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Synthesis and Antitumor Activity of Duocarmycin Derivatives: Modification at the C-7 Position of Segment-A of A-Ring Pyrrole Compounds

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Abstract—A series of the C7-substituted A-ring pyrrole derivatives of duocarmycin were synthesized, and evaluated for in vitro anticellular activity against HeLa S_3 cells and in vivo antitumor activity against murine sarcoma 180 in mice. All of the C7-substituted A-ring pyrrole compounds decreased potency in vitro and in vivo. However, some showed strong antitumor activity with T/C values less than 0.3. Among them, the 7-formyl compound **5d** showed remarkable potent in vivo antitumor activity and low peripheral blood toxicity, which were equal to **2c**.

Introduction

A new class of antitumor antibiotics produced by *Streptomyces* sp., including duocarmycin(DUM)s A (**1a**), B1, B2 (**1b**), C1, C2 (**1c**) and SA possess exceptionally potent cytotoxicity (Fig. 1).[1,2] Since DUMB1, B2 (**1b**), C1, and C2 (**1c**) readily yield DUMA (**1a**) in an aqueous solution, DUMA is thought to be an active form among these antibiotics. DUMA (**1a**) and DUMSA have a unique cyclopropane ring responsible for the sequence-selective alkylation of double-stranded DNA mediating N3 adenine covalent adduct formation.[3] This mechanism is similar to that of CC-1065 which has been reported to show high cytotoxicity.[4,5] KW-2189 (**2a**),[6] selected as the best compound in analogues of A-ring pyrrole derivatives of DUMB2 (**1b**), showed good stability in the culture medium and aqueous solubility greater than 10 mg/mL.[7,8] It showed strong activities against murine ascitic and human solid tumors.[6b] KW-2189 (**2a**) is currently under phase II clinical evaluation.

The segment-A (Seg-A) containing a spirocyclopropylhexadienone moiety is necessary for the formation of covalent bonding with DNA. Our previous results indicate that the Seg-A structure influences the electrophilicity of cyclopropane.[6c] On the other hand, the segment-B (Seg-B) of DUM has been considered to play an important role for noncovalent binding to the minor groove of DNA.[9] We have previously synthesized a series of DUM analogues bearing the simplified DNA-binding moieties.[10] Among them, A-ring pyrrole DUM bearing cinnamoyl[10b] or heteroarylacryloyl[10c] groups showed remarkable potent in vivo antitumor activity and low peripheral blood toxicity.

In the previous papers,[11] we have reported our investigation into the synthesis, anticellular and antitumor activity, and toxicity of C3-substituted A-ring pyrrole DUM derivatives containing various modifications. Our study showed a new approach in the modification of C7-substituted A-ring pyrrole derivatives of DUM. The modification at the C-7 position of Seg-A containing cyclopropane ring has been little reported as DUM or CC-1065 analogues, and would affect electrophilicity of the cyclopropane ring. Interestingly, the substituents at the C-7 position of Seg-A would influence the reversible DNA-alkylating activity of DUM analogues.[12]

Chemistry

First, we have investigated various reaction conditions to compound **2b** or the 8-*O*-protected DUMB2 of A-ring pyrrole, however, the modification at the C-7 position of Seg-A was unsuccessful. Next, Seg-A moiety (**3a**) was treated with NBS or NCS to yield the 7-bromo (**4a**) or 7-chloro (**4b**) compound (Scheme 1). The Seg-A

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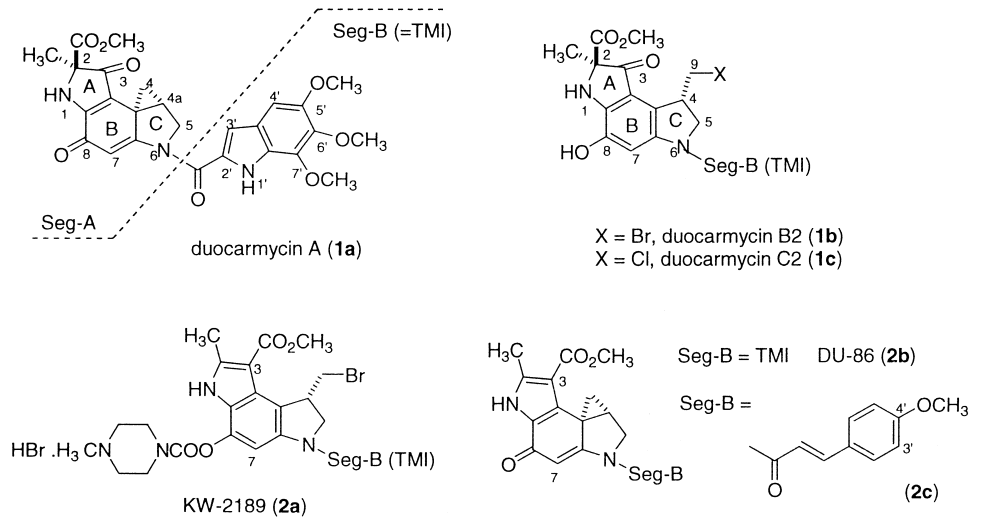
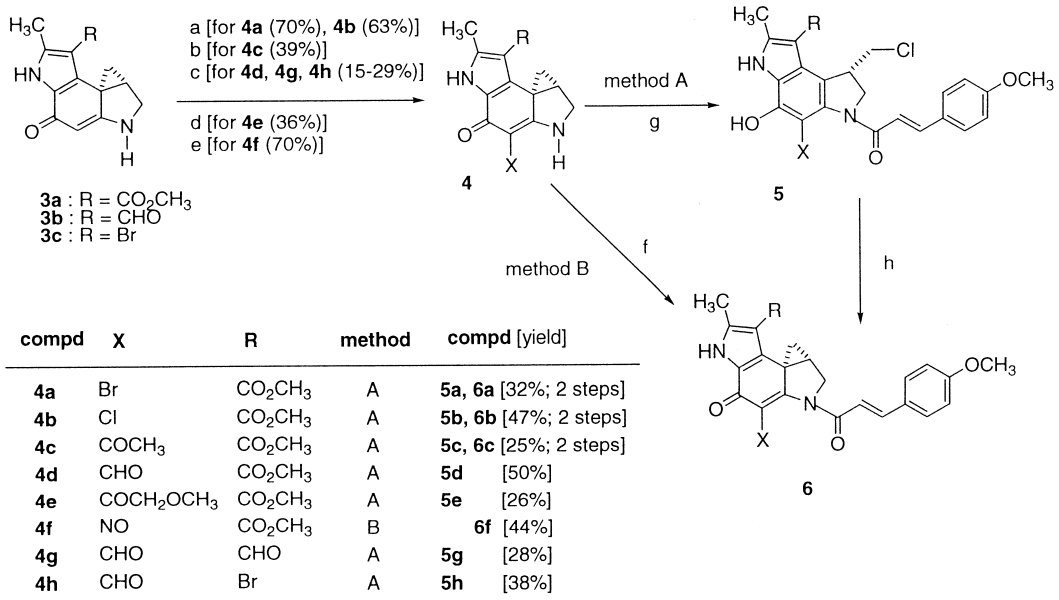


Figure 1. Structure of duocarmycins and duocarmycin derivatives.



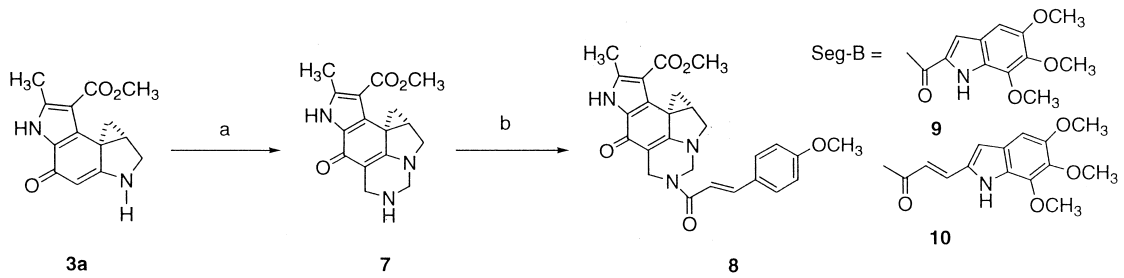
compd	X	R	method	compd [yield]
4a	Br	CO_2CH_3	A	5a, 6a [32%; 2 steps]
4b	Cl	CO_2CH_3	A	5b, 6b [47%; 2 steps]
4c	$COCH_3$	CO_2CH_3	A	5c, 6c [25%; 2 steps]
4d	CHO	CO_2CH_3	A	5d [50%]
4e	$COCH_2OCH_3$	CO_2CH_3	A	5e [26%]
4f	NO	CO_2CH_3	B	6f [44%]
4g	CHO	CHO	A	5g [28%]
4h	CHO	Br	A	5h [38%]

Scheme 1. (a) NBS or NCS, CCl_4, rt; (b) Ac_2O, $BF_3{\cdot}Et_2O$, rt; (c) Cl_2CHOMe, $TiCl_4$, CH_2Cl_2, rt; (d) $MeOCH_2COCl$, $AlCl_3$, CH_2Cl_2, rt; (e) $NaNO_2$, AcOH, dioxane, −20 °C; (f) 1) NaH, 2) *p*-nitrophenyl 4-methoxycinnamate, DMF, −20 °C; (g) 1) HCl, 2) 4-methoxycinnamic acid, EDCI, rt; (h) DBU, CH_3CN, rt.

derivatives **3a–c** were treated with electrophilic reaction, such as Friedel–Crafts reaction using Lewis acids, to afford the 7-acetyl (**4c**), 7-formyl (**4d**, **g**, **h**), 7-methoxymethylcarbonyl (**4e**) compound. Moreover, the treatment of **3a** with $NaNO_2$ in acetic acid and 1,4-dioxane afforded the 7-nitroso (**4f**) compound in 70% yield. Then, the preparation of *N*-4′-methoxycinnamoyl derivatives have been investigated by two approaches.

Compounds **5a–e**, **5g**, **5h** were prepared by the reaction of **4a–e**, **4g**, **4h** with 4 N HCl in CH_3CN followed by the addition of 4-methoxycinnamic acid in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), respectively. The obtained compounds **5a–c** were converted to **6a–c** upon treatment with DBU in CH_3CN (method A). The cyclopropane compounds **6d**, **e**, **g**, **h** were not obtained, because compounds **5d**, **e**, **g**, **h** decomposed under the same condition. Compound **4f** was allowed to react with *p*-nitrophenyl 4-methoxycinnamate in the presence of NaH to yield the corresponding 4′-methoxycinnamate (**6f**) in reasonable yield (method B).

Since the formation of the 7-formyl compounds **4d**, **g**, **h** had low yields (15–29%), we have investigated other methods. Treatment of **3a** with $POCl_3$ in DMF (Vilsmeier reaction) didn't afford the desired 7-formyl (**4d**) but *N*-formyl compound of **3a**. Furthermore, compound **3a** was treated with hexamethylenetetramine (HMT) in TFA, and the four rings compound **7** was produced (Scheme 2). It was assumed that the produce of compound **7** was caused by the intermediate attacking the amine moiety of C-ring part as an electrophilic reaction. The obtained compound **7** was allowed to



Scheme 2. (a) HMT, TFA, rt, (57%); (b) 4-methoxycinnamic acid (70%) or 5,6,7-trimethoxy-2-indolecarboxylic acid (76%) or 3-(5,6,7-trimethoxy-2-indole)acrylic acid (43%), EDCI, CH_2Cl_2, rt.

react with the corresponding carboxylic acid in the presence of EDCI to yield compounds **8**, **9**, **10**.

Results and Discussion

The antitumor activity of some representative derivatives was evaluated primarily by assays of the inhibition of HeLa S_3 cells growth (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 represents means of tumor volume in treated and control mice, respectively. All of the C7-substituted A-ring pyrrole compounds bearing a 4′-methoxycinnamoyl group showed inferior anticellular activity to **2c**.[10b] Moreover, all of the C7-substituted A-ring pyrrole compounds bearing a 4′-methoxycinnamoyl group generally exhibited sufficient efficacy in vivo at 10–20 times higher dose than that of **2c**.[10b] These results suggest that the electron-withdrawing C7-substituents decrease the alkylation reactivity of cyclopropane ring to DNA. However, compounds **6b**, **5d**, **5g**, **5h** showed more potent antitumor activity than **2c**. Among them, compound **5d** showed excellent activity in vivo with T/C values of 0.14. Moreover, the peripheral blood toxicity (reduction of the number of peripheral blood platelets) of compound **5d** was lower than that of compound **2b** bearing Seg-B of natural type, and was equal to that of compound **2c**. Similarly, the other C7-substituted A-ring pyrrole compounds bearing a 4′-methoxycinnamoyl group showed low peripheral blood toxicity.

We have expected that the four rings compounds **8**, **9**, **10** would increase biological activity by the binding of these compounds to a different position of DNA minor groove. However, the four rings compounds **8**, **9**, **10** showed extremely low anticellular and antitumor activity. These results suggest that the four rings compounds show low affinity to the minor groove of DNA.

Table 1. Anticellular activity, antitumor activity and hematotoxicity of duocarmycin derivatives

No.	HeLa S_3 IC_{50} (nM)[a]		Sarcoma 180 (s.c.–i.v.)[b]		Hematotoxicity	
	1 h	72 h	Dose (mg/kg)	T/C[c]	WBC[d] (%)	PL[e] (%)
5a	74	6.0	16	0.35	30	NT[g]
6a	1600	41	16	0.49	26	NT[g]
6b	800	19	8	0.23	40	109
6c	84	30	4	0.38	23	77
5d	190	29	16	0.14	28	75
5e	35	9.9	8	0.56	42	104
6f	3200	930	4	0.72	100	111
5g	180	94	8	0.28(1)[f]	56	95
5h	920	0.72	8	0.24	28	76
8	3500	57	8	0.94	100	93
9	> 10000	2200	8	0.63	105	77
10	> 10000	600	8	0.91	105	93
2b	0.045	0.0052	0.25	0.21	22	38
2c	2.9–7.0	0.26–0.94	0.83	0.34	50	63

[a]Drug concentration required to inhibit the growth of HeLa S_3 cells by 50%.
[b]Mice (5 mice in group) were implanted subcutaneously (sc) with tumor cells, and the drug was dosed (mg/kg) intravenously (iv).
[c]T and C are the values of mean tumor volume of treated and control mice, respectively.
[d]Number of white blood cells of tumor-bearing mice on day 4 (% of control).
[e]Number of peripheral platelets of normal mice on day 7 (% of control).
[f]Mortality (5 mice in group).
[g]Not tested.

Conclusions

A series of the C7-substituted A-ring pyrrole derivatives of duocarmycin were synthesized, and evaluated for in vitro anticellular activity against HeLa S_3 cells and in vivo antitumor activity against murine sarcoma 180 in mice. All of the C7-substituted A-ring pyrrole compounds decreased potency in vitro and in vivo. However, some showed strong antitumor activity with T/C values less than 0.3. Among them, the 7-formyl compound **5d** showed remarkable potent in vivo antitumor activity and low peripheral blood toxicity, which were equal to **2c**. Modification at the C-7 position of Seg-A was shown to be a promising approach for preparing new candidates in DUM derivatives. Further detailed studies of the structure–activity relationships (SAR) of these derivatives are in progress.

Experimental

Infrared spectra (IR) were recorded on a JASCO IR-810 spectrometer. ^1H NMR spectra were measured on JEOL JNM-EX270 spectrometers and are reported in δ units. Mass spectra were measured with JEOL JMS-DX303 and SHIMAZU QP-1000 spectrometers. Elemental analyses were performed with a Perkin–Elmer 2400 C, H, N analyzer. For column chromatography, silica gel (SiO_2, Merck Kieselgel 60 F_{254}) was used.

Preparative TLC (PTLC) was carried out on glass plates coated with Merck Kieselgel 60 F_{254s}. The usual work-up refers to washing of organic layers with brine, drying over anhydrous Na_2SO_4, and evaporating off the solvents under reduced pressure.

2-Methyl-3-methoxycarbonyl-7-bromo-A-ring pyrrole-DUM-Segment A (4a). To a solution of **3a** (20 mg, 0.078 mmol) in CCl_4 (1 mL) was added NBS (15 mg, 0.085 mmol). The mixture was stirred at room temperature for 3 h 40 min, and 0.01 M-phosphoric buffer (pH 7) was added to the mixture. Then, the whole was extracted with $CHCl_3$ and the usual work-up was conducted. Thereafter, the residue was purified by PTLC ($CHCl_3$:MeOH, 15:1) to give 18 mg (70%) of **4a**: ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 3.69 (1 H, dd, $J = 11.2$, 5.3 Hz), 3.64 (3H, s), 3.57 (1H, d, $J = 10.9$ Hz), 3.49–3.55 (1H, m), 2.38 (3H, s), 2.04 (1H, dd, $J = 7.8$, 3.1 Hz), 1.04 (1H, dd, $J = 4.6$, 3.6 Hz). FABMS: m/z 339, 337 $(M + H)^+$.

2-Methyl-3-methoxycarbonyl-7-chloro-A-ring pyrrole-DUM-Segment A (4b). Yield 63%; ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 3.78 (1H, dd, $J = 10.9$, 5.3 Hz), 3.72 (3H, s), 3.66 (1H, d, $J = 10.9$ Hz), 3.55–3.61 (1H, m), 2.46 (3H, s), 2.15 (1H, dd, $J = 7.8$, 3.1 Hz), 1.14 (1H, dd, $J = 5.0$, 3.3 Hz). FABMS: m/z 293 $(M + H)^+$.

2-Methyl-3-methoxycarbonyl-7-acetyl-A-ring pyrrole-DUM-Segment A (4c). To a solution of BF_3-Et_2O (0.057 mL, 0.47 mmol) in Ac_2O (1.2 mL) was added **3a** (40 mg, 0.16 mmol). The mixture was stirred at room temperature for 2 h, and aqueous $NaHCO_3$ was added to the mixture. Then, the whole was extracted with $CHCl_3$ and the usual work-up was conducted. Thereafter, the residue was purified by PTLC ($CHCl_3$:MeOH, 15:1) to give 18 mg (39%) of **4c**: ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 3.91 (1H, dd, $J = 12.4$, 5.8 Hz), 3.81 (1H, d, $J = 12.2$ Hz), 3.76 (3H, s), 3.58–3.65 (1H, m), 2.64 (3H, d, $J = 1.0$ Hz), 2.52 (3H, d, $J = 1.0$ Hz), 2.06 (1H, dd, $J = 7.8$, 3.5 Hz), 1.02 (1H, dd, $J = 4.3$, 4.0 Hz). FABMS: m/z 301 $(M + H)^+$.

2-Methyl-3-methoxycarbonyl-7-formyl-A-ring pyrrole-DUM-Segment A (4d). To a solution of $TiCl_4$ (0.025 mL, 0.35 mmol) and Cl_2HCOMe (0.032 mL, 0.35 mmol) in CH_2Cl_2 (1.2 mL) was added **3a** (30 mg, 0.12 mmol). The mixture was stirred at room temperature for 4 h 20 min, and aqueous $NaHCO_3$ was added to the mixture. Then, the whole was extracted with AcOEt and the usual work-up was conducted. Thereafter, the residue was purified by PTLC ($CHCl_3$:MeOH, 15:1) to give 9.5 mg (29%) of **4d**: ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 9.92 (1H, s), 3.89 (1H, dd, $J = 12.5$, 5.6 Hz), 3.80 (1H, d, $J = 12.5$ Hz), 3.71 (3H, s), 3.58–3.62 (1H, m), 2.46 (3H, s), 2.10 (1H, dd, $J = 7.9$, 3.3 Hz), 1.03 (1H, dd, $J = 4.6$, 3.6 Hz). FABMS: m/z 287 $(M + H)^+$.

2-Methyl-3-methoxycarbonyl-7-methoxymethylcarbonyl-A-ring pyrrole-DUM-Segment A (4e). To a solution of $AlCl_3$ (62 mg, 0.47 mmol) and **3a** (28 mg, 0.11 mmol) in CH_2Cl_2 (2.4 mL) was added $MeOCH_2COCl$ (0.043 mL, 0.47 mmol). The mixture was stirred at room temperature for 1 h, and aqueous $NaHCO_3$ was added to the mixture. Then, the whole was extracted with $CHCl_3$ and the usual work-up was conducted. Thereafter, the residue was purified by PTLC ($CHCl_3$:MeOH, 15:1) to give 28 mg (36%) of **4e**: ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 4.70 (2H, s), 3.89 (1H, dd, $J = 12.5$, 5.6 Hz), 3.80 (1H, d, $J = 12.5$ Hz), 3.70 (3H, s), 3.55–3.62 (1H, m), 3.39 (3H, s), 2.47 (3H, s), 2.02 (1H, dd, $J = 7.9$, 3.3 Hz), 0.99 (1H, dd, $J = 4.3$, 4.0 Hz). FABMS: m/z 331 $(M + H)^+$.

2-Methyl-3-methoxycarbonyl-7-nitrosyl-A-ring pyrrole-DUM-Segment A (4f). To a solution of **3a** (20 mg, 0.078 mmol) in AcOH (0.4 mL) and 1,4-dioxane (0.4 mL) was added $NaNO_2$ (11 mg, 0.16 mmol). The mixture was stirred at −20 °C for 1 h 20 min, and aqueous $NaHCO_3$ was added to the mixture. Then, the whole was extracted with $CHCl_3$ and the usual work-up was conducted. Thereafter, the residue was purified by column chromatography ($CHCl_3$:MeOH, 20:1) to give 16 mg (70%) of **4f**: ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 4.02 (1H, dd, $J = 14.8$, 5.6 Hz), 3.93 (1H, dd, $J = 14.8$, 1.3 Hz), 3.74 (3H, s), 3.64–3.71 (1H, m), 2.52 (3H, s), 2.14 (1H, dd, $J = 7.9$, 3.6 Hz), 1.00 (1H, dd, $J = 5.0$, 4.0 Hz). FABMS: m/z 288 $(M + H)^+$.

2-Methyl-3, 7-diformyl-A-ring pyrrole-DUM-Segment A (4g). Yield 15%; ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 9.93 (1H, s), 9.67 (1H, s), 3.91 (1H, dd, $J = 12.9$, 5.6 Hz), 3.82 (1H, d, $J = 12.2$ Hz), 3.56–3.60 (1H, m), 2.52 (3H, s), 2.06 (1H, dd, $J = 7.8$, 3.5 Hz), 1.07 (1H, dd, $J = 4.6$, 4.0 Hz). FABMS: m/z 257 $(M + H)^+$.

2-Methyl-3-bromo-7-formyl-A-ring pyrrole-DUM-Segment A (4h). Yield 16%; ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 9.92 (1H, s), 3.93 (1H, dd, $J = 12.5$, 5.6 Hz), 3.84 (1H, d, $J = 12.2$ Hz), 3.26–3.33 (1H, m), 2.21 (3H, s), 2.08 (1H, dd, $J = 7.8$, 4.1 Hz), 1.06 (1H, dd, $J = 4.6$, 4.6 Hz). FABMS: m/z 309, 307 $(M + H)^+$.

4′-Methoxycinnamoyl 2-methyl-3-methoxycarbonyl-7-bromo-A-ring pyrrole-DUMC2 (5a). Method A: to a solution of **4a** (66 mg, 0.20 mmol) in AcOEt (3 mL) was added 6.86 N HCl in EtOH (0.085 mL, 0.59 mmol), and the mixture was stirred at room temperature for 1 h. After concentrating in vacuo, 4-methoxycinnamic acid (104 mg, 0.585 mmol) and EDCI (112 mg, 0.585 mmol) were added to a solution of the residue in DMF (3 mL), and the mixture was stirred at room temperature for 22 h 15 min. 0.01 M phosphate buffer (pH 7) was added to the mixture. Then, the whole was extracted with AcOEt and the usual work-up was conducted. Thereafter, the residue was purified by column chromatography ($CHCl_3$:MeOH, 100:1–80:1) to give 47 mg (45%) of **5a**: ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 7.59 (1H, d, $J = 15.5$ Hz), 7.40 (2H, d, $J = 8.6$ Hz), 6.79 (2H, d, $J = 8.6$ Hz), 6.58 (1H, d, $J = 15.8$ Hz), 4.47 (1H, d, $J = 10.6$ Hz), 4.11 (1H, dd, $J = 10.6$, 6.6 Hz), 4.01 (1H, m), 3.97 (3H, s), 3.80 (1H, dd, $J = 9.2$, 2.6 Hz), 3.78 (3H, s), 3.15 (1H, dd, $J = 10.6$, 10.2 Hz), 2.56 (3H, s). IR (KBr): 1645, 1603, 1574, 1512, 1446, 1421, 1360, 1254, 1207, 1173, 1095 cm^{-1}. FABMS: m/z 535, 533 $(M + H)^+$. Anal. calcd for $C_{24}H_{22}BrClN_2O_5 \cdot 0.5H_2O$: C, 53.11; H, 4.27; N, 5.16; found C, 52.95; H, 4.15; N, 4.64.

4′-Methoxycinnamoyl 2-methyl-3-methoxycarbonyl-7-bromo-A-ring pyrrole-DUMA (6a). To a solution of **5a** (11 mg, 0.020 mmol) in CH_3CN (0.82 mL) was added DBU (0.015 mL, 0.10 mmol). The mixture was stirred at room temperature for 1 h 20 min, and 0.01 M phosphate buffer (pH 7) was added to the mixture. Then, the whole was extracted with $CHCl_3$ and the usual work-up was conducted. Thereafter, the residue was purified by column chromatography ($CHCl_3$:acetone, 12:1) to give 7.0 mg (70%) of **6a**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.56 (1H, brs), 7.77 (1H, d, J = 15.5 Hz), 7.51 (2H, d, J = 8.9 Hz), 6.89 (2H, d, J = 8.9 Hz), 6.51 (1H, d, J = 15.5 Hz), 4.48 (1H, d, J = 11.5 Hz), 4.16 (1H, dd, J = 11.4, 4.5 Hz), 3.83 (3H, s), 3.83 (3H, s), 3.40–3.46 (1H, m), 2.72 (3H, s), 2.62 (1H, dd, J = 7.4, 3.8 Hz), 1.34 (1H, dd, J = 5.3, 4.0 Hz); IR (KBr): 1705, 1601, 1512, 1454, 1362, 1284, 1230, 1215, 1173, 1117, 1026 cm^{-1}. FABMS: m/z 499, 497 $(M+H)^+$. Anal. calcd for $C_{24}H_{21}BrN_2O_5 \cdot 0.2CHCl_3$: C, 55.77; H, 4.10; N, 5.37; found C, 55.68; H, 4.06; N, 4.69.

4′-Methoxycinnamoyl 2-methyl-3-methoxycarbonyl-7-chloro-A-ring pyrrole-DUMA (6b). Method A: yield 47%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.96 (1H, brs), 7.78 (1H, d, J = 15.2 Hz), 7.50 (2H, d, J = 8.6 Hz), 6.88 (2H, d, J = 8.9 Hz), 6.48 (1H, d, J = 15.2 Hz), 4.46 (1H, d, J = 11.6 Hz), 4.15 (1H, dd, J = 11.6, 4.3 Hz), 3.83 (3H, s), 3.82 (3H, s), 2.71 (3H, s), 2.62 (1H, dd, J = 7.3, 4.0 Hz), 1.33 (1H, dd, J = 5.0, 4.3 Hz). IR (KBr): 1664, 1616, 1603, 1512, 1454, 1369, 1281, 1232, 1205, 1173, 1120 cm^{-1}. FABMS: m/z 453 $(M+H)^+$. Anal. calcd for $C_{24}H_{21}ClN_2O_5 \cdot 0.5H_2O$: C, 62.41; H, 4.80; N, 6.06; found C, 62.75; H, 4.59; N, 5.75.

4′-Methoxycinnamoyl 2-methyl-3-methoxycarbonyl-7-methylcarbonyl-A-ring pyrrole-DUMA (6c). Method A: yield 25%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.54 (1H, brs), 7.73 (1H, d, J = 15.2 Hz), 7.49 (2H, d, J = 8.9 Hz), 6.90 (2H, d, J = 8.9 Hz), 6.48 (1H, d, J = 15.2 Hz), 4.33 (1H, dd, J = 10.1, 4.8 Hz), 4.21 (1H, d, J = 10.2 Hz), 3.85 (3 H, s), 3.81 (3H, s), 3.55–3.61 (1H, m), 2.69 (3H, s), 2.62 (3H, s), 2.48 (1H, dd, J = 7.4, 3.5 Hz), 1.27 (1H, dd, J = 4.9, 4.3 Hz). IR (KBr): 1697, 1603, 1512, 1379, 1286, 1255, 1232, 1173, 1124, 1074 cm^{-1}. FABMS: m/z 461 $(M+H)^+$. FAB–HRMS calcd for $C_{26}H_{25}N_2O_6$ $(M+H)^+$ m/z 461.1712, found 461.1735. Anal. calcd for $C_{26}H_{24}N_2O_6 \cdot 0.4CHCl_3$: C, 62.39; H, 4.84; N, 5.51; found C, 62.60; H, 4.75; N, 5.26.

4′-Methoxycinnamoyl 2-methyl-3-methoxycarbonyl-7-formyl-A-ring pyrrole-DUMC2 (5d). Method A: yield 50%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 12.29 (1H, s), 9.89 (1H, s), 9.80 (1H, brs), 7.81 (1H, d, J = 15.2 Hz), 7.54 (2H, d, J = 8.6 Hz), 6.93 (2H, d, J = 8.6 Hz), 6.77 (1H, d, J = 15.5 Hz), 4.58 (1H, d, J = 10.6 Hz), 4.33 (1H, dd, J = 10.6, 7.6 Hz), 4.20–4.26 (1H, m), 3.92 (3H, s), 3.86 (1H, dd, J = 10.2, 3.0 Hz), 3.85 (3 H, s), 3.27 (1H, dd, J = 10.2, 10.2 Hz), 2.72 (3H, s). IR (KBr): 1701, 1662, 1655, 1601, 1512, 1421, 1352, 1281, 1254, 1173 cm^{-1}. FABMS: m/z 483 $(M+H)^+$. Anal. calcd for $C_{25}H_{23}ClN_2O_6 \cdot 1.5H_2O$: C, 58.88; H, 5.14; N, 5.49; found C, 59.26; H, 4.99; N, 5.24.

4′-Methoxycinnamoyl 2-methyl-3-methoxycarbonyl-7-methoxymethylcarbonyl-A-ring pyrrole-DUMC2 (5e). Method A: yield 26%; ^{1}H NMR (270 MHz, $CDCl_3$ + CD_3OD): δ 7.68 (1H, d, J = 15.5 Hz), 7.49 (2H, d, J = 8.6 Hz), 6.88 (2H, d, J = 8.6 Hz), 6.74 (1H, d, J = 15.5 Hz), 4.49 (1H, d, J = 9.9 Hz), 4.28 (2H, s), 4.20–4.33 (2H, m), 3.84 (3H, s), 3.78 (3H, s), 3.75 (1H, brd, J = 7.8 Hz), 3.29 (3H, s), 3.13 (1H, dd, J = 10.6, 9.2 Hz), 2.62 (3H, s). IR (KBr): 1655, 1599, 1512, 1423, 1306, 1257, 1198, 1173, 1092, 825 cm^{-1}. FABMS: m/z 527 $(M+H)^+$. FAB–HRMS calcd for $C_{27}H_{28}{}^{35}ClN_2O_7$ $(M+H)^+$ m/z 527.1585, found 527.1599.

4′-Methoxycinnamoyl 2-methyl-3-methoxycarbonyl-7-nitrosyl-A-ring pyrrole-DUMA (6f). Method B: to a solution of NaH (60%, 6.4 mg, 0.16 mmol) in DMF (0.3 mL) was added a DMF solution (0.5 mL) of **4f** (40 mg, 0.14 mmol). The mixture was stirred under Ar atomosphere at −20 °C for 2 h 20 min. Then, a solution of the *p*-nitrophenyl ester of 4-methoxycinnamic acid (44 mg, 0.15 mmol) in DMF (0.5 mL) was added and stirred for 1 h 40 min. 0.01 M-phosphoric buffer (pH 7) was added to the mixture. Then, the whole was extracted AcOEt and the usual work-up was conducted. Thereafter, the residue was purified by PTLC ($CHCl_3$: MeOH, 15:1) to give 27 mg (44%) of **6f**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.35 (1H, brs), 7.88 (1H, d, J = 15.8 Hz), 7.52 (2H, J = 8.9 Hz), 6.91 (2H, d, J = 8.6 Hz), 6.46 (1H, d, J = 16.2 Hz), 4.36 (2H, d, J = 4.0 Hz), 3.85 (3H, s), 3.80 (3H, s), 3.32–3.36 (1H, m), 2.66 (3H, s), 2.18 (1H , dd, J = 8.3, 3.6 Hz), 0.92 (1H, dd, J = 5.0, 4.0 Hz). IR (KBr): 1709, 1641, 1601, 1512, 1448, 1288, 1255, 1171, 1080, 962 cm^{-1}. FABMS: m/z 448 $(M+H)^+$. Anal. calcd for $C_{24}H_{21}N_3O_6 \cdot 0.8H_2O$: C, 62.41; H, 4.93; N, 9.10; found C, 62.45; H, 4.89; N, 8.90.

4′-Methoxycinnamoyl 2-methyl-3, 7-diformyl-A-ring pyrrole-DUMC2 (5g). Method A: yield 28%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 12.26 (1H, brs), 10.05 (1H, s), 9.39 (1H, s), 9.55 (1H, brs), 7.81 (1H, d, J = 15.5 Hz), 7.56 (2H, d, J = 8.6 Hz), 6.94 (2H, d, J = 8.6 Hz), 6.78 (1H, d, J = 15.2 Hz), 4.59 (1H, d, J = 10.6 Hz), 4.39 (1H, dd, J = 10.2, 8.2 Hz), 4.25–4.32 (1H, m), 3.86 (3H, s), 3.85 (1H, brd, J = 9.4 Hz), 3.39 (1H, dd, J = 10.2, 8.6 Hz), 2.78 (3H, s). IR (KBr): 1653, 1601, 1512, 1473, 1421, 1360, 1304, 1254, 1173 cm^{-1}. FABMS: m/z 453 $(M+H)^+$. FAB–HRMS calcd for $C_{24}H_{22}{}^{35}ClN_2O_5$ $(M+H)^+$ m/z 453.1217, found 453.1232. Anal. calcd for $C_{24}H_{21}ClN_2O_5 \cdot 1.0H_2O$: C, 61.21; H, 4.92; N, 5.95; found C, 60.91; H, 5.21; N, 5.41.

4′-Methoxycinnamoyl 2-methyl-3-bromo-7-formyl-A-ring pyrrole-DUMC2 (5h). Method A: yield 38%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 12.55 (1 H, s), 9.92 (1H, s), 9.06 (1 H, brs), 7.81 (1H, d, J = 15.5 Hz), 7.54 (2H, d, J = 8.3 Hz), 6.93 (2H, d, J = 8.6 Hz), 6.76 (1H, d, J = 15.5 Hz), 4.62 (1H, d, J = 10.9 Hz), 4.37 (1H, dd, J = 10.6, 7.6 Hz), 3.95–4.02 (2H, m), 3.85 (3H, s), 3.36 (1H, dd, J = 11.2 Hz), 2.45 (3H, s). IR (KBr): 1655, 1601, 1541, 1510, 1421, 1348, 1252, 1171 cm^{-1}. FABMS: m/z 505, 503 $(M+H)^+$. Anal. calcd for $C_{23}H_{20}BrClN_2O_4 \cdot 0.5H_2O$: C, 53.87; H, 4.13; N, 5.46; found C, 53.88; H, 4.17; N, 5.10.

2-Methyl-3-methoxycarbonyl-6,7-(methanoiminomethano)-A-ring pyrrole-DUM-Segment-A (7). To a solution of **3a**

(236 mg, 0.915 mmol) in TFA (7 mL) was added HMT (257 mg, 1.83 mmol). The mixture was stirred at room temperature for 3 h 40 min, and aqueous $NaHCO_3$ was added to the mixture. Then, the whole was extracted with $CHCl_3$ and the usual work-up was conducted. Thereafter, the residue was purified by column chromatography ($CHCl_3$:MeOH, 15:1) to give 155 mg (57%) of 7: ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.73 (1H, brs), 4.21 (1H, d, J = 11.9 Hz), 4.02 (1H, d, J = 10.9 Hz), 3.83 (1H, d, J = 16.8 Hz), 3.78 (3H, s), 3.75 (1H, d, J = 14.2 Hz), 3.47–3.55 (2H, m), 3.39–3.45 (1H, m), 2.59 (3H, s), 2.28 (1H, dd, J = 7.6, 2.6 Hz), 1.21 (1H, dd, J = 4.6, 3.0 Hz). FABMS: m/z 300 $(M+H)^+$.

4′-Methoxycinnamoyl 2-methyl-3-methoxycarbonyl-6,7-(methanoiminomethano)-A-ring pyrrole-DUMA (8). 4-Methoxycinnamic acid (27 mg, 0.15 mmol) and EDCI (29 mg, 0.15 mmol) were added to a solution of **7** (15 mg, 0.050 mmol) in CH_2Cl_2 (1 mL). The mixture was stirred at room temperature for 3 h 40 min, and 0.01 M phosphate buffer (pH 7) was added to the mixture. Then, the whole was extracted with $CHCl_3$ and the usual work-up was conducted. Thereafter, the residue was purified by PTLC ($CHCl_3$:MeOH, 20:1) to give 16 mg (70%) of **8**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 10.97 (1H, brs), 7.70 (1H, d, J = 15.5 Hz), 7.50 (2H, d, J = 8.9 Hz), 6.90 (2H, d, J = 8.6 Hz), 6.89 (1H, d, J = 17.8 Hz), 5.53 (1H, d, J = 10.6 Hz), 4.75 (1H, d, J = 15.5 Hz), 4.49 (1H, d, J = 15.5 Hz), 4.24 (1H, d, J = 10.9 Hz), 3.84 (3H, s), 3.78 (3H, s), 3.69 (1H, d, J = 9.9 Hz), 3.46–3.57 (2H, m), 2.58 (3H, s), 2.34 (1H, dd, J = 7.3, 3.0 Hz), 1.36 (1H, dd, J = 4.0, 3.6 Hz). IR (KBr): 1601, 1576, 1512, 1456, 1427, 1281, 1252, 1173, 1120, 1084 cm^{-1}. FABMS: m/z 460 $(M+H)^+$. FAB–HRMS calcd for $C_{26}H_{26}N_3O_5$ $(M+H)^+$ m/z 460.1873, found 460.1894. Anal. calcd for $C_{26}H_{25}N_3O_5 \cdot 1.0H_2O$: C, 65.40; H, 5.70; N, 8.80; found C, 65.50; H, 5.73; N, 8.24.

5′,6′,7′-Trimethoxyindole-2′-carboxyl 2-methyl-3-methoxycarbonyl-6,7-(methanoiminomethano)-A-ring pyrrole-DUMA (9). Yield 76%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.92 (1H, brs), 9.42 (1H, brs), 6.89 (1H, s), 6.79 (1H, s), 5.51 (1H, d, J = 10.6 Hz), 5.13 (1H, d, J = 15.8 Hz), 4.68 (1H, d, J = 14.8 Hz), 4.34 (1H, d, J = 10.9 Hz), 4.03 (3H, s), 3.91 (3H, s), 3.90 (3H, s), 3.76 (3H, s), 3.66 (1H, d, J = 9.2 Hz), 3.46–3.54 (2H, m), 2.54 (3H, s), 2.33 (1H, dd, J = 7.3, 2.3 Hz), 1.32 (1H, dd, J = 4.3, 3.2 Hz). IR (KBr): 1701, 1635, 1579, 1464, 1429, 1306, 1257, 1223, 1196, 1119 cm^{-1}. FABMS: m/z 533 $(M+H)^+$. Anal. calcd for $C_{28}H_{28}N_4O_7 \cdot 0.5H_2O$: C, 62.10; H, 5.40; N, 10.35; found C, 61.82; H, 5.46; N, 9.68.

β-(5′,6′,7′-Trimethoxy-2′-indole) acryloyl 2-methyl-3-methoxycarbonyl-6,7-(methanoiminomethano)-A-ring pyrrole-DUMA (10). Yield 43%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.10 (1H, brs), 10.35 (1H, brs), 7.80 (1H, d, J = 15.2 Hz), 7.14 (1H, d, J = 15.5 Hz), 6.79 (1H, s), 6.66 (1H, d, J = 2.0 Hz), 5.49 (1H, d, J = 10.9 Hz), 4.88 (1H, d, J = 15.5 Hz), 4.55 (1H, d, J = 15.5 Hz), 4.20 (1H, d, J = 10.9 Hz), 4.06 (3H, s), 3.90 (3H, s), 3.87 (3H, s), 3.72 (3H, s), 3.56 (1H, d, J = 9.2 Hz), 3.41–3.51 (2H, m), 2.52 (3H, s), 2.28 (1H, dd, J = 7.3, 2.6 Hz), 1.23 (1H, m). IR (KBr): 1647, 1630, 1618, 1578, 1466, 1448, 1313, 1213, 1200, 1122 cm^{-1}. FABMS: m/z 559 $(M+H)^+$. Anal. calcd for $C_{30}H_{30}N_4O_7 \cdot 1.0H_2O$: C, 62.49; H, 5.59; N, 9.72; found C, 62.64; H, 5.57; N, 9.48.

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+}- and 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, 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 using a Microcell Counter (CC-180A, 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) were kindly supplied by the National Cancer Center (Tokyo, Japan). Sarcoma 180 cells were passaged and used for the experiment in adult male ddY mice. Human xenografts were passaged and used in adult male BALB/c-*nu*/*nu* mice. Murine solid tumor was inoculated subcutaneously (sc) at the axillary region of mice. Human xenografts were inoculated sc in the flank of nude mice. Drugs were administered intravenously (iv) beginning 1 day after tumor inoculation. Antitumor efficacy is 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.[13]

The criteria for effectiveness against murine solid tumors were the percentage T/C values with 42% and less, and statistical significance was 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 on the day of evaluation and V_0 is the tumor volume on the day of initial drug treatment. The criteria for effectiveness were T/C values with 50% and less, and statistical significance was determined by the Mann–Whitney U test ($P < 0.01$, one-sided).[14]

Hematotoxicity (effect of compounds on peripheral blood (PB) platelet counts and white blood cell counts)

Effect on PB platelet counts. Each drug was dissolved with saline and was administered into the tail vein of

normal male ddY mice (mean weight 20 ± 1 g). After 7 days, peripheral blood was obtained from the orbital vein to measure the platelet counts using a microcell counter (CC-180A, Toa Medical Electronics Co., Ltd., Kobe, Japan). Results are presented as percentage of the absolute value of the treated group versus that of control (percent of control).

Effect on PB white blood cell counts. Drugs were administered intravenously (iv) beginning 1 day after tumor inoculation. After 4 days, peripheral blood was obtained from the orbital vein of tumor-bearing mice to measure the white blood cell counts using a microcell counter (CC-180A, Toa Medical Electronics Co., Ltd., Kobe, Japan). Results are presented as percentage of the absolute value of the treated group versus that of control (percent of control).

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