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Synthesis and Antitumor Activity of Duocarmycin Derivatives: A-Ring Pyrrole Compounds Bearing β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl Group

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Abstract—A series of A-ring pyrrole derivatives of duocarmycin bearing β-(5′,6′,7′-trimethoxy-2′-indolyl)acryloyl group 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. New Seg-B analogues bearing β-(5′,6′,7′-trimethoxy-2′-indolyl)acryloyl group containing double bond as spacer had lower peripheral blood toxicity than the derivatives bearing 5′,6′,7′-trimethoxyindole-2′-carboxyl group in Seg-B of the natural type. Moreover, most of them exhibited potent antitumor activity against in vivo murine tumor models.

Introduction

A new class of antitumor antibiotics produced by *Streptomyces* sp., including duocarmycins (DUMs) A (**1a**), B1, B2 (**1b**), C1, C2 (**1c**) and SA possess exceptionally potent cytotoxicity (Fig. 1).[1,2] DUMA (**1a**), which is considered to be an active form among these DUMs, possesses a unique cyclopropane ring with the alkylating ability of DNA. DUMA (**1a**) and DUMSA have been reported to show this cytotoxicity through a sequence-selective alkylation of double-stranded DNA mediating N3 adenine covalent adduct formation.[3] KW-2189 (**2**),[4] 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.[5,6] It showed strong activities against murine ascitic and human solid tumors.[4b] KW-2189 (**2**) was designed as a prodrug which requires enzymatic hydrolysis followed by regeneration of DU-86 (**3a**) as an active metabolite.[7]

The segment-A (Seg-A) containing a spirocyclopropylhexadienone moiety is necessary for the formation of covalent bonding with DNA.[4c] On the other hand, the segment-B (Seg-B) has been considered to play an important role for noncovalent binding to the minor groove of DNA.[8] We have previously synthesized a series of DUM analogues bearing the simplified DNA-binding moieties.[9] Among them, A-ring pyrrole DUMs bearing cinnamoyl (**3b**)[9b] or heteroarylacryoyl groups[10] showed remarkably potent in vivo antitumor activity and low peripheral blood toxicity, compared with the A-ring pyrrole derivatives having 5′,6′,7′-trimethoxyindole-2′-carboxyl group in Seg-B. The previous structure–activity relationships (SAR) of the Seg-B analogues suggest that our approach to synthesize the new Seg-B analogues bearing β-(5′,6′,7′-trimethoxy-2′-indolyl)acryloyl group containing double bond as spacer may enhance the potency and decrease toxicity. This paper describes the synthesis, in vitro anticellular and in vivo antitumor activity, and hematotoxicity of new Seg-B duocarmycin analogues.

Chemistry

Initially, the 2-methyl-3-methoxycarbonyl A-ring pyrrole compound (**3a**) was treated with NaOMe in MeOH to afford compound **4a** (Seg-A) and methyl 5,6,7-trimethoxyindole-2-carboxylate (**5**), quantitatively (Scheme 1).[9b,11] The obtained compound **5** was allowed to reduce using DIBAL-H and then to oxidize using CrO_3–Py to

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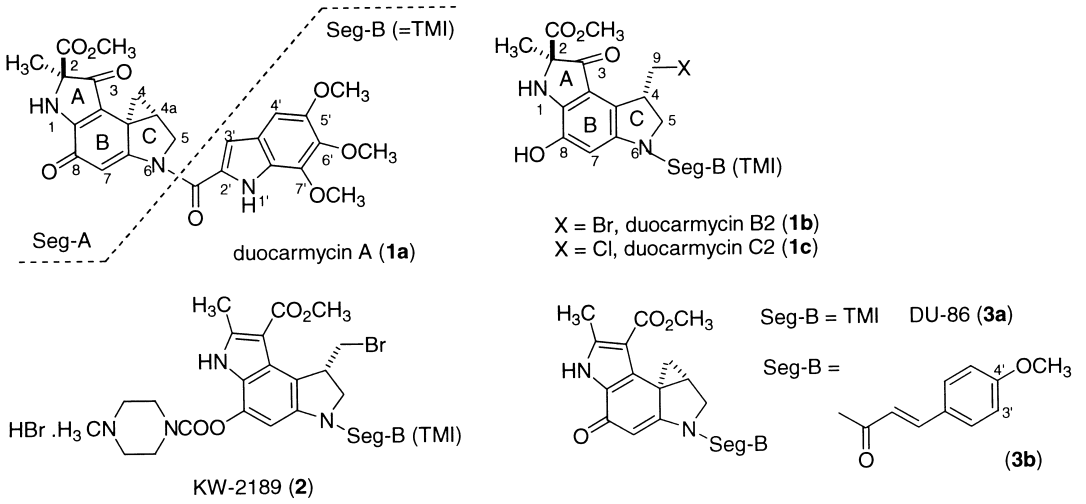
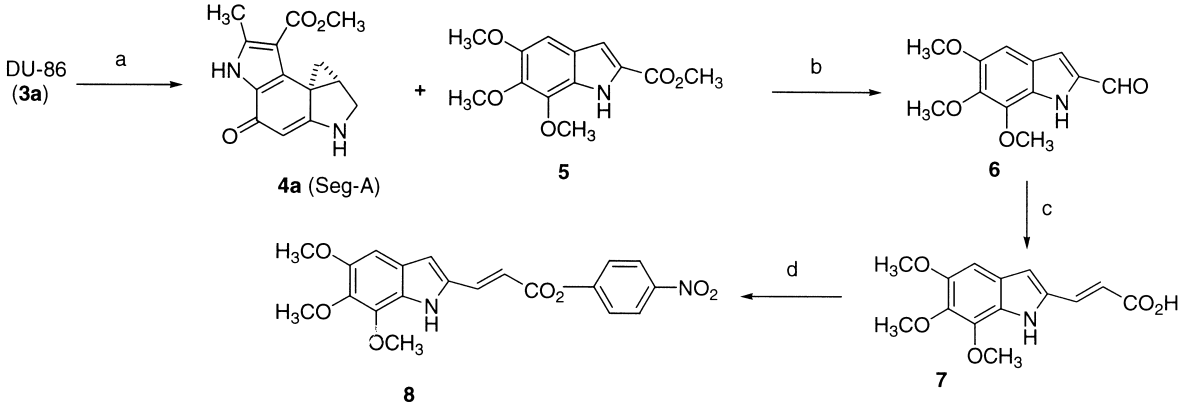


Figure 1. Structure of duocarmycins and duocarmycin derivatives.



Scheme 1. (a) NaOMe, MeOH, rt (76%); (b) (1) DIBAL-H, THF, 0 °C; (2) CrO_3–Py, CH_2Cl_2, 0 °C–rt (36%); (c) (1) NaH, $(EtO)_2P(=O)CH_2CO_2Et$; (2) 4 N KOH, MeOH, 50 °C (92%); (d) *p*-nitrophenol, Et_3N, 2-chloro-1-methylpyridinium iodide, CH_2Cl_2, reflux (94%).

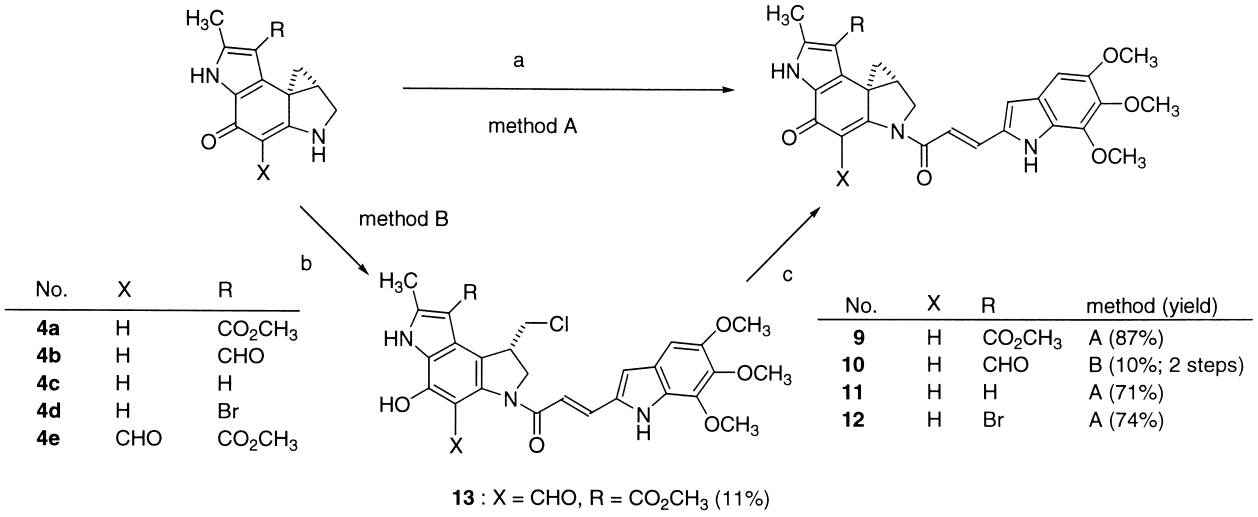
yield 2-formyl-5,6,7-trimethoxyindole (**6**). Ethyl 3-(5,6,7-trimethoxy-2-indolyl)acrylate was synthesized from compound **6** and triethyl phosphonoacetate by Horner–Emmons reaction.[12] The ethyl 3-(5,6,7-trimethoxy-2-indolyl)acrylate was treated with 4 N KOH to afford compound **7**, which was converted to *p*-nitrophenyl esters (**8**) by the reaction with *p*-nitrophenol using 1-methy-2-chloropyridinium iodide (Mukaiyama's method).[13]

The preparation of compounds **9**–**13** has been investigated by two approaches.[14] Compounds **4a**, **4c**, **4d** were allowed to react with (*E*)-*p*-nitrophenyl 3-(5,6,7-trimethoxy-2-indolyl)acrylate (**8**) in the presence of NaH to yield the corresponding cyclopropane compounds (**9**, **11**, **12**) in reasonable yield (see Scheme 2, method A). On the other hand, the intermediate of **10** and the compound **13** were prepared by the reaction of **4b**, **4e** with 4 N HCl in AcOEt followed by the addition of compound **7** in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), respectively (see Scheme 2, method B). The obtained intermediate of **10** was converted to **10** upon treatment with DBU in CH_3CN. However, compound **13** caused decomposition under the same conditions.

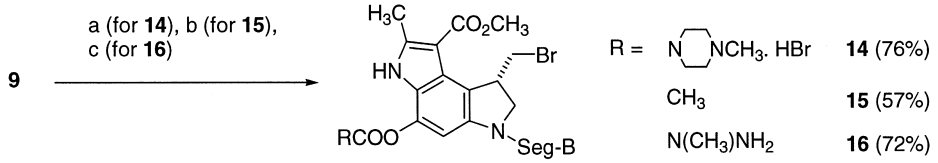
In order to enhance in vivo antitumor activities of β-(5′,6′,7′-trimethoxy-2′-indoyl)acrylate (**9**), the 8-*O*-substituted derivatives were prepared (as described in preceding papers).[4a,15] The preparation of the 8-*O*-substituted analogues is outlined in Scheme 3. The 8-*O*-acetate (**15**) was prepared by the reaction of **9** with HBr in CH_3CN followed by the addition of acetic anhydride in the presence of 4-dimethylaminopyridine (DMAP). Compound **9** was treated with HBr, followed by conversion to the 4-nitrophenylcarbonate by the reaction with 4-nitrophenyl chloroformate in the presence of triethylamine. The carbonate was allowed to react with *N*-methylpiperazine or methylhydrazine to produce the 8-*O*-[(*N*-methylpiperazinyl)carbonyl] (the free base of **14**) and 8-*O*-(2-methylcarbazoyl) (**16**) compounds. The obtained 8-*O*-[(*N*-methylpiperazinyl)carbonyl] derivative was converted to the HBr salt (**14**) upon treatment with HBr in AcOEt. The aqueous solubility of this salt (**14**) was found to be 1.2 mg/mL.

Results and Discussion

The antitumor activity of some representative derivatives was evaluated primarily by assays of the inhibition



Scheme 2. (a) NaH, **8**, DMF, −20 °C; (b) (1) 4 N HCl, AcOEt; (2) **7**, EDCl, DMF, rt; (c) DBU, CH_3CN, rt.



Scheme 3. (a) (1) HBr, CH_3CN, rt; (2) 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, −78 °C; (3) *N*-methylpiperazine, 0 °C; (4) HBr; (b) (1) HBr, CH_3CN, rt; (2) Ac_2O, DMAP, CH_2Cl_2, 0 °C; (c) (1) HBr, CH_3CN, rt; (2) 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, −78 °C; (3) $MeHNNH_2$.

of HeLa S_3 cell 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 represent means of tumor volume in treated and control mice, respectively. The 3-formyl (**10**) and 3-bromo (**12**) compounds exhibited strong anticellular activity with IC_{50} values below 0.1 nM at 1 h exposure, which was almost equivalent to compound **3a** (DU-86). This tendency was identical with SAR of 3-substituted A-ring pyrrole DUM analogues in a previous paper.[16]

Peripheral blood toxicity (reduction of the number of peripheral blood platelets) of β-(5′,6′,7′-trimethoxy-2′-indolyl)acrylates (**9**–**16**) was lower than that of the derivative bearing Seg-B of natural type. This tendency was similar to that of cinnamates (**3b**)[9b] and heteroarylacrylates.[10] These results suggest that new Seg-B analogues bearing β-(5′,6′,7′-trimethoxy-2′-indolyl)acryloyl group containing double bond as spacer decrease peripheral blood toxicity by noncovalent binding site change attributable to double bond as spacer.

The cyclopropane compounds (**9**–**12**) showed strong antitumor activity against murine solid tumor, which was almost equivalent to that of compound **3a**. In contrast, 7-formyl compound (**13**) showed inferior anticellular and antitumor activity to that of 7-hydro compounds (**9**–**12**). All of the 8-*O*-substituted derivatives (**14**–**16**) of **9** exhibited sufficient efficacy with low peripheral blood toxicity. Among them, 8-*O*-(2-methylcarbazoyl) compound (**16**) showed excellent activity in vivo with T/C values of 0.07. Compound **16** was evaluated in vivo for efficacy in nude mice bearing human xenograft St-4 (poor differentiated stomach adenocarcinoma). Compound **16** showed effective activity T/C values of 0.34 (3 mg/kg dose). Its potency against St-4 human stomach tumor xenograft was nearly comparable

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

No.	HeLa S_3 IC_{50} (nM)[a]		Sarcoma 180 (sc-iv)[b]		Hematotoxicity	
	1 h	72 h	Dose (mg/kg)	T/C[c]	WBC[d] (%)	PL[e] (%)
9	0.36	0.19	0.25	0.25	20	76
10	0.044	0.091	0.13	0.20	32	74
11	0.49	0.14	0.25	0.24	21	58
12	0.058	0.022	0.13	0.20	26	82
13	71	56	8.0	0.42	50	91
14	59	3.2	4.0	0.19	22	94
15	0.64	0.082	0.5	0.22	29	87
16	4.2	0.27	2.0	0.07	26	91
3a	0.045	0.0052	0.25	0.21	22	38
2	53	1.6	0.63	0.15	24	10

[a]Drug concentration required to inhibit the growth of HeLa S_3 cells by 50%.
[b]Mice (five mice/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).

to the potency of our clinical candidate KW-2189 (**2**) ($T/C = 0.12$ (0.63 mg/kg dose)).[4]

Conclusions

A series of A-ring pyrrole compounds of duocarmycin bearing β-(5′,6′,7′-trimethoxy-2′-indolyl)acryloyl group were prepared and evaluated for their anticellular activity against Hela S_3 cells and for antitumor activity against sarcoma 180 murine solid tumor and St-4 human stomach tumor xenograft. The A-ring pyrrole derivatives bearing β-(5′,6′,7′-trimethoxy-2′-indolyl)-acryloyl group containing double bond as spacer to Seg-B of the natural type showed weaker peripheral blood toxicity than derivatives having the Seg-B of the natural type. Moreover, most of them exhibited potent antitumor activity against in vivo murine tumor models.

Experimental

Infra-red spectra (IR) were recorded on a JASCO IR-810 spectrometer. ^{1}H NMR spectra were measured on JEOL JNM-EX270 and Hitachi R-90H 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.

(*E*)-*p*-Nitrophenyl 3-(5,6,7-trimethoxy-2-indolyl)acrylate (8). To a solution of **5** (265 mg, 1.00 mmol) in dry THF (15 mL) was added DIBAL-H in toluene (1 M, 3 mL, 3 mmol), and the mixture was stirred at 0 °C for 3 h. The reaction was quenched by addition of water, and then the whole was extracted with AcOEt. The usual work up afforded 237 mg (100%) of 2-hydroxymethyl-5,6,7-trimethoxyindole. CrO_3 (348 mg, 3.48 mmol) was added to pyridine (1.2 mL), and the mixture was stirred at room temperature for 20 min. After adding CH_2Cl_2 (2 mL) to this mixture, a solution of 2-hydroxymethyl-5,6,7-trimethoxyindole (276 mg, 1.16 mmol) in CH_2Cl_2 (8 mL) was added to the mixture and stirred from 0 °C to the room temperature for 17 h. 0.5 N HCl was added to the mixture, and then the whole was filtered. The filtrate was extracted with AcOEt and then worked up as usual. Thereafter, the residue was purified by column chromatography (hexane:AcOEt, 5:1) to give 98 mg (36%) of **6**: ^{1}H NMR (90 MHz, $CDCl_3$): δ 9.75 (1H, s), 9.14 (1H, brs), 7.13 (1H, d, $J = 2.2$ Hz), 6.85 (1H, s), 4.06 (3H, s), 3.94 (3H, s), 3.90 (3H, s).

To a solution of NaH (60%, 30 mg, 0.75 mmol) in THF (1 mL) was added a solution of triethyl phosphonoacetate (168 mg, 0.751 mmol) in THF (0.5 mL). The mixture was stirred under Ar atmosphere at 0 °C. After 20 min, a solution of **6** (98 mg, 0.42 mmol) in THF (1.5 mL) was added, and the mixture was stirred for 1 h 20 min. The reaction was quenced by addition of water, and the resulting mixture was poured into 0.5 N HCl. The whole was extracted with AcOEt and worked up as usual. Thereafter, the residue was purified by column chromatography (hexane:AcOEt, 7:1–5:1) to give 117 mg (93%) of ethyl 3-(5,6,7-trimethoxy-2-indolyl)-acrylate. To a solution of ethyl 3-(5,6,7-trimethoxy-2-indolyl)-acrylate (135 mg, 0.445 mmol) in MeOH (3 mL) was added 4 N KOH (0.33 mL, 1.34 mmol). The mixture was stirred at 50 °C for 1 h 30 min. The reaction mixture was poured into 0.5 N HCl, and the combine was extracted AcOEt. The usual work up afforded 121 mg (99%) of **7**: ^{1}H NMR (90 MHz, $CDCl_3$): δ 8.44 (1H, brs), 7.72 (1H, d, $J = 16.0$ Hz), 6.79 (1H, s), 6.75 (1H, d, $J = 2.0$ Hz), 6.20 (1H, d, $J = 15.8$ Hz), 4.09 (3H, s), 3.93 (3H, s), 3.89 (3H, s).

To a solution of **7** (500 mg, 3.24 mmol) in CH_2Cl_2 (20 mL) were added *p*-nitrophenol (766 mg, 5.51 mmol), triethylamine (1.54 mL, 11.0 mmol), 2-chloro-1-methylpyridinium iodide (1.41 g, 5.51 mmol) and the mixture was heated under reflux (bath temperature, 50 °C) for 2 h 30 min. The reaction mixture was poured into aqueous $NaHCO_3$, and the combine was extracted with $CHCl_3$ and worked up as usual. Thereafter, the residue was purified by column chromatography ($CHCl_3$) to give 842 mg (94%) of **8**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 8.44 (1H, brs), 8.30 (2H, d, $J = 8.9$ Hz), 7.84 (1H, d, $J = 15.8$ Hz), 7.37 (2H, d, $J = 9.2$ Hz), 6.82 (1H, d, $J = 2.0$ Hz), 6.80 (1H, s), 6.34 (1H, d, $J = 15.8$ Hz), 4.10 (3H, s), 3.94 (3H, s), 3.90 (3H, s); FAB–MS *m*/*z* 399 $(M + H)^+$.

β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl 2-methyl-3-methoxycarbonyl-A-ring pyrrole duocarmycin (9). To a solution of NaH (60%, 39 mg, 0.93 mmol) in DMF (1 mL) was added a DMF solution (3.5 mL) of **4a** (Seg-A) (200 mg, 0.775 mmol), and the mixture was stirred under Ar atomosphere at −20 °C for 2 h 40 min. Then, a solution of **8** (340 mg, 0.853 mmol) in DMF (3.5 mL) was added and stirred for 3 h 10 min. 0.01 M phosphate buffer (pH 7) was added to the mixture. The whole was extracted with AcOEt and then worked up as usual. Thereafter, the residue was purified by column chromatography ($CHCl_3$:MeOH, 100:1–80:1) to give 347 mg (87%) of **9**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.88 (1H, br), 10.64 (1H, brs), 7.65 (1H, d, $J = 15.5$ Hz), 7.23 (1H, d, $J = 17.2$ Hz), 6.73 (1H, s), 6.58 (1H, s), 6.43 (1H, brs), 4.23–4.27 (2H, br), 3.86 (3H, s), 3.84 (3H, s), 3.80 (3H, s), 3.64 (3H, s), 3.37 (1H, br), 2.35 (3H, s), 2.22 (1H, br), 1.53 (1H, br). IR (KBr): 1705, 1660, 1608, 1464, 1390, 1360, 1294, 1219, 1109 cm^{-1}. FAB–MS: *m*/*z* 518 $(M + H)^+$. FAB–HRMS calcd for $C_{28}H_{28}N_3O_7$ $(M + H)^+$ *m*/*z* 518.1927, found 518.1939. Anal. calcd for $C_{28}H_{27}N_3O_7 \cdot 1.7H_2O$: C, 61.35; H, 5.59; N, 7.67; found: C, 61.30; H, 5.21; N, 7.02.

β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl 2-methyl-3-formyl-A-ring pyrrole duocarmycin (10). To a solution of **4b** (Seg-A) (15 mg, 0.066 mmol) in AcOEt (1 mL) was added 4 N HCl in AcOEt (0.082 mL, 0.33 mmol), and

the mixture was stirred at room temperature for 40 min. After concentrating in vacuo, **7** (54 mg, 0.20 mmol) and EDCI (38 mg, 0.20 mmol) were added to a solution of the residue in DMF (1 mL), and the mixture was stirred at room temperature for 22 h. 0.01 M phosphate buffer (pH 7) was added to the mixture. The whole was extracted with AcOEt and then worked up as usual. Thereafter, the residue was purified by PTLC ($CHCl_3$: MeOH, 10:1) to give 7.5 mg (22%) of β-(5′,6′,7′-trimethoxy-2′-indolyl)acryloyl 8-hydroxy 2-methyl-3-formyl-A-ring pyrrole duocarmycin C2: ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 9.79 (1H, s), 7.73 (1H, s), 7.59 (1H, d, $J = 15.2$ Hz), 6.88 (1H, d, $J = 15.5$ Hz), 6.69 (1H, s), 6.62 (1H, s), 4.41 (1H, d, $J = 10.2$ Hz), 4.31–4.33 (1H, m), 4.23 (1H, dd, $J = 9.6$, 8.9 Hz), 4.00 (3H, s), 3.84 (3H, s), 3.80 (3H, s), 3.47 (1H, br), 3.28 (1H, dd, $J = 8.6$, 7.6 Hz), 2.59 (3H, s). FAB–MS: m/z 524 $(M+H)^+$.

To a solution of β-(5′,6′,7′-trimethoxy-2′-indolyl)acryloyl 8-hydroxy 2-methyl-3-formyl-A-ring pyrrole duocarmycin C2 (7.5 mg, 0.014 mmol) in CH_3CN (0.8 mL) was added DBU (0.0064 mL, 0.043 mmol), and the mixture was stirred at room temperature for 40 min. 0.01 M phosphate buffer (pH 7) was added to the mixture. The whole was extracted with $CHCl_3$ and then worked up as usual. Thereafter, the residue was purified by PTLC ($CHCl_3$:acetone, 10:1) to give 3.3 mg (47%) of **10**: ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 9.74 (1H, s), 7.69 (1H, d, $J = 15.5$ Hz), 6.76 (1H, d, $J = 15.5$ Hz), 6.73 (1H, s), 6.70 (1H, s), 4.23 (1H, d, $J = 10.9$ Hz), 4.13 (1H, dd, $J = 11.2$, 5.0 Hz), 4.00 (3H, s), 3.86 (3H, s), 3.83 (3H, s), 3.46–3.50 (1H, m), 2.57 (3H, s), 2.31 (1H, dd, $J = 7.3$, 3.3 Hz), 1.29 (1H, dd, $J = 5.0$, 3.6 Hz). IR (KBr): 1666, 1606, 1466, 1390, 1340, 1279, 1240, 1221, 1169, 1124 cm^{-1}. FAB–MS: m/z 488 $(M+H)^+$. Anal. calcd for $C_{27}H_{25}N_3O_6 \cdot 0.5H_2O \cdot 0.5CHCl_3$: C, 59.38; H, 4.80; N, 7.55; found: C, 59.34; H, 5.10; N, 7.24.

β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl 2-methyl-A-ring pyrrole duocarmycin (11). Method A: Yield 71%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 11.22 (1H, brs), 10.85 (1H, brs), 7.63 (1H, d, $J = 15.5$ Hz), 7.30 (1H, d, $J = 17.2$ Hz), 6.73 (1H, s), 6.56 (1H, s), 6.34 (1H, brs), 5.34 (1H, s), 4.24 (1H, d, $J = 11.6$ Hz), 4.17 (1H, dd, $J = 11.7$, 4.5 Hz), 3.86 (3H, s), 3.83 (3H, s), 3.81 (3H, s), 2.49 (1H, m), 2.12 (3H, s), 1.43 (1H, dd, $J = 7.1$, 3.8 Hz), 1.25 (1H, m). IR (KBr): 1659, 1608, 1535, 1479, 1392, 1244, 1221, 1103 cm^{-1}. FAB–MS: m/z 460 $(M+H)^+$. Anal. calcd for $C_{26}H_{25}N_3O_5 \cdot 1.2H_2O$: C, 64.91; H, 5.74; N, 8.73; found: C, 64.90; H, 5.68; N, 8.48.

β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl 2-methyl-3-bromo-A-ring pyrrole duocarmycin (12). Method A: Yield 74%; ^{1}H NMR (270 MHz, $CDCl_3 + CD_3OD$): δ 7.56 (1H, d, $J = 15.2$ Hz), 6.74 (1H, d, $J = 15.2$ Hz), 6.64 (1H, s), 6.59 (1H, s), 6.55 (1H, br), 4.14 (1H, d, $J = 10.9$ Hz), 4.03 (1H, dd, $J = 11.1$, 4.8 Hz), 3.89 (3H, s), 3.76 (3H, s), 3.73 (3H, s), 3.05–3.11 (1H, m), 2.23 (1H, dd, $J = 7.6$, 4.0 Hz), 2.15 (3H, s), 1.16 (1H, dd, $J = 4.6$, 4.6 Hz). IR (KBr): 1666, 1606, 1464, 1392, 1356, 1277, 1221, 1174, 1051 cm^{-1}. FAB–MS: m/z 540, 538 $(M+H)^+$. Anal. calcd for $C_{26}H_{24}BrN_3O_5 \cdot 0.5H_2O$: C, 57.05; H, 4.60; N, 7.68; found: C, 57.09; H, 4.61; N, 7.49.

β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl 7-formyl 8-hydroxy 2-methyl-3-methoxycarbonyl-A-ring pyrrole duocarmycin C2 (13). Method B: Yield 11%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 12.32 (1H, brs), 9.90 (1H, s), 9.55 (1H, brs), 8.71 (1H, brs), 7.81 (1H, d, $J = 15.2$ Hz), 6.79 (1H, s), 6.75 (1H, s), 6.69 (1H, d, $J = 15.8$ Hz), 4.59 (1H, d, $J = 9.9$ Hz), 4.30 (1H, dd, $J = 9.6$, 9.6 Hz), 4.20–4.30 (1H, m), 4.10 (3H, s), 3.93 (6H, s), 3.90 (3H, s), 3.85 (1H, br), 3.29 (1H, dd, $J = 10.2$, 9.9 Hz), 2.71 (3H, s). IR (KBr): 1606, 1576, 1464, 1392, 1277, 1221, 1097, 1051 cm^{-1}. FAB–MS: m/z 582 $(M+H)^+$. FAB–HRMS calcd for $C_{29}H_{29}{}^{35}ClN_3O_8$ $(M+H)^+$ m/z 582.1644, found 582.1643. Anal. calcd for $C_{29}H_{28}ClN_3O_8 \cdot 2.0H_2O$: C, 56.36; H, 5.22; N, 6.80; found: C, 56.53; H, 5.12; N, 6.36.

β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl 8-*O*-[(*N*-methylpiperazinyl)carbonyl] 2-methyl-3-methoxycarbonyl-A-ring pyrrole duocarmycin B2 hydrobromide (14). Hydrobromic acid in methanol (5%, 250 mg, 0.154 mmol) was added to a solution of **9** (20 mg, 0.039 mmol) in CH_2Cl_2 (1.3 mL), and the mixture was stirred at room temperature for 1 h 10 min. After the mixture was cooled at −78 °C, *p*-nitrophenyl chloroformate (23 mg, 0.12 mmol) and triethylamine (0.022 mL, 0.15 mmol) were added to the mixture, and the mixture was stirred at −78 °C for 30 min. Then, *N*-methypiperazine (0.015 mL, 0.14 mmol) was added, and the mixture was stirred continually at 0 °C from −78 °C for 20 min. The mixture was extracted with $CHCl_3$ and washed with aqueous $NaHCO_3$ and worked up as usual. Thereafter, the residue was purified by PTLC ($CHCl_3$:MeOH, 15:1) to give 21 mg (76%) of the free base of **14**. A solution of the free base of **14** (16 mg, 0.022 mmol) in AcOEt (1.6 mL) was treated with anhydrous hydrobromic acid in methanol (5%, 108 mg) at room temperature for 30 min. The mixture was concentrated under reduced pressure to give 17 mg of **14**: ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.00 (1H, s), 11.49 (1H, s), 9.87 (1H, br), 8.13 (1H, s), 7.56 (1H, d, $J = 15.2$ Hz), 7.30 (1H, d, $J = 15.2$ Hz), 6.86 (1H, s), 6.79 (1H, d, $J = 1.7$ Hz), 4.37–4.59 (3H, m), 4.00 (3H, s), 3.86 (3H, s), 3.80 (6H, s), 3.71 (1H, br), 3.53–3.58 (5H, br), 3.15–3.34 (4H, br), 2.90 (3H, d, $J = 4.0$ Hz), 2.69 (3H, s). IR (KBr): 1699, 1695, 1645, 1470, 1435, 1423, 1416, 1254, 1219, 1095 cm^{-1}. FAB–MS (the free base): m/z 726, 724 $(M+H)^+$. FAB–HRMS (the free base) calcd for $C_{34}H_{39}{}^{79}BrN_5O_8$ $(M+H)^+$ m/z 724.1982, found 724.1957. Anal. calcd for $C_{34}H_{38}BrN_5O_8 \cdot HBr \cdot 5.0H_2O$: C, 45.60; H, 5.51; N, 7.82; found: C, 45.76; H, 5.38; N, 7.75.

β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl 8-*O*-acetyl 2-methyl-3-methoxycarbonyl-A-ring pyrrole duocarmycin B2 (15). Hydrobromic acid in methanol (5%, 244 mg, 0.151 mmol) was added to a solution of **9** (20 mg, 0.038 mmol) in CH_2Cl_2 (1.3 mL), and the mixture was stirred at room temperature for 60 min. After the mixture was cooled at 0 °C, Ac_2O (0.011 mL, 0.011 mmol) and DMAP (14 mg, 0.011 mmol) were added to the

stirred solution, and the mixture was stirred at 0 °C for 1 h. 0.01 M phosphate buffer (pH 7) was added to the mixture. The whole was extracted with $CHCl_3$, and the organic layer was worked up as usual. Thereafter, the reside was purified by HPLC (HPLC conditions: column: YMC-Pack ODS, S-5 μm, 120A, 250 mm×20 mm (YMC, Co, Ltd, Japan). Mobile Phase: CH_3CN:H_2O = 70:30. Detection: 330 nm. Flow rate: 30 mL/min) to give 14 mg (57%) of **15**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 8.68 (1H, brs), 8.55 (1H, brs), 7.56 (1H, d, J = 15.2 Hz), 6.79 (1H, s), 6.60 (1H, s), 6.46 (1H, d, J = 15.2 Hz), 4.42–4.53 (1H, m), 4.33 (1H, d, J = 10.2 Hz), 4.23 (1H, dd, J = 9.9, 8.6 Hz), 4.11 (3H, s), 3.96 (3H, s), 3.95 (3H, s), 3.90 (3H, s), 3.78 (1H, dd, J = 9.7, 2.5 Hz), 3.31 (1H, dd, J = 10.2, 10.2 Hz), 2.44 (3H, s), 2.34 (3H, s). IR (KBr): 1678, 1643, 1468, 1416, 1306, 1217, 1201, 1122, 1107, 1088 cm^{-1}. FAB–MS: m/z 642, 640 $(M+H)^+$. FAB–HRMS calcd for $C_{30}H_{31}$ $^{79}BrN_3O_8$ $(M+H)^+$ m/z 640.1295, found 640.1318.

β-(5′,6′,7′-Trimethoxy-2′-indolyl)acryloyl 8-*O*-(2-methylcarbazoyl) 2-methyl-3-methoxycarbonyl-A-ring pyrrole duocarmycin B2 (16). Hydrobromic acid in methanol (5%, 376 mg, 0.232 mmol) was added to a solution of **9** (30 mg, 0.058 mmol) in CH_2Cl_2 (1.4 mL), and the mixture was stirred at room temperature for 50 min. After the mixture was cooled at −78 °C *p*-nitrophenyl chloroformate (35 mg, 0.17 mmol) and triethylamine (0.040 mL, 0.29 mmol) were added to a stirred solution at −78 °C for 25 min. Methylhydrazine (0.015 mL, 0.29 mmol) was added to the mixture, and the mixture was attained at 0 °C for 30 min. The mixture was diluted with $CHCl_3$, and washed with aqueous $NaHCO_3$. The usual work up and purification by PTLC ($CHCl_3$: MeOH, 25:1) gave 28 mg (72%) of **16**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 10.52 (1H, brs), 9.98 (1H, brs), 8.11 (1H, s), 7.46 (1H, d, J = 15.2 Hz), 6.72 (1H, s), 6.66 (1H, d, J = 15.5 Hz), 6.53 (1H, s), 4.27–4.39 (1H, br), 4.30 (1H, d, J = 10.2 Hz), 4.10 (1H, dd, J = 9.9, 9.2 Hz), 4.02 (3H, s), 3.89 (3H, s), 3.86 (3H, s), 3.83 (3H, s), 3.70 (1H, dd, J = 9.6, 2.3 Hz), 3.28 (3H, brs), 3.20 (1H, dd, J = 10.2, 9.9 Hz), 2.30 (3H, brs). IR (KBr): 1701, 1697, 1645, 1466, 1414, 1350, 1306, 1219, 1159, 1109, 1090 cm^{-1}. FAB–MS: m/z 672, 670 $(M+H)^+$. FAB–HRMS calcd for $C_{30}H_{33}$$^{79}BrN_5O_8$ $(M+H)^+$ m/z 670.1513, found 670.1492. Anal. calcd for $C_{30}H_{32}BrN_5O_8 \cdot 2.5H_2O$: C, 50.36; H, 5.21; N, 9.79; found: C, 50.42; H, 4.83; N, 9.45.

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) cells 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 tumor volume (mm^3) = length (mm)×[width (mm)]^2/2 according to the method of the National Cancer Institute.[17]

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).[18]

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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