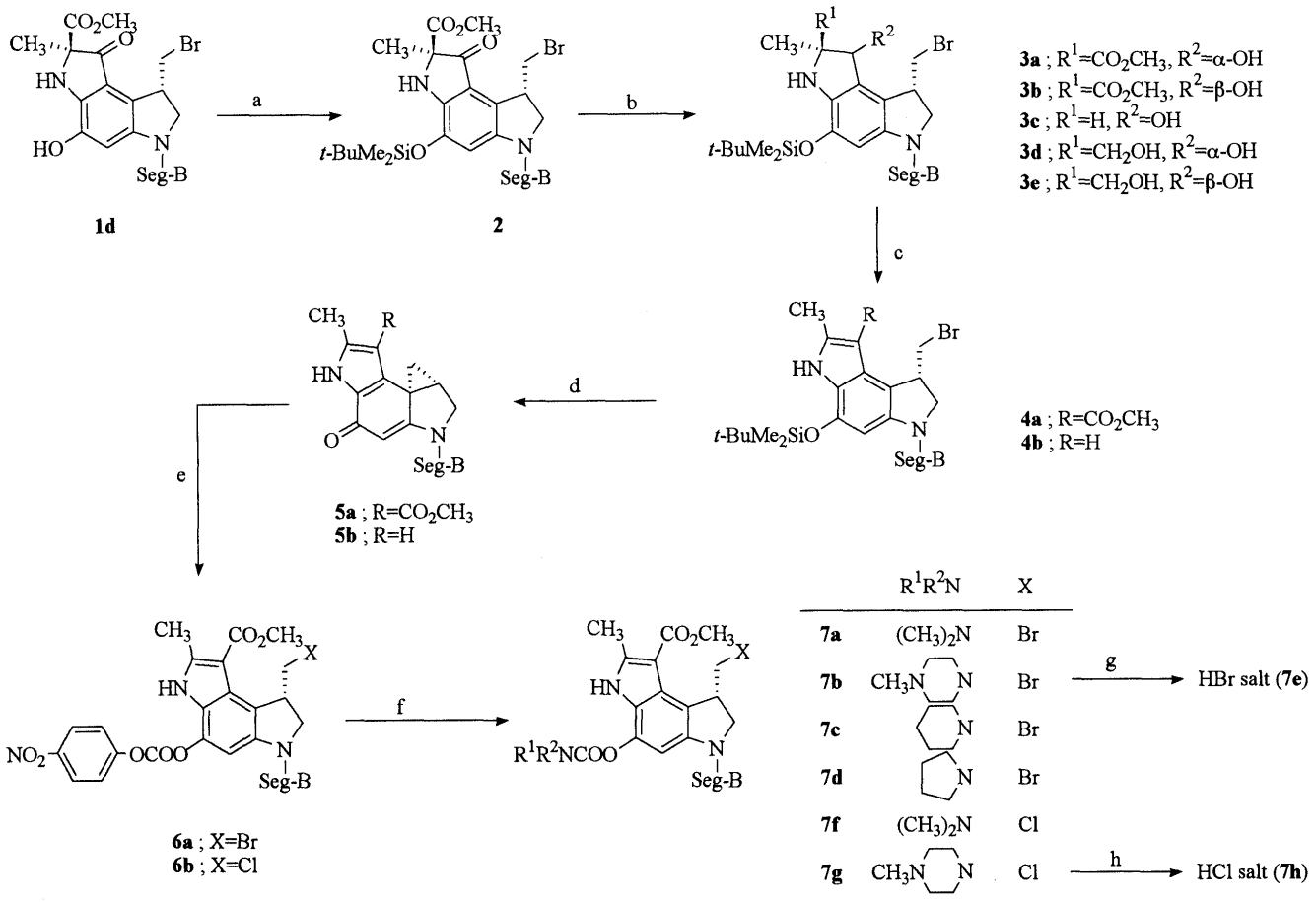


Chart 1

(a) *tert*-$BuMe_2SiCl$, imidazole, DMF; (b) $NaBH_4$, allyl alcohol or MeOH; (c) CSA, toluene; (d) *n*-Bu_4NF, THF; (e) HBr or HCl, CH_3CN, then 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2; (f) R^1R^2NH; (g) HBr, Me_2CO–MeOH; (h) HCl, EtOH.

Table 1. Anticellular Activity, Antitumor Activity and Stability Tests of Duocarmycin Derivatives

No.	Stability[a] $T_{1/2}$ (h)		HeLa S_3 IC_{50} (nM)[b]		S-180 (s.c.-i.v.)[c]	
	in aqueous solution	in calf serum	1 h	72 h	Dose (mg/kg)	T/C[d]
4a	2	2	0.17	0.002	0.5	0.15
7a	19	35	55	7.3	1.0	0.055
7b	16	40	210	1.3	0.5	0.24
7c	26	45	22	2.6	1.0	0.098
7d	20	37	2.6	0.3	1.0	0.088
7e	16	30	53	1.6	0.5	0.14
7f	158		730	3.3		
7h	130		>10	5.9	8.0	0.13
8	34		190	3.4	1.0	0.095
9a			>10000	>10000		
9b			>1000	>1000	16.0	0.57
5a	130	13	0.045	0.0052	0.25	0.21
5b	324	20	0.0088	0.0004	0.063	0.40
1a	1	<1	0.0055	0.0058	0.0075	0.26
1h	75		1900	56	1.0	0.087
1i	61		170	15	1.0	0.13

a) Half-life at 35 °C. 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) intraveneously (i.v.). *d*) *T* and *C* are the values of mean tumor volume of treated and control mice, respectively.

1i) in aqueous solution, and were hydrolyzed to the 9-hydroxy compounds. We also examined the stability of **8** under the same conditions. It gave the same degradation product **9b** as that of **7e**. These results indicate that the A-ring pyrrole analogs decompose through an intermediate with unusual reactivity, such as **10** (Fig. 2).

Biological Results and Discussion

The antitumor activity of some representative derivatives was evaluated primarily by assays of growth in-

hibition against HeLa S_3 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 in treated and control mice, respectively.

Compounds **5a** and **5b**, which were considered as active forms of A-ring pyrrole analogs, exhibited exceptionally potent anticellular activity almost equal to that of **1a**. The IC_{50} values of **5a** and **5b** at 72 h exposure were 0.0052 and 0.0004 nM, respectively. The anticellular activity of the synthetic intermediate **4a** protected by a silyl group increased with increasing exposure time. The IC_{50} value of 72 h exposure was approximately 100-fold smaller than that of 1 h exposure, and was similar to that of **5a** ($IC_{50}=0.002$ nM). Compound **4a** was readily converted to **5a** in aqueous solution and in calf serum, as described above.

Table 2. Antitumor Activity of **7a** and **7e** against Murine Tumors and Human Xenografted Carcinomas

	T/C[a]			
	7a	**7e**	**1d**	Cisplatin
	Dose (mg/kg)			
	0.64	0.63	0.25	11.0
M5076 sarcoma	0.02[b]	0.03	0.23	0.05
B16 malanoma	0.10[b]	0.07	0.36	0.03
Colon 26 adenocarcinoma	0.08[c]	0.19	0.54	0.22
St-4 (stomach)	0.15 (1)[d]	0.12	0.53	0.62
Co-3 (colon)	0.25	0.27	N.T.[e]	0.50
LC-6 (lung)	0.024	0.04	0.70	0.20

a) T and C are the values of mean tumor volume of treated and control mice, respectively. *b*) The dose was 0.41 mg/kg. *c*) The dose was 1.0 mg/kg. *d*) Mortality (5 mice in a group). *e*) Not tested.

On the other hand, the 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**7a**—**e**) showed decreased anticellular activity, about 1000 times inferior to that of **5a** (72 h exposure), but they exhibited promising *in vivo* antitumor activity against murine sarcoma 180 ($T/C=0.055$—0.24). The C_9-Cl derivative (**7h**) also exhibited high efficacy *in vivo* ($T/C=0.13$), but the optimal dose was higher than those of the C_9-Br derivatives. In contrast, the C_9-OH derivatives (**9a**, **9b**) which were produced by hydrolysis of **7a** and **7e**, respectively, did not show significant anticellular and antitumor activity.

Consequently, the 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**7a**, **7e**) were selected for further evaluation against several murine solid tumors (M5076 sarcoma, B-16 melanoma and Colon 26 adenocarcinoma) and human solid tumors (St-4, Co-3, LC-6). As shown in Table 2, they showed statistically significant antitumor activity against murine solid tumors with T/C values less than 0.2, and possessed high activity against human solid tumors that were insensitive to most chemotherapeutic drugs ($T/C=0.04$—0.27). Furthermore, tumor regression was observed in mice bearing St-4 and LC-6 carcinomas.[14]

The 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**7a**—**h**) were designed as stable prodrugs requiring enzymatic hydrolysis of the carbamoyl moiety, followed by regeneration of **5a**.[9] However, these carbamoyl derivatives were not as stable as **1h** or **1i** in aqueous solution, and were hydrolyzed to single C_9-OH compounds. The C_9-OH compounds were produced by a nucleophilic substitution reaction of water. We observed the interaction between these carbamoyl derivatives and DNA by means of circular dichroism (CD) studies (data not shown).[15] The results suggested that the carbamoyl derivatives of A-ring pyrrole compounds may directly alkylate DNA.[16] In practice, the

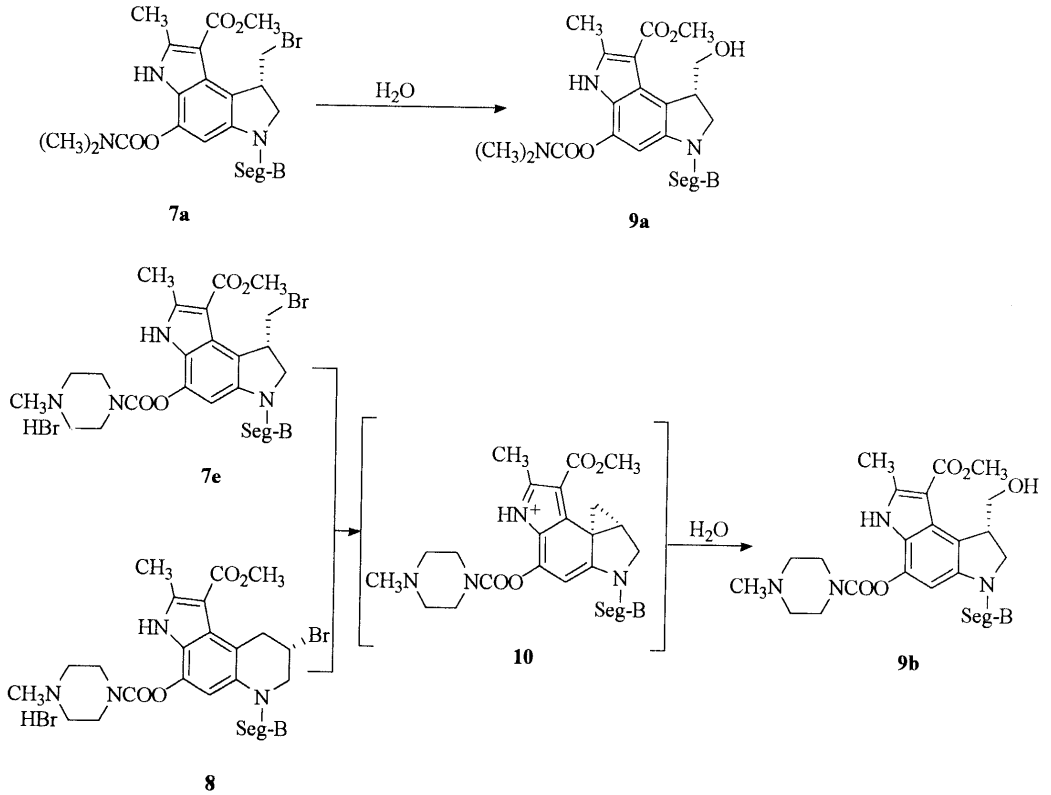


Fig. 2. Degradation Pathway of **7a**, **7e** and **8** in Aqueous Solution

8-*O*-*N*,*N*-dialkylcarbamoyl derivatives (**7a**—**h**) exhibited 10-fold higher anticellular potency than **1h** or **1i**, though the anticellular activity of **5a** was almost equal to that of DUMA (**1a**). We propose that DNA alkylation by **7e** occurs through a labile intermediate such as compound **10**, as depicted in Fig. 2. This is supported by the observation that **8** afforded the same decomposition product (**9b**) as did **7e**.

Although it is not certain that DNA alkylation ability by itself is necessary for *in vivo* antitumor activity, the 8-*O*-*N*,*N*-dialkylcarbamoyl derivatives of A-ring pyrrole compound have exceptionally high efficacy against several tumors and they do not cause delayed death, which is induced by **1b**.[17] Among these analogs, **7e** was selected for clinical trial as KW-2189, based on its improved antitumor activity and water solubility. Further research on **7e** is under way with respect to mechanisms of antitumor activity.[18,19]

Experimental

All melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a JASCO IR-810. ^{1}H-NMR spectra were measured on a JEOL JNM-GX270 (270 MHz) or a Bruker AM-400 (400 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Elemental analyses were performed with a Perkin-Elmer 2400 C, H, N analyzer. Mass spectra were measured with a Hitachi B-80 and a Shimadzu QP-1000. For column chromatography, silica gel (SiO_2, Wako C-200) was used. Analytical thin-layer chromatography (TLC) was performed on Silica gel 60 F_{254} plates (Merck).

8-*O*-*tert*-Butyldimethylsilyl-DUMB2 (2) *tert*-Butyldimethylsilyl chloride (50 mg, 0.33 mmol) was added to a solution of DUMB2 (123 mg, 0.21 mmol) and imidazole (43 mg, 0.63 mmol) in DMF (3 ml), and the mixture was stirred at room temperature for 4.5 h. Then, 2 N HCl was added to the reaction mixture, and the mixture was extracted with EtOAc twice. The combined extracts were washed with aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was subjected to column chromatography (hexane–EtOAc, 3 : 1) to give 140 mg (95%) of **2** as a light-tan powder. mp 120—130 °C (dec.) ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.35 (3H, s), 0.36 (3H, s), 1.06 (9H, s), 1.69 (3H, s), 3.57 (1H, dd, J=10.3, 9.1 Hz), 3.78 (3H, s), 3.91 (3H, s), 3.94 (3H, s), 4.06 (1H, dd, J=10.3, 3.0 Hz), 4.06 (3H, s), 4.17 (1H, m), 4.54 (1H, dd, J=10.6, 4.4 Hz), 4.62 (1H, dd, J=10.6, 9.1 Hz), 5.04 (1H, br s), 6.87 (1H, s), 6.95 (1H, d, J=2.2 Hz), 7.91 (1H, s), 9.38 (1H, br s). IR (KBr): 1745, 1700, 1618, 1497, 1293, 837 cm^{-1}. SI-MS *m/z*: 704 702 $(M+H)^+$, 470 468, 234. *Anal.* Calcd for $C_{32}H_{40}BrN_3O_8Si$: C, 54.70; H, 5.74; N, 5.98. Found: C, 54.82; H, 5.93; N, 5.75.

8-*O*-*tert*-Butyldimethylsilyl-3α-hydroxy-DUMB2 (3a) $NaBH_4$ (25 mg, 0.66 mmol) was added to a solution of **2** (155 mg, 0.22 mmol) in allyl alcohol (7 ml), and the mixture was stirred at 0 °C for 2.5 h. Then, 2 N HCl was added, and the resulting mixture was extracted with $CHCl_3$. The combined extracts were washed with aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was subjected to column chromatography (hexane–EtOAc, 1 : 1) to give 115 mg (74%) of **3a** as a white powder. mp 124—125 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.30 (3H, s), 0.32 (3H, s), 1.04 (9H, s), 1.60 (3H, s), 2.09 (1H, br s), 3.49 (1H, dd, J=10.3, 9.8 Hz), 3.72 (3H, s), 3.91 (3H, s), 3.92 (1H, m), 3.93 (3H, s), 4.05 (3H, s), 4.07 (1H, dd, J=10.3, 3.2 Hz), 4.50 (1H, dd, J=10.6, 3.9 Hz), 4.57 (1H, dd, J=10.6, 8.9 Hz), 5.31 (1 H, br s), 6.86 (1H, s), 6.91 (1H, d, J=2.2 Hz), 7.91 (1 H, s), 9.43 (1H, br s). IR (KBr): 3406, 1734, 1621, 1485, 1111, 838 cm^{-1}. SI-MS *m/z*: 706 704 $(M+H)^+$, 234. *Anal.* Calcd for $C_{32}H_{42}BrN_3O_8Si$: C, 54.54; H, 6.01; N, 5.96. Found: C, 54.47; H, 6.19; N, 5.71.

8-*O*-*tert*-Butyldimethylsilyl-3β-hydroxy-DUMB2 (3b) Reduction of **2** by $NaBH_4$ was carried out in MeOH to afford **3a** (40%), **3b** (22%), **3c** (7%), **3d** (6%) and **3e** (7%). The whole was chromatographed on silica gel with hexane–EtOAc (1 : 1) in **3b**: Yield: 22% (a white powder). mp 129—130 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.29 (3H, s), 0.30 (3H, s), 1.02 (9H, s), 1.61 (3H, s), 3.24 (1H, br s), 3.54 (1H, dd, J=10.1, 9.4 Hz), 3.79 (3H, s), 3.82 (1H, dd, J=10.1, 3.4 Hz), 3.91 (3H, s), 3.93 (3H, s), 4.06 (3H, s), 4.08 (1H, m), 4.44 (1H, dd, J=10.6, 4.9 Hz), 4.61 (1H, dd, J=10.6, 9.4 Hz), 5.08 (1H, br s), 6.86 (1H, s), 6.91 (1H, d, J=2.2 Hz), 7.88 (1H, s), 9.40 (1H, br s). IR (KBr): 1732, 1600, 1485, 1111 cm^{-1}. EI-MS *m/z*: 705 703 $(M)^+$. *Anal.* Calcd for $C_{32}H_{42}BrN_3O_8Si$: C, 54.54; H, 6.01; N, 5.96. Found: C, 54.57; H, 6.20; N, 5.81.

8-*O*-*tert*-Butyldimethylsilyl-2-decarbomethoxy-3-hydroxy-DUMB2 (3c) Yield: 7% (a white solid). mp 140—142 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.29 (3H, s), 0.30 (3H, s), 1.01 (9H, s), 1.35 (3H, s), 1.37 (1H, d, J=6.4 Hz), 3.49 (1H, dd, J=10.1, 9.6 Hz), 3.71 (1H, m), 3.81 (1H, dd, J=10.1, 3.4 Hz), 3.89 (3H, s), 3.93 (3H, s), 4.05 (3H, s), 4.05 (1H, m), 4.40 (1H, dd, J=10.8, 5.2 Hz), 4.55 (1H, dd, J=10.8, 9.3 Hz), 4.90 (1H, d, J=6.4 Hz), 5.11 (1H, br s), 6.85 (1H, s), 6.88 (1H, d, J=2.2 Hz), 7.87 (1H, br s), 9.43 (1H, br s). IR (KBr): 3450, 2934, 1618, 1486, 1309, 840 cm^{-1}. EI-MS *m/z*: 629 627 $(M+H_2O)^+$. *Anal.* Calcd for $C_{30}H_{40}BrN_3O_6Si$: C, 55.72; H, 6.23; N, 6.50. Found: C, 55.92; H, 6.63; N, 6.82.

8-*O*-*tert*-Butyldimethylsilyl-2-hydroxymethyl-3α-hydroxy-DUMB2 (3d) Yield: 6% (a white solid). mp 135—138 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.28 (3H, s), 0.30 (3H, s), 1.01 (9H, s), 1.34 (3H, s), 2.01 (1H, br s), 2.08 (1H, d, J=8.9 Hz), 3.47 (1H, dd, J=10.1, 10.1 Hz), 3.48 (2H, br s), 3.62 (1H, br s), 3.86 (1H, m), 3.91 (3H, s), 3.94 (3H, s), 4.06 (3H, s), 4.14 (1H, dd, J=10.1, 3.0 Hz), 4.53 (1H, dd, J=10.8, 8.4 Hz), 4.58 (1H, dd, J=10.8, 3.9 Hz), 5.04 (1H, d, J=8.9 Hz), 6.87 (1H, s), 6.92 (1H, d, J=2.2 Hz), 7.87 (1H, br s), 9.41 (1H, br s). IR (KBr): 3400, 2934, 1619, 1473, 1313, 839 cm^{-1}. SI-MS *m/z*: 678 676 $(M+H)^+$, 234. *Anal.* Calcd for $C_{31}H_{42}BrN_3O_7Si$: C, 55.02; H, 6.26; N, 6.21. Found: C, 55.13; H, 6.16; N, 6.01.

8-*O*-*tert*-Butyldimethylsilyl-2-hydroxymethyl-3β-hydroxy-DUMB2 (3e) Yield: 7% (a white solid). mp 135—138 °C. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 0.30 (6H, s), 0.95 (9H, s), 1.30 (3H, s), 3.20—3.80 (3H, m), 3.85 (3H, s), 3.90 (3H, s), 4.05 (3H, s), 4.00—5.10 (3H, m), 6.84 (1H, s), 6.90 (1H, d, J=2.0 Hz), 7.94 (1H, s), 9.40 (1H, br s). SI-MS *m/z*: 678 676 $(M+H^+)$, 234. *Anal.* Calcd for $C_{31}H_{42}BrN_3O_7Si$: C, 55.02; H, 6.26; N, 6.21. Found: C, 55.33; H, 6.19; N, 5.70.

8-*O*-*tert*-Butyldimethylsilyl-2-methyl-3-methoxycarbonyl-A-ring Pyrrole-DUMB2 (4a) CSA (1.6 g, 6.81 mmol) was added to a solution of **3a** (1.6 g, 2.27 mmol) in dry toluene (30 ml), and the reaction mixture was stirred for 1 h at 50 °C. Then, the mixture was poured into aqueous $NaHCO_3$ and the whole was extracted with EtOAc. The extract was washed with brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with hexane–EtOAc (4 : 1) to give 0.95 g (61%) of **4a** as a white powder. mp 140—142 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.37 (3H, s), 0.39 (3H, s), 1.07 (9H, s), 2.76 (3H, s), 3.21 (1H, dd, J=9.9, 9.9 Hz), 3.80 (1H, dd, J=9.9, 2.1 Hz), 3.92 (3H, s), 3.95 (3H, s), 3.98 (3H, s), 4.07 (3H, s), 4.52 (1H, m), 4.54 (1H, br d, J=8.5 Hz), 4.73 (1H, br d, J=8.9 Hz), 6.89 (1H, s), 6.99 (1H, d, J=2.3 Hz), 7.98 (1H, s), 8.30 (1H, br s), 9.40 (1H, br s). IR (KBr): 2934, 1696, 1628, 1493, 1412, 1305, 1213, 1112, 837 cm^{-1}. SI-MS *m/z*: 688 686 $(M+H)^+$, 454 452, 359, 234. *Anal.* Calcd for $C_{32}H_{40}BrN_3O_7Si$: C, 55.97; H, 5.87; N, 6.12. Found: C, 55.86; H, 6.03; N, 5.91.

8-*O*-*tert*-Butyldimethylsilyl-2-methyl-A-ring Pyrrole-DUMB2 (4b) CSA (32.7 mg, 0.141 mmol) was added to a solution of **3c** (30.5 mg, 0.047 mmol) in dry toluene (5 ml), and the reaction mixture was stirred for 1 h at 50 °C. Then, the mixture was poured into aqueous $NaHCO_3$, and the whole was extracted with EtOAc. The extract was washed with brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with hexane–EtOAc (4 : 1) to give 22.9 mg (77%) of **4b** as a white powder. mp 110—115 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.36 (6H, s), 1.07 (9H, s), 2.48 (3H, s), 3.38 (1H, dd, J=10.3, 10.3 Hz), 3.90 (1H, m), 3.91 (3H, s), 3.94 (3H, s), 4.06 (3H, s), 4.50 (2H, m), 4.85 (1H, m), 6.14 (1H, q, J=1.1 Hz), 6.88 (1H, s), 6.95 (1H, d, J=2.4 Hz), 7.83 (1H, s), 7.90 (1H, br s), 9.44 (1H, br s). IR (KBr): 2934, 1631, 1609, 1493, 1413, 1307, 839 cm^{-1}. EI-MS *m/z*: 629 627 $(M)^+$, 396 394, 234. *Anal.* Calcd for $C_{30}H_{38}BrN_3O_5S \cdot 0.5H_2O$: C, 56.51; H, 6.16; N, 6.59. Found: C, 56.65; H, 6.27; N, 6.56.

2-Methyl-3-methoxycarbonyl-A-ring Pyrrole-DUMA (5a) A solution of **4a** (500 mg, 0.73 mmol) in dry THF (50 ml) was stirred at room temperature. A 1.0 M solution in THF of tetrabutylammonium fluoride (1.1 ml, 1.1 mmol) was added, and the mixture was stirred for 1 h. Then, 0.01 M phosphate buffer (pH 7) was added to the resulting mixture, and the whole was extracted with $CHCl_3$. The organic layer was washed with

brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (50 : 1) to give 290 mg (81%) of **5a** as a white powder. mp 185—188 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.37 (1H, br d, J=5.4 Hz), 2.38 (1H, dd, J=7.5, 3.3 Hz), 2.63 (3H, s), 3.67 (1H, m), 3.82 (3H, s), 3.90 (3H, s), 3.94 (3H, s), 4.08 (3H, s), 4.45 (2H, m), 6.81 (1H, s), 6.95 (1H, d, J=2.3 Hz), 7.12 (1H, s), 9.40 (1H, br s), 11.58 (1H, br s). IR (KBr): 2934, 1700, 1637, 1525, 1487, 1459, 1385, 1295, 1264, 1106 cm^{-1}. SI-MS m/z: 492 $(M+H)^+$, 234. *Anal.* Calcd for $C_{26}H_{25}N_3O_7 \cdot 1.0H_2O$: C, 61.29; H, 5.34; N, 8.25. Found: C, 61.01; H, 5.20; N, 7.93.

2-Methyl-A-ring Pyrrole-DUMA (5b) The procedure was the same as that of **5a** except for the use of **4b** (20 mg, 0.032 mmol). The crude product was purified by silica gel chromatography to afford 12.4 mg (90%) of **5b** as a white powder. mp 176—182 °C (dec.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.53 (1H, t, J=4.6 Hz), 1.70 (1H, m), 2.36 (3H, s), 2.69 (1H, m), 3.89 (3H, s), 3.93 (3H, s), 4.07 (3H, s), 4.36 (1H, dd, J=10.4, 10.4 Hz), 4.40 (1H, dd, J=10.4, 4.5 Hz), 5.67 (1H, m), 6.78 (1H, s), 6.92 (1H, s), 6.94 (1H, d, J=2.3 Hz), 9.31 (1H, br s), 9.99 (1H, br s). IR (KBr): 3455, 3495, 2938, 1636, 1477, 1388, 1305, 1265 cm^{-1}. SI-MS m/z: 434 $(M+H)^+$, 234. *Anal.* Calcd for $C_{24}H_{23}N_3O_5 \cdot 1.0H_2O$: C, 63.85; H, 5.58; N, 9.31. Found: C, 63.97; H, 5.69; N, 9.29.

8-*O*-*N*, *N*-Dimethylcarbamoyl-2-methyl-3-methoxycarbonyl-A-ring Pyrrole-DUMB2 (7a) Hydrobromic acid (48%, 5 ml) was added to a solution of **5a** (285 mg, 0.58 mmol) in CH_3CN (15 ml), and the mixture was stirred for 2 h at room temperature. It was poured into 1 N HBr and the whole was extracted with $CHCl_3$. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. 4-Nitrophenyl chloroformate (235 mg, 1.17 mmol) and triethylamine (0.16 ml, 1.17 mmol) were added to a stirred solution of the residue in dry methylene chloride (10 ml) at −78 °C, and the resulting mixture was stirred at the same temperature for 0.5 h. Then an aqueous solution of 50% dimethylamine (0.52 ml, 5.8 mmol) was added to the solution, and the mixture was stirred at 0 °C for 1 h. The mixture was diluted with $CHCl_3$ and the whole was washed with 0.01 M phosphate buffer (pH 7) and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (50 : 1) to give 303 mg (81%) of **7a** as a white powder. mp 180—182 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.59 (3H, s), 3.07 (3H, s), 3.20 (3H, s), 3.22 (1H, dd, J=9.9, 9.9 Hz), 3.81 (1H, dd, J=9.9, 2.3 Hz), 3.92 (3H, s), 3.95 (3H, s), 3.97 (3H, s), 4.08 (3H, s), 4.58 (2H, m), 4.73 (1H, br d, J=9.7 Hz), 6.90 (1H, s), 7.00 (1H, d, J=2.3 Hz), 8.14 (1H, s), 9.09 (1H, br s), 9.37 (1H, br s). IR (KBr): 3470, 3300, 2946, 1701, 1411, 1313, 1217, 1167, 1109 cm^{-1}. SI-MS m/z: 645 643 $(M+H)^+$, 565, 411 409, 234. *Anal.* Calcd for $C_{29}H_{31}BrN_4O_8 \cdot 1.5CH_3OH$: C, 52.97; H, 5.39; N, 8.10. Found: C, 53.15; H, 5.46; N, 7.70.

8-*O*-(4-Methyl-1-piperazinylcarbamoyl)-2-methyl-3-methoxycarbonyl-A-ring Pyrrole-DUMB2 (7b) Hydrobromic acid (48%, 7.5 ml) was added to a solution of **5a** (900 mg, 1.83 mmol) in CH_3CN (20 ml), and the mixture was stirred for 2 h at room temperature. The resulting mixture was poured into 1 N HBr and the whole was extracted with $CHCl_3$. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. 4-Nitrophenyl chloroformate (740 mg, 3.67 mmol) and triethylamine (0.51 ml, 3.67 mmol) were added to a stirred solution of the residue in dry methylene chloride (15 ml) at −78 °C, then the resulting mixture was stirred at the same temperature for 0.5 h. 1-Methylpiperazine (0.51 ml, 4.61 mmol) was added, and the whole was stirred at 0 °C for 1 h. It was diluted with $CHCl_3$ and washed with 0.01 M phosphate buffer (pH 7) and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (10 : 1) to give 1.03 g (81%) of **7b** as a white powder. mp 160—163 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.37 (3H, s), 2.50 (4H, br s), 2.70 (3H, s), 3.23 (1H, dd, J=10.0, 10.0 Hz), 3.64 (2H, br s), 3.78 (2H, br s), 3.82 (1H, dd, J=10.0, 2.2 Hz), 3.92 (3H, s), 3.95 (3H, s), 3.97 (3H, s), 4.08 (3H, s), 4.54 (1H, m), 4.63 (1H, m), 4.74 (1H, dd, J=10.2, 1.2 Hz), 6.90 (1H, s), 6.99 (1H, d, J=2.3 Hz), 8.15 (1H, s), 8.81 (1H, br s), 9.34 (1H, br s). IR (KBr): 3475, 3232, 2944, 1698, 1491, 1410, 1313, 1217, 1110 cm^{-1}. SI-MS m/z: 700 698 $(M+H)^+$, 466 464, 339, 234. *Anal.* Calcd for $C_{32}H_{36}BrN_5O_8 \cdot 1.0H_2O$: C, 53.64; H, 5.34; N, 9.77. Found: C, 53.31; H, 5.30; N, 9.45.

8-*O*-Piperidinylcarbamoyl-2-methyl-3-methoxycarbonyl-A-ring Pyrrole-DUMB2 (7c) The procedure was the same as that for **7a** except that piperidine was used. The crude product was purified by silica gel chromatography to afford **7c** (65%) as a white solid. mp 134—137 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.68 (6H, br s), 2.59 (3H, s), 3.22 (1H, dd, J=10.1, 10.1 Hz), 3.54 (2H, br s), 3.69 (2H, br s), 3.81 (1H, dd, J=10.1, 2.1 Hz), 3.92 (3H, s), 3.95 (3H, s), 3.96 (3H, s), 4.07 (3H, s), 4.61 (2H, m), 4.74 (1H, dd, J=10.3, 1.0 Hz), 6.90 (1H, s), 7.00 (1H, d, J=2.4 Hz), 8.14 (1H, s), 9.09 (1H, br s), 9.38 (1H, br s, NH). IR (KBr): 3470, 3250, 2940, 2858, 1698, 1491, 1410, 1312, 1255, 1214, 1165, 1109 cm^{-1}. SI-MS m/z: 685 683 $(M+H)^+$, 234. *Anal.* Calcd for $C_{32}H_{35}BrN_4O_8 \cdot 0.5H_2O$: C, 55.50; H, 5.24; N, 8.09. Found: C, 55.78; H, 5.30; N, 7.90.

8-*O*-Pyrrolidinylcarbamoyl-2-methyl-3-methoxycarbonyl-A-ring Pyrrole-DUMB2 (7d) The procedure was the same as that for **7a** except that pyrrolidine was used. The crude product was purified by silica gel chromatography to afford **7d** (65%) as a white powder. mp 152—160 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.99 (4H, m), 2.65 (3H, s), 3.22 (1H, dd, J=10.1, 10.1 Hz), 3.52 (2H, t, J=6.6 Hz), 3.67 (2H, t, J=6.6 Hz), 3.81 (1H, dd, J=10.1, 2.1 Hz), 3.92 (3H, s), 3.95 (3H, s), 3.97 (3H, s), 4.08 (3H, s), 4.55 (1H, dd, J=10.2, 2.4 Hz), 4.63 (1H, m), 4.74 (1H, dd, J=10.2, 1.0 Hz), 6.90 (1H, s), 7.00 (1H, d, J=2.3 Hz), 8.16 (1H, s), 9.06 (1H, br s), 9.36 (1H, br s). IR (KBr): 3230, 2942, 1699, 1490, 1415, 1312, 1216, 1109 cm^{-1}. SI-MS m/z: 671 669 $(M+H)^+$, 591, 234. *Anal.* Calcd for $C_{31}H_{33}BrN_4O_8 \cdot 1.5H_2O \cdot 1.0\ CH_3OH$: C, 52.75; H, 5.53; N, 7.69. Found: C, 52.63; H, 5.20; N, 7.31.

8-*O*-(4-Methyl-1-piperazinylcarbamoyl)-2-methyl-3-methoxycarbonyl-A-ring Pyrrole-DUMB2 Hydrobromide (7e) A solution of **7b** (6.69 g, 9.57 mmol) in acetone (60 ml) and methanol (270 ml) was treated with 48% hydrobromic acid (1.7 ml, 9.95 mmol) at room temperature for 4 h. The resulting mixture was evaporated *in vacuo* to give 6.42 g (86%) of **7e** as a white crystalline compound. mp 207—213 °C (dec.). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.69 (3H, s), 2.89 (3H, s), 3.26 (4H, br s), 3.41 (1H, dd, J=9.0, 9.0 Hz), 3.53 (3H, m), 3.80 (3H, s), 3.82 (3H, s), 3.85 (3H, s), 3.94 (3H, s), 4.20 (1H, m), 4.47 (3H, m), 4.65 (1H, dd, J=10.5, 8.5 Hz), 6.97 (1H, s), 7.00 (1H, d, J=2.1 Hz), 7.94 (1H, s), 9.81 (1H, br s), 11.30 (1H, d, J=2.1 Hz), 11.97 (1H, s). IR (KBr): 1717, 1692, 1608, 1525, 1490, 1409, 1310, 1218, 1167, 1108 cm^{-1}. SI-MS m/z: 700 698 $(M+H)^+$, 466 464, 339, 234. *Anal.* Calcd for $C_{32}H_{36}BrN_5O_8 \cdot HBr \cdot 1.0H_2O$: C, 48.19; H, 4.93; N, 8.78. Found: C, 47.92; H, 5.10; N, 8.49.

8-*O*-*N*,*N*-Dimethylcarbamoyl-2-methyl-3-methoxycarbonyl-9-chloro-A-ring Pyrrole-DUM (7f) Hydrochloric acid (6 N, 1 ml) was added to a solution of **5a** (14 mg, 0.029 mmol) in CH_3CN (2 ml), and the mixture was stirred for 2 h at room temperature. It was poured into 1 N HCl and the whole was extracted with $CHCl_3$. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. 4-Nitrophenyl chloroformate (11.7 mg, 0.058 mmol) and triethylamine (8 ml, 0.058 mmol) were added to a stirred solution of the residue in dry methylene chloride (2 ml) at −78 °C, and the resulting mixture was stirred at the same temperature for 0.5 h. Then, an aqueous solution of 50% dimethylamine (26 ml, 0.29 mmol) was added, and the mixture was stirred at 0 °C for 1 h. It was diluted with $CHCl_3$ and the whole was washed with 0.01 M phosphate buffer (pH 7) and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (80 : 1) to give 15 mg (86%) of **7f** as a white powder. mp 158—159 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.67 (3H, s), 3.07 (3H, s), 3.20 (3H, s), 3.35 (1H, dd, J=10.0, 10.0 Hz), 3.92 (3H, s), 3.94 (4H, br s), 3.95 (3H, s), 4.09 (3H, s), 4.55 (2H, m), 4.77 (1H, br d, J=9.1 Hz), 6.89 (1H, s), 6.99 (1H, d, J=2.3 Hz), 8.16 (1H, s), 8.86 (1H, br s), 9.34 (1H, br s). IR (KBr): 2940, 1702, 1697, 1490, 1382, 1313, 1219, 1175, 1110 cm^{-1}. SI-MS m/z: 599 $(M+H)^+$, 366, 294, 234. *Anal.* Calcd for $C_{29}H_{31}ClN_4O_8 \cdot 0.5H_2O$: C, 57.28; H, 5.30; N, 9.21. Found: C, 57.45; H, 5.31; N, 8.64.

8-*O*-(4-Methyl-1-piperazinylcarbamoyl)-2-methyl-3-methoxycarbonyl-9-chloro-A-ring Pyrrole-DUM (7g) The procedure was the same as that for **7f** except that 1-methylpiperazine was used. The crude product was purified by silica gel chromatography to afford **7g** (66%) as a white powder. mp 170—172 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.61 (3H, s), 2.70 (3H, s), 2.89 (4H, br s), 3.34 (1H, dd, J=10.2, 10.2 Hz), 3.80 (5H, m), 3.91 (3H, s), 3.93 (3H, s), 3.95 (3H, s), 4.09 (3H, s), 4.50 (2H, m), 4.70 (1H, dd, J=10.1, 10.1 Hz), 6.88 (1H, s), 6.97 (1H, d, J=2.2 Hz), 8.12 (1H, s), 9.35 (1H, br s), 9.52 (1H, br s). IR (KBr): 2940, 1698, 1637, 1491, 1410, 1314, 1218, 1154, 1109 cm^{-1}. SI-MS m/z: 654 $(M+H)^+$, 420, 234. *Anal.* Calcd for $C_{32}H_{36}ClN_5O_8 \cdot 0.5H_2O$: C, 57.96; H, 5.62; N, 10.56. Found: C, 58.17; H, 5.62; N, 10.52.

8-*O*-(4-Methyl-1-piperazinylcarbamoyl)-2-methyl-3-methoxycarbonyl-9-chloro-A-ring Pyrrole-DUM Hydrochloride (7h) **7g** (17 mg, 0.026 mmol) was dissolved in EtOH (1 ml), and the resulting mixture was treated with anhydrous 5.8 N HCl in EtOH (9 ml) at room temperature

for 1 h. The whole was evaporated *in vacuo* to give 18 mg (100%) of **7h** as a white crystalline compound. mp 219—224 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.69 (3H, s), 2.84 (3H, s), 3.51 (5H, m), 3.61 (2H, br s), 3.80 (3H, s), 3.82 (3H, s), 3.85 (3H, s), 3.94 (3H, s), 4.15 (2H, br s), 4.43 (3H, m), 4.65 (1H, dd, J=10.2, 9.6 Hz), 6.96 (1H, s), 7.00 (1H, d, J=2.0 Hz), 7.93 (1H, s), 10.72 (1H, br s), 11.30 (1H, br s), 12.13 (1H, br s). IR (KBr): 2946, 1700, 1609, 1527, 1491, 1410, 1313, 1217, 1172, 1109, 1090 cm^{-1}. SI-MS m/z: 654 $(M+H)^+$, 420, 234. *Anal.* Calcd for $C_{32}H_{36}ClN_5O_8 \cdot HCl \cdot 3.5H_2O$: C, 51.00; H, 5.88; N, 9.29. Found: C, 51.11; H, 5.83; N, 9.15.

8-*O*-*N*,*N*-Dimethylcarbamoyl-2-methyl-3-methoxycarbonyl-9-hydroxy-A-ring Pyrrole-DUM (9a) Aqueous $NaHCO_3$ (5 ml) was added to a solution of **7a** (50 mg, 0.078 mmol) in CH_3CN (5 ml), and the mixture was stirred at 60 °C for 48 h, then extracted with $CHCl_3$. The extract was washed with brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–Me_2CO (3 : 1) to give 39 mg (84%) of **9a** as a white powder. mp 165—170 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.26 (1H, s), 2.60 (3H, s), 3.07 (3H, s), 3.19 (3H, s), 3.57 (1H, dd, J=10.5, 7.4 Hz), 3.86 (1H, dd, J=10.5, 4.5 Hz), 3.90 (3H, s), 3.91 (3H, s), 3.94 (3H, s), 4.07 (3H, s), 4.44 (1H, m), 4.52 (1H, dd, J=10.0, 8.4 Hz), 4.68 (1H, dd, J=10.0, 1.2 Hz), 6.86 (1H, s), 6.99 (1H, d, J=2.4 Hz), 8.16 (1H, s), 8.96 (1H, br s), 9.37 (1H, br s). IR (KBr): 2934, 1704, 1606, 1493, 1413, 1314, 1217, 1164, 1110 cm^{-1}. SI-MS m/z: 581 $(M+H)^+$, 234. *Anal.* Calcd for $C_{29}H_{32}N_4O_9 \cdot 2.0H_2O$: C, 56.49; H, 5.88; N, 9.09. Found: C, 56.61; H, 5.91; N, 8.91.

8-*O*-(4-Methyl-1-piperazinylcarbamoyl)-2-methyl-3-methoxycarbonyl-9-hydroxy-A-ring Pyrrole-DUM (9b) Phosphate buffer (0.05 M, pH 7, 20 ml) was added to a solution of **7e** (18 mg, 0.025 mmol) in CH_3CN (6 ml), and the mixture was stirred at 35 °C for 48 h, then extracted with $CHCl_3$. The extract was washed with brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (10 : 1) to give 13 mg (82%) of **9b** as a pale yellow compound. mp 219—224 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.25 (3H, s), 2.40 (2H, br s), 2.43 (2H, br s), 2.65 (3H, s), 3.11 (1H, ddd, J=10.3, 9.6, 5.3 Hz), 3.47 (2H, br s), 3.62 (1H, ddd, J=10.3, 5.3, 5.3 Hz), 3.69 (2H, br s), 3.79 (3H, s), 3.82 (3H, s), 3.82 (3H, s), 3.94 (3H, s), 4.14 (1H, m), 4.48 (1H, dd, J=10.3, 10.3 Hz), 4.53 (1H, dd, J=10.3, 1.6 Hz), 4.84 (1H, t, J=5.3 Hz), 6.95 (1H, s), 7.01 (1H, d, J=2.1 Hz), 7.87 (1H, br s), 11.26 (1H, br s), 11.81 (1H, br s). IR (KBr): 2938, 1700, 1611, 1526, 1493, 1411, 1314, 1217, 1111 cm^{-1}. SI-MS m/z: 636 $(M+H)^+$, 234. *Anal.* Calcd for $C_{32}H_{37}N_5O_9 \cdot 0.5H_2O$: C, 56.62; H, 5.94; N, 10.86. Found: C, 56.65; H, 5.90; N, 10.79.

Stability in Aqueous Solution and in Calf Serum The stability of the DUMB2 derivatives under aqueous conditions was examined by chromatography on a UNISIL pack 5C18 reversed-phase HPLC column (GL Science Co. Ltd., Tokyo, Japan). A test compound (1 mg) was dissolved in acetonitrile (10 ml). This solution (2 ml) was diluted with aqueous solution or calf serum (each 8 ml). Aqueous solution was composed of 0.01 M phosphate buffer (pH 7). 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 the absorbance at 330 nm.

Biological Studies Human uterine cervix carcinoma HeLa S_3 cells were obtained from American Type Culture Collection through Dainippon Pharmaceutical Co. (Osaka, Japan). The cells (2×10^4/well) were precultured in the culture medium in 24-well multidishes (Nunc, Roskilde, Denmark) for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. For the pulse exposure experiment, cells were treated with each compound for 1 h, washed with Dulbecco's phosphate-buffered saline [Ca^{2+}-, Mg^{2+}-free; PBS(−)] and further incubated in fresh medium for 71 h. For the continuous exposure experiment, cells were treated with each compound for 72 h. Then the cells were treated with PBS(−) containing 0.05% trypsin (Difco Laboratories, Detroit, MI) and 0.02% EDTA (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan) and counted by using a Microcell Counter (Toa Medical Electronics Co., Ltd., Kobe, Japan). The IC_{50} values (drug concentration required for 50% inhibition of the cell growth) were determined.

Sarcoma 180, St-4 (poorly differentiated stomach adenocarcinoma), Co-3 (well-differentiated colon adenocarcinoma) and LC-6 (large cell lung adenocarcinoma) were kindly supplied by the National Cancer Center (Tokyo, Japan). M5076 reticulum cell sarcoma, B-16 melanoma and Colon 26 adenocarcinoma were supplied by the Japanese Foundation for Cancer Research (Tokyo, Japan). Sarcoma 180 cells were passaged and used for the experiment in adult male ddY mice. B-16 melanoma and M5076 reticulum cell sarcoma cells were passaged and used in adult male C57BL/6 mice. Colon 26 adenocarcinoma cells were passaged and used in adult male BALB/c mice. Human xenografts were passaged and used in adult male BALB/c-*nu*/*nu* mice. All murine solid tumors were inoculated subcutaneously (s.c.) at the axillary region of mice. Human xenografts were inoculated s.c. in the flank of nude mice. 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. The length and width of the tumors were measured, and tumor volume was calculated as

$$\text{tumor volume (mm}^3\text{)} = \text{length (mm)} \times [\text{width (mm)}]^2/2$$

according to the method of the National Cancer Institute.[20]

The criteria for effectiveness against murine solid tumors were a T/C value of 42% or less, and statistical significance determined by the Mann–Whitney U test ($p<0.05$). Drug efficacy against human xenografts was expressed as the percentage of mean V/V_0 value against that of the control group, where V is the tumor volume at the day of evaluation and V_0 is the tumor volume at the day of initial drug treatment. The criteria for effectiveness were a T/C value of 50% or less, and statistical significance determined by the Mann–Whitney U test ($p<0.01$, one-sided).[21]

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