Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 381–391

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Antitumor Activity of Duocarmycin Derivatives: Modification at C-8 position of A-Ring Pyrrole Compounds Bearing the Simplified DNA-Binding Groups

Nobuyoshi Amishiro,* Satoru Nagamura, Chikara Murakata, Akihiko Okamoto, Eiji Kobayashi, Masao Asada, Katsushige Gomi, Tatsuya Tamaoki, Masami Okabe, Naoko Yamaguchi, Kazuo Yamaguchi and Hiromitsu Saito

Pharmaceutical Research Institute, Kyowa Hakko Kogyo Company, Ltd., 1188 Shimotogari, Nagaizumi, Sunto, Shizuoka, 411-8731, Japan

Received 9 September 1999; accepted 29 October 1999

Abstract—A series of the 8-*O*-substituted A-ring pyrrole derivatives of duocarmycin bearing the simplified DNA-binding moieties such as cinnamoyl or heteroarylacryloyl groups 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. In addition, the stability of the 8-*O*-substituted analogues in aqueous solution and the conversion to their active form (cyclopropane compound) from the 8-*O*-substituted analogues in mice or human serum were examined. The 8-*O*-substituted A-ring pyrrole derivatives bearing the simplified DNA-binding moieties showed remarkably potent in vivo antitumor activity and low peripheral blood toxicity compared with the 8-*O*-substituted A-ring pyrrole derivatives having the trimethoxyindole skeleton in segment-B (Seg-B), which were equal to 8-*O*-[(*N*-methylpiperazinyl)carbonyl] derivatives of 4′-methoxycinnamates and 4′-methoxy-β-heteroarylacrylates. Moreover, among 8-*O*-substituted analogues, several compounds can be chemically or enzymatically converted to their active form in human serum. This result indicated that new 8-*O*-substituted derivatives were different prodrugs from KW-2189 and 8-*O*-substituted analogues being the same type of prodrug as KW-2189.

Introduction

Duocarmycin(DUM)s (A, **1a**; SA, **1b**; B1, **1e**; B2, **1c**; C1, **1f**; C2, **1d**) are novel antitumor antibiotics isolated from the culture broth of *Streptomyces* sp. (Fig. 1).[1–8] DUMs are known to exhibit potent growth-inhibitory activity against human uterine cervix carcinoma HeLa S_3 in vitro, and also exhibit modest broad antitumor spectrum against murine transplantable solid tumor.[9–13] Since DUMB1 (**1e**), B2 (**1c**), C1 (**1f**), and C2 (**1d**) readily yield DUMA (**1a**) in aqueous solution, DUMA is thought to be an active form among these antibiotics. DUMA and DUMSA (**1b**) have a unique cyclopropane ring responsible for the sequence-selective alkylation of double-stranded DNA resulting in N3 adenine covalent adduct formation.[14–19] This mechanism is similar to that of CC-1065 (**1g**) which has been reported to show high cytotoxicity.[20–25] KW-2189 (**2b**),[26–29] selected as the best compound in analogues of A-ring pyrrole derivatives of duocarmycin B2, showed good stability in the culture medium and aqueous solubility greater than 10 mg/mL.[30–35] It showed strong activities against murine ascitic and human solid tumors.[27] KW-2189 (**2b**) is currently under phase II clinical evaluation.

The segment-A (Seg-A) has the electrophilic cyclopropane ring necessary for the formation of covalent bonding with DNA.[38] 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.[36–38] With the objective to identify novel promising candidates, we have previously synthesized a series of DUM analogues bearing the simplified DNA-binding moieties.[39–41] Among these Seg-B derivatives, some of A-ring pyrrole compounds bearing cinnamoyl[40] or heteroarylacryloyl[41] groups as Seg-B showed strong antitumor activity and low peripheral blood toxicity.[41] Moreover, the 8-*O*-(*N*,*N*-dialkylcarbamoyl) cinnamates having an amino group at the 3′-position, and 8-*O*-[(*N*-methylpiperazinyl)carbonyl]

Keywords: antibiotics; antitumor compounds; DNA; natural products; prodrug.

*Corresponding author.

0968-0896/00/$ - see front matter
PII: S0968-0896(99)00293-X

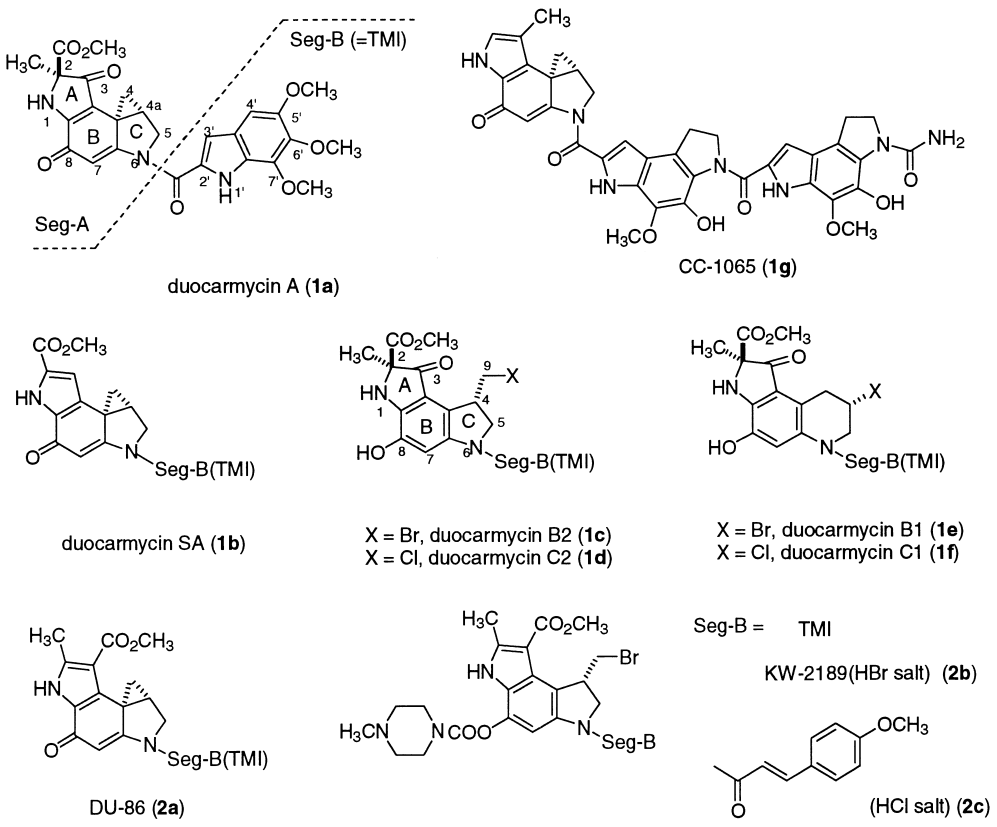


Figure 1. Structure of duocarmycins, CC-1065, and duocarmycins derivatives.

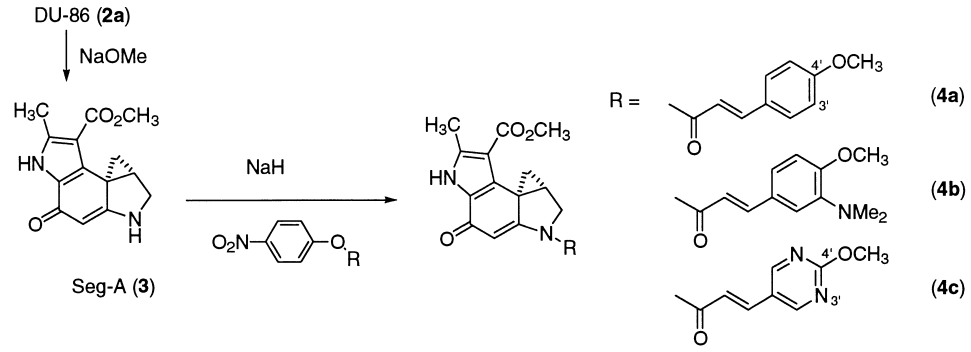
derivatives of 6-membered *N*-heteroarylacrylates were found to possess adequate water solubility in excess of 10 mg/mL.[40,41]

In the previous papers,[42] we have reported the synthesis of A-ring pyrrole derivatives modified to the various substituent at the C-8 phenolic hydroxyl group of Seg-A. We also have studied the structure–activity relationships (SAR) of their analogues, and the stability and solubility of their analogues under aqueous conditions. The results suggest that 8-*O*-substituted A-ring pyrrole analogues need to release an active compound **2a** (DU-86) chemically or enzymatically to exhibit any significant in vivo antitumor activity. It seems that DU-86 (**2a**) as a metabolite of A-ring pyrrole analogues is responsible for the in vivo antitumor activity of KW-2189 (**2b**). KW-2189 itself seems not to be dominant in its antitumor potency.[42–46] KW-2189 was activated by carboxyl esterase.[43,45,46] However, the conversion of KW-2189 to its active form was influenced by carboxyl esterase activity. As an example, KW-2189 was not activated in human serum, but in mouse serum. For prediction of the clinical effect, it is necessary that new prodrug be able to release to an active form in human serum. Unlike KW-2189 (**2b**), A-ring pyrrole derivatives bearing the simplified DNA-binding moieties further modified at the C-8 phenolic hydroxyl group are expected to release an active form in human serum. In this paper, we describe the synthesis of A-ring pyrrole derivatives bearing a cinnamoyl or heteroarylacryloyl group modified at the C-8 phenolic hydroxyl group to thiocarbonate, ester, and hydrazinocarboxylate, and also describe the evaluation of their anticellular and antitumor activities, hematotoxicity. In addition, we examined the stability and solubility of their analogues under aqueous conditions, and in vitro metabolism of their analogues in mice or human serum.

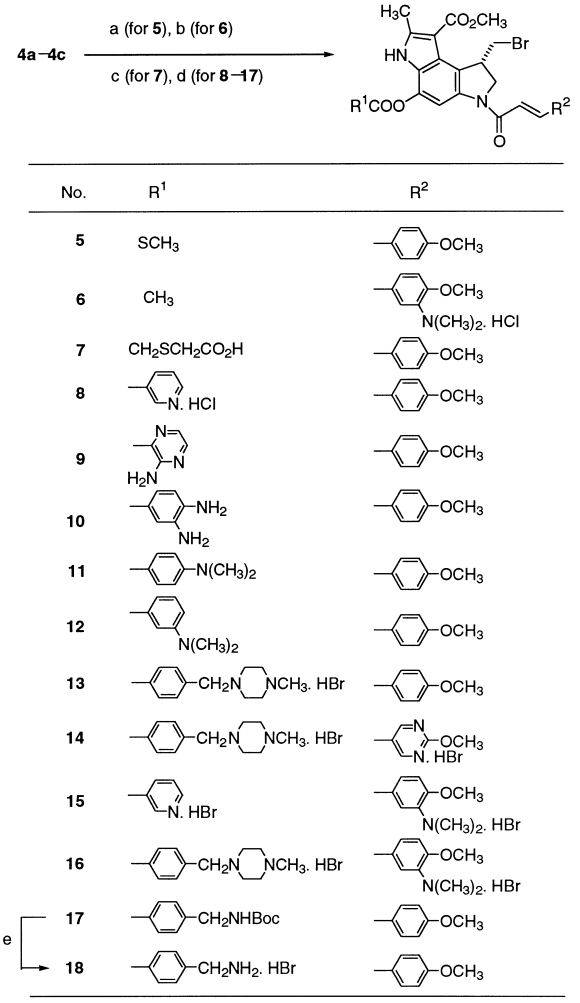
Chemistry

The 2-methyl-3-methoxycarbonyl A-ring pyrrole compound **2a** (DU-86) was initially prepared by employing the Wagner–Meerwein type rearrangement of the 8-*O*-protected-3-hydroxyduocarmycin B2 followed by deprotection of the protecting group under basic conditions.[40,41,47,48] Compound **2a** was treated with NaOMe in MeOH to quantitatively afford compound **3** (Seg-A) and methyl trimethoxyindole-2-carboxylate as shown in Scheme 1. The obtained compound **3** was allowed to react with substituted-cinnamic acid or substituted-heteroarylacrlic acid *p*-nitrophenyl esters in the presence of NaH to yield the corresponding cyclopropane compounds **4a**–**4c** in reasonable yield, as described previously.[40,41]

The 8-*O*-thiocarbonate (**5**) was prepared by the reaction of **4a** with 48% HBr in CH_3CN followed by the addition of methyl chlorothioformate in the presence of triethylamine (see Scheme 2). The 8-*O*-acetate (the free base of **6**) and the 8-*O*-carboxylmethylsulfanylacetate (**7**) were prepared by the reaction of **4a** or **4b** with 48% HBr in CH_3CN followed by the addition of acetic anhydride or thioglycolic anhydride in the presence of



Scheme 1.



Scheme 2. (a) (1) HBr, CH_3CN, (2) CH_3SCOCl, Et_3N, CH_2Cl_2; (b) (1) HBr, CH_3CN, (2) Ac_2O, 4-dimethylaminopyridine (DMAP), CH_2Cl_2, (3) HCl; (c) (1) HBr, CH_3CN, (2) thiodiglycolic anhydride, DMAP, CH_2Cl_2, (d) (1) HBr, CH_3CN, (2) R^1CO_2H, DCC, DMAP, CH_2Cl_2, (3) HCl for **8** or HBr for **13**–**16**; (e) (1) HBr, $ClCH_2CH_2Cl$, 60 °C, (2) HBr.

4-dimethylaminopyridine (DMAP).[42] Further, we prepared the 8-*O*-benzoates (**10**–**12**, **17**, the free base of **13**, **14**, **16**, **18**) and the 8-*O*-heteroaryl esters (**9**, the free base of **8**, **15**) with a hydrophilic moiety by the reaction with the corresponding carboxylic acid in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP).[42] Deprotection of the Boc group of **17** using HBr afforded the aminomethylbenzoate (**18**). The free bases of compound **6**, **8**, **13**–**16**, **18** were converted to the HCl salts (**6**, **8**) or HBr salts (**13**–**16**, **18**) upon treatment with HCl or HBr.

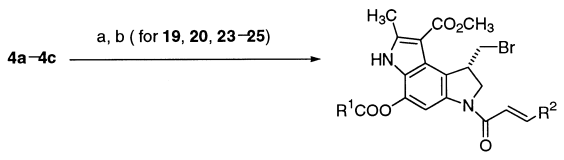
Compounds **4a**–**4c** were converted to the 4-nitrophenyl carbonate by the reaction with 4-nitrophenyl chloroformate in the presence of triethylamine, which was treated with various hydrazine, 1-amino-4-methylpiperazine and 1-amino-4-piperidinopiperidine to produce carbazoyl (**21**, the free base of **25**, **22**), piperazinylaminocarbonyl (the free base of **19**, **23**, **24**) and piperidinylaminocarbonyl (the free base of **20**) derivatives in reasonable yield (see Scheme 3). The free bases of **19**, **20**, **23**–**25** were converted to HBr salts (**19**, **20**, **23**–**25**) by the same method as shown in Scheme 2.

Results and Discussion

The aqueous solubility of HCl or HBr salts was shown in Table 1. The HCl or HBr salts (**6**, **14**–**16**, **23**–**25**) of 8-*O*-substituted derivatives of **4b** and **4c** were found to possess adequate water solubility in excess of 10 mg/mL. In contrast, the HCl or HBr salts (**8**, **13**, **18**) of 8-*O*-substituted derivatives of **4a** exhibited poor aqueous solubility below 0.1 mg/mL. For improving aqueous solubility, formation of organic acid salts of the free base of **13** was examined. The results was found that the conversion of the free base of **13** to $2MeSO_3H$ salt has improved aqueous solubility (>5 mg/mL). Furthermore, HBr salts (**19**, **20**) of piperazinylaminocarbonyl and piperidinylaminocarbonyl derivatives of **4a** were more aqueous soluble than HCl salt (**2c**) of piperazinylcarbamoyl derivative of **4a**.

The stability of compounds was measured in 0.05 M phosphate buffer (pH 7) containing 50% DMF by HPLC analysis (Table 1). The thioester (**5**), carboxylmethylsulfanylacetate (**7**), and heteroaryl esters (**8**, **9**) mainly decomposed to a cyclopropane compound (**4a**), which was different from the substituted benzoates (**10**, **11**–**13**). Similarly the heterocyclic aminocarbonyl (**19**, **20**) and carbazoyl (**21**, **22**) compounds mainly decomposed to a cyclopropane compound (**4a**).

Next, we examined in vitro metabolism of 8-*O*-substituted derivatives in mice or human serum (Table 3).



No.	R^1	R^2
19	NHN⟨⟩NCH3. 2HBr	⟨⟩OCH3
20	NHN⟨⟩N⟨⟩.2HBr	⟨⟩OCH3
21	$N(NH_2)CH_3$	⟨⟩OCH3
22	$N(CH_3)NHCH_3$	⟨⟩OCH3
23	NHN⟨⟩NCH3. HBr	N⟨⟩N OCH3
24	NHN⟨⟩NCH3. HBr	⟨⟩OCH3 N(CH3)2. HBr
25	$N(NH_2)CH_3$. HBr	⟨⟩OCH3 N(CH3)2. HBr

Scheme 3. (a) (1) HBr, CH_3CN, (2) 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, (3) The free base of R^1H; (b) HBr.

Compounds **2b** (KW-2189), **6**, **13**, **19**, **20**, **25** were treated with mice or human serum, respectively. The results indicated that compounds **6**, **13**, **19**, **20**, **25** released the corresponding cyclopropane compounds in mice and human serum, which were different from KW-2189 (**2b**). Enzymatic conversion ratio of these analogues in mice serum was nearly equal. In contrast, the enzymatic conversion ratio of these analogues in human serum was different, and the order of conversion was **6** > **19** > **20** > **13**≫**25**. No conversion to cyclopropane compound (DU-86, **2a**) from KW-2189 (**2b**) in human serum was observed at all. These results indicated that new 8-*O*-substituted analogues were different prodrugs from KW-2189.

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. A compound **2c** having the same 8-*O*-substituent as KW-2189 (**2b**) showed weak anticellular activity, about 1000 times inferior to that of **4a** (1 h exposure), which was the same tendency that showed KW-2189 (**2b**) was less potent than **2a** (DU-86). On the other hand, it was assumed that new 8-*O*-substituted analogues, of which the IC_{50} values at 1 h exposure were equal to that of the corresponding active form, converted rapidly to cyclopropane compounds in the culture medium. Moreover, all of new 8-*O*-substituted analogues exhibited potent antitumor activity in vivo. This result also indicated that these compounds were chemically or enzymatically converted to their active form in vivo. Furthermore, most of the derivatives showed lower peripheral blood toxicity (reduction of the number of peripheral blood platelets) than that of KW-2189, which showed a similar tendency in A-ring pyrrole derivatives having the simplified DNA-binding moieties.[41]

Several analogues demonstrating a different activated form from KW-2189 and adequate water solubility were evaluated in vivo for efficacy in nude mice bearing human xenograft St-4 (poor differentiated stomach adenocarcinoma) (Table 2). Compounds **13**, **19**, **20**, **23**, **24** showed excellent activity in vivo (T/C <0.3). These activities against St-4 human stomach tumor xenogaraft were nearly comparable to our clinical candidate KW-2189.

Conclusions

A series of the 8-*O*-substituted A-ring pyrrole derivatives bearing the simplified DNA-binding moieties such as cinnamoyl or heteroarylacryloyl groups were prepared and evaluated for in vitro anticellular activity against HeLa S_3 cells and in vivo antitumor activity against sarcoma 180 murine solid tumor and St-4 human stomach tumor xenograft. In addition, the stability of the 8-*O*-substituted analogues in aqueous solution and the conversion to their active form (cyclopropane compound) from the 8-*O*-substituted analogues in mice or human serum were examined. Some showed remarkably potent in vivo antitumor activity and low peripheral blood toxicity compared with derivatives bearing the trimethoxyindole skeleton in Seg-B, which were equal to 8-*O*-[(*N*-methylpiperazinyl)carbonyl] derivatives of 4′-methoxycinnamates and 4′-methoxy-β-heteroarylacrylates. Moreover, among 8-*O*-substituted analogues, several compounds can be chemically or enzymatically converted to their active form in human serum. This result indicated that new 8-*O*-substituted derivatives were different prodrugs from KW-2189 and 8-*O*-substituted analogues being the same type of prodrug as KW-2189.

Experimental

Infrared spectra (IR) were recorded on a JASCO IR-810 spectrometer. ^1H NMR spectra were measured on JEOL JNM-EX270 spectrometer. 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}.

4′-Methoxycinnamoyl 8-*O*-methylthiocarbonyl-A-ring pyrrole-duocarmycin B2 (5). Hydrobromic acid (48%, 0.0054 mL) was added to a solution of **4a** (10 mg, 0.024 mmol) in CH_3CN (0.61 mL), and the mixture was stirred at room temperature for 40 min. The mixture was poured into 1 N HBr, and the whole was extracted with $CHCl_3$. The combined organic extracts were washed with brine and concentrated in vacuo. Methyl

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

No	HeLa S_3 IC_{50} (nM)[a]		Sarcoma 180 (s.c.–i.v.)[b]		Hematotoxicity		Stability $t_{1/2}$ in aq. soln.[f] (h)	Water solubility (mg/mL)
	1 h	72 h	Dose (mg/kg)	T/C[c]	WBC[d] (%)	PL[e] (%)		
5	5.5	0.43	1	0.36	49	93	22[i]	< 0.1
6	3.2	0.54	1	0.20	27	84	N.T.[j]	> 10
7	5.8	0.33	2	0.17	28	79	8[i]	N.T.
8	7.7	0.40	2	0.33	21	82	4[i]	< 0.1
9	3.9	0.38	4	0.17	19	68	1[i]	N.T.
10	52	2.7	2	0.21	29	83	22	N.T.
11	770	5.5	2	0.13	21	79	35	N.T.
12	91	0.43	2	0.31	22	82	4	N.T.
13	11	0.83	2	0.21	25	85	30	< 0.1
14	9.3	1.3	1	0.25	32	69	N.T.	> 10
15	7.6	0.43	1	0.28	33	75	N.T.	> 10
16	7.9	1.1	2	0.21	19	29	N.T.	> 10
17	59	0.52	2	0.28	28	85	N.T.	N.T.
18	11	1.0	4	0.35	17	56 (2)[g]	N.T.	< 0.1
19	12	0.91	8	0.21	30	97	< 1[i]	0.3
20	11	0.8	8	0.10	22	61	< 1[i]	3
21	15	0.94	2	0.22	30	89	12[i]	N.T.
22	18	1.5	4	0.14	22	70	15[i]	N.T.
23	14	1.6	4	0.15	20	83	N.T.	> 10
24	12	0.94	4	0.15	41	76	N.T.	> 10
25	23	1.2	2	0.07	20	69	N.T.	> 10
2a	0.045	0.0052	0.25	0.21	22	38	N.T.	N.T.
2b	53	1.6	0.63	0.15	24	10	N.T.	> 10
2c	1800	37	4	0.20	25	44[h]	23	< 0.1
4a	2.9–7.0	0.26–0.94	0.83	0.34	50	63	N.T.	N.T.
4b	2.5	0.92	0.50	0.22	41	86	N.T.	N.T.
4c	2.5	0.90	2	0.45	28	86	N.T.	N.T.

[a]Drug concentration required to inhibit the growth of HeLa S_3 cells by 50%.
[b]Mice (5 mice/group) were implanted subcutaneously (s.c.) with tumor cells, and the drug was dosed (mg/kg) intravenously (i.v.).
[c]T and C are the values of the mean of tumor volume of treated and control mice, respectively.
[d]Number of white blood cells of tumor-bearing mice on day 4 (percentage of control).
[e]Number of peripheral platelets of nomial mice on day 7 (percentage of control).
[f]A half-life at 35 °C. Drug concentration was 0.05 mg/mL. See Experimental.
[g]Mortality (5 mice/group).
[h]Dose of 5.33 mg/kg.
[i]Cyclopropane compound was detected as a main decomposition product.
[j]Not tested.

Table 2. Antitumor activity against human xenografted solid tumor

No.	St-4 (s.c.–i.v.)[a]	
	Dose (mg/kg)	T/C[b]
6	2.3	0.31
13	4.0	0.23
15	2.4	0.34
16	2.7	0.24 (1)[c]
19	18	0.12
20	12	0.11
23	12	0.19
24	12	0.17
25	2.7	0.44
2b	0.63	0.12
2c	12	0.15

[a]Mice (5 mice/group) were implanted subcutaneously (s.c.) with tumor cells, and the drug was dosed (mg/kg) intravenously (i.v.).
[b]T and C are the values of the mean of tumor volume of treated and control mice, respectively.
[c]Mortality (5 mice/group).

chlorothiolformate (0.0064 mL, 0.074 mmol) and triethylamine (0.010 mL, 0.072 mmol) were added to a stirred solution of the residue in dry CH_2Cl_2 (1.1 mL) at −78 °C. Then, the resulting mixture was stirred at −78 °C for 1 h 20 min. The mixture was diluted with $CHCl_3$, and washed with aqueous $NaHCO_3$ and brine. The organic extracts were concentrated in vacuo. The residue was purified by PTLC ($CHCl_3$:CH_3OH, 15:1) to give 12 mg (84%) of **5**: ^1H NMR (270 MHz, $CDCl_3$): δ 8.80 (1 H, s), 8.37 (1 H, brs), 7.80 (1H, d, J = 15.2 Hz), 7.56 (2 H, d, J = 8.6 Hz), 6.93 (2H, d, J = 8.9 Hz), 6.79 (1H, d, J = 15.2 Hz), 4.50–4.59 (1H, m), 4.47 (1H, d, J = 10.6 Hz), 4.30 (1H, dd, J = 9.2, 9.2 Hz), 3.95 (3H, s), 3.85 (3H, s), 3.79 (1H, dd, J = 10.2, 3.4 Hz), 3.22 (1H, dd, J = 10.2, 10.2 Hz), 2.68 (3H, s), 2.44 (3H, s). IR (KBr): 1701, 1610, 1506, 1404, 1294, 1265, 1234, 1196, 1176, 1111 cm^{-1}. FABMS: m/z 575, 573 $(M+H)^+$. FAB-HRMS calcd for $C_{26}H_{26}{}^{79}BrN_2O_6S$ $(M+H)^+$ m/z 573.0695, found 573.0703. Anal. calcd for $C_{26}H_{25}BrN_2O_6S{\cdot}0.5\ H_2O$: C, 53.61; H, 4.50; N, 4.81; found C, 53.75; H, 4.50; N, 4.51.

3′-(Dimethylamino)-4′-methoxycinnamoyl 8-*O*-acetyl-A-ring pyrrole-duocarmycin B2 hydrochoride (6). Hydrobromic acid (48%, 0.017 mL) was added to a solution of **4b** (23 mg, 0.050 mmol) in CH_3CN (2 mL), and the mixture was stirred at room temperature for 40 min. The reaction mixture was concentrated in vacuo. Acetic anhydride (0.015 mL, 0.16 mmol) and DMAP (20 mg, 0.16 mmol) were added to a stirred solution of the residue in dry CH_2Cl_2 (2 mL) at 0 °C. Then, the resulting

Table 3. In vitro metabolism of duocarmycin derivatives in serum

Compound	Metabolite	Enzymatic conversion (non-enzymatic conversion)[a]	
		Mouse serum	Human serum
6	**4b**	122 (23)	114 (31)
13	**4a**	118 (4)	14 (4)
19	**4a**	116 (–)	89 (–)
20	**4a**	108 (–)	27 (–)
25	**4b**	102 (2)	2 (2)
2b	**2a**	98 (–)	– (–)

[a]pmol/30 min/mg protein.

mixture was stirred at 0 °C for 10 min. The mixture was diluted with $CHCl_3$, and washed with 0.01 M phosphate buffer (pH 7) and brine. The organic extracts were concentrated in vacuo. The residue was purified by PTLC ($CHCl_3$:CH_3OH, 15:1) to give 28 mg (95%) of the free base of **6**. A solution of the free base of **6** (20 mg, 0.035 mmol) in AcOEt (1.7 mL) was treated with anhydrous 6.86 N HCl in ethanol (0.010 mL) at room temperature for 3 h. The mixture concentrated under reduced pressure to give 25 mg of **6**: ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.07 (1H, s), 8.06 (1H, brs), 7.95 (1H, br), 7.80–7.83 (1H, br), 7.63 (1H, d, J = 15.5 Hz), 7.27 (1H, d, J = 8.6 Hz), 7.15 (1H, d, J = 15.2 Hz), 4.43–4.53 (3H, br), 3.98 (3H, s), 3.85 (3H, s), 3.79 (1H, br), 3.09 (6H, s), 2.66 (3H, s), 2.39 (3 H, s). IR (KBr): 1759, 1693, 1651, 1514, 1437, 1414, 1277, 1203, 1090, 1014 cm^{-1}. FABMS (the free base): m/z 586, 584 $(M+H)^+$. FAB-HRMS (the free base) calcd for $C_{28}H_{31}{}^{79}BrN_3O_6$ $(M+H)^+$ m/z 584.1396, found 584.1400. Anal. calcd for $C_{28}H_{30}BrN_3O_6$·HCl·1.5 H_2O: C, 51.90; H, 5.29; N, 6.49; found C, 51.73; H, 5.31; N, 6.02.

4′-Methoxycinnamoyl 8-*O*-carboxylmethylsulfanylacetyl-A-ring pyrrole-duocarmycin B2 (7). Hydrobromic acid (48%, 0.034 mL) was added to a solution of **4a** (25 mg, 0.060 mmol) in CH_3CN (1.5 mL), and the mixture was stirred at room temperature for 1 h. The mixture was poured into 1 N HBr, and the whole was extracted with $CHCl_3$. The combined organic extracts were washed with brine and concentrated in vacuo. Thioglycolic anhydride (25 mg, 0.19 mmol) and DMAP (23 mg, 0.19 mmol) were added to a stirred solution of the residue in dry CH_2Cl_2 (1.5 mL) at 0 °C. The resulting mixture was stirred at 0 °C for 3 h. 1 N HBr was added to the reaction mixture. The mixture was diluted with $CHCl_3$, and washed with saturated $NaHCO_3$ and brine. Then, the organic extracts were concentrated in vacuo. The residue was purified by PTLC ($CHCl_3$:CH_3OH:AcOH, 4:1:0.1) to give 26 mg (69%) of **7**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 10.40 (1H, brs), 8.02 (1H, s), 7.66 (1H, d, J = 15.2 Hz), 7.49 (2H, d, J = 8.3 Hz), 6.88 (2H, d, J = 8.3 Hz), 6.63 (1H, d, J = 15.5 Hz), 4.36–4.46 (1H, m), 4.29 (1H, d, J = 10.6 Hz), 4.11 (1H, dd, J = 9.9, 9.2 Hz), 3.89 (3H, s), 3.82 (3H, s), 3.64 (1H, dd, J = 9.9, 2.3 Hz), 3.59 (2H, s), 3.40 (1H, d, J = 14.8 Hz), 3.33 (1H, d, J = 14.8 Hz), 3.07 (1H, dd, J = 10.2, 9.9 Hz), 2.50 (3H, s). IR (KBr): 1697, 1637, 1603, 1512, 1437, 1416, 1252, 1219, 1174, 1105 cm^{-1}. FABMS: m/z 633, 631 $(M+H)^+$; FAB-HRMS calcd for $C_{28}H_{28}{}^{79}BrN_2O_8S$ $(M+H)^+$ m/z 631.0750, found 631.0725. Anal. calcd for $C_{28}H_{27}BrN_2O_8S$·0.4 $CHCl_3$: C, 50.22; H, 4.06; N, 4.12; found C, 50.07; H, 4.26; N, 3.95.

4′-Methoxycinnamoyl 8-*O*-(pyridine-2-carbonyl)-A-ring pyrrole-duocarmycin B2 hydrochloride (8). Hydrobromic acid (48%, 0.011 mL) was added to a solution of **4a** (20 mg, 0.048 mmol) in CH_3CN (1.2 mL), and the mixture was stirred at room temperature for 50 min. The mixture was poured into 1 N HBr, and the whole was extracted with $CHCl_3$. The combined organic extracts were washed with brine and concentrated in vacuo. DCC (30 mg, 0.14 mmol), DMAP (18 mg, 0.14 mmol) and nicotinic acid (18 mg, 0.14 mmol) were added to a stirred solution of the residue in dry CH_2Cl_2 (1.1 mL) at −20 °C. Then, the resulting mixture was stirred at room temperature for 18 h 30 min. The mixture was diluted with $CHCl_3$, and washed with aqueous $NaHCO_3$ and brine. The organic extracts were concentrated in vacuo. The residue was purified by PTLC ($CHCl_3$:CH_3OH, 30:1) to give 19 mg (65%) of the free base of **8**. A solution of the free base of **8** (20 mg, 0.033 mmol) in AcOEt (1.6 mL) was treated with anhydrous 6.86 N HCl in ethanol (0.0096 mL) at room temperature for 3 h. The mixture concentrated under reduced pressure to give 20 mg of **8**: ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.14 (1H, brs), 9.37 (1H, s), 8.96 (1H, brd, J = 4.0 Hz), 8.58 (1H, dt, J = 7.9, 2.0 Hz), 8.23 (1H, s), 7.75 (2H, d, J = 8.6 Hz), 7.71–7.74 (1H, m), 7.60 (1 H, d, J = 15.5 Hz), 7.07 (1H, d, J = 15.5 Hz), 7.00 (2H, d, J = 8.6 Hz), 4.41–4.59 (3H, m), 3.86 (4H, s), 3.81 (3H, s), 2.64 (3H, s). IR (KBr): 1697, 1645, 1601, 1512, 1408, 1281, 1252, 1217, 1174, 1097 cm^{-1}. FABMS (the free base): m/z 606, 604 $(M+H)^+$. FAB-HRMS (the free base) calcd for $C_{30}H_{27}{}^{79}BrN_3O_6$ $(M+H)^+$ m/z 604.1083, found 604.1069. Anal. calcd for $C_{30}H_{26}BrN_3O_6$·HCl·1.0 H_2O: C, 54.68; H, 4.44; N, 6.38; found C, 54.39; H, 4.44; N, 5.68.

4′-Methoxycinnamoyl 8-*O*-(3-amino-pyrazine-2-carbonyl)-A-ring pyrrole-duocarmycin B2 (9). Yield 64%; ^{1}H NMR (270 MHz, $CDCl_3$ + CD_3OD): δ 8.54 (1H, s), 8.29 (1H, d, J = 2.0 Hz), 7.92 (1H, d, J = 2.0 Hz), 7.73 (1H, d, J = 15.5 Hz), 7.53 (2H, d, J = 8.6 Hz), 6.89 (2H, d, J = 8.9 Hz), 6.76 (1H, d, J = 14.8 Hz), 4.48–4.59 (1H, m), 4.44 (1H, d, J = 10.2 Hz), 4.29 (1H, dd, J = 9.9, 8.6 Hz), 3.91 (3H, s), 3.81 (3H, s), 3.79 (1H, dd, J = 8.6, 2.0 Hz), 3.21 (1H, dd, J = 10.2, 9.9 Hz), 2.65 (3H, s). IR (KBr): 1716, 1697, 1647, 1597, 1512, 1408, 1296, 1248, 1174, 1092 cm^{-1}. FABMS: m/z 622, 620 $(M+H)^+$. FAB-HRMS calcd for $C_{29}H_{27}{}^{79}BrN_5O_6$ $(M+H)^+$ m/z 620.1145, found 620.1138. Anal. calcd for $C_{29}H_{26}BrN_5O_6$·1.0 H_2O·0.6 $CHCl_3$: C, 50.07; H, 4.06; N, 9.86; found C, 50.38; H, 4.06; N, 9.61.

4′-Methoxycinnamoyl 8-*O*-(3,4-diaminobenzoyl)-A-ring pyrrole-duocarmycin B2 (10). Yield 36%; ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.06 (1H, brs), 8.07 (1H, brs), 7.75 (2H, d, J = 8.6 Hz), 7.59 (1H, d, J = 15.5 Hz), 7.37 (1H, s), 7.35 (1H, dd, J = 8.2, 2.0 Hz), 7.06 (1H, d, J = 15.5 Hz), 6.99 (2H, d, J = 8.6 Hz), 6.63 (1H, d, J = 7.9 Hz), 5.51 (2H, brs), 4.82 (2H, br), 4.39–4.57 (3H, m), 3.85 (3H, s), 3.83 (1H, brd, J = 11.2 Hz), 3.81 (3H, s), 3.45 (1H, dd, J = 8.9, 8.9 Hz), 2.62 (3H, s). IR (KBr):

1701, 1693, 1645, 1593, 1512, 1410, 1306, 1250, 1205, 1092 cm^{-1}. FABMS *m/z* 635, 633 $(M+H)^+$. FAB-HRMS calcd for $C_{31}H_{30}{}^{79}BrN_4O_6$ $(M+H)^+$ *m/z* 633.1349, found 633.1363. Anal. calcd for $C_{31}H_{29}BrN_4O_6 \cdot 1.5\ H_2O$: C, 56.37; H, 4.88; N, 8.48; found C, 56.07; H, 4.80; N, 8.09.

4′-Methoxycinnamoyl 8-*O*-(4-dimethylaminobenzoyl)-A-ring pyrrole-duocarmycin B2 (11). Yield 58%; ^1H NMR (270 MHz, $CDCl_3$): δ 9.44 (1H, brs), 8.28 (1H, brs), 7.92 (2H, d, *J* = 8.9 Hz), 7.75 (1H, d, *J* = 15.2 Hz), 7.54 (2H, d, *J* = 8.6 Hz), 6.92 (2H, d, *J* = 8.2 Hz), 6.77 (1H, d, *J* = 15.5 Hz), 6.51 (2H, d, *J* = 8.6 Hz), 4.47–4.58 (1H, m), 4.43 (1H, d, *J* = 10.2 Hz), 4.25 (1H, dd, *J* = 9.2, 8.2 Hz), 3.94 (3H, s), 3.84 (3H, s), 3.82 (1H, brd, *J* = 10.6 Hz), 3.19 (1H, dd, *J* = 10.2, 9.9 Hz), 3.01 (6H, s), 2.50 (3H, s); R (KBr): 1697, 1647, 1603, 1512, 1406, 1267, 1174, 1155, 1088 cm^{-1}. FABMS *m/z* 648, 646 $(M+H)^+$. FAB-HRMS calcd for $C_{33}H_{33}{}^{79}BrN_3O_6$ $(M+H)^+$ *m/z* 646.1553, found 646.1541. Anal. calcd for $C_{33}H_{32}BrN_3O_6 \cdot 1.5\ H_2O$: C, 58.84; H, 5.25; N, 6.24; found C, 59.19; H, 4.98; N, 5.92.

4′-Methoxycinnamoyl 8-*O*-(3-dimethylaminobenzoyl)-A-ring pyrrole-duocarmycin B2 (12). Yield 51%; ^1H NMR (270 MHz, $CDCl_3$): δ 9.57 (1H, brs), 8.30 (1H, brs), 7.73 (1H, d, *J* = 15.2 Hz), 7.53 (2H, d, *J* = 8.6 Hz), 7.42 (1H, d, *J* = 7.9 Hz), 7.34 (1H, brs), 7.18 (1H, dd, *J* = 7.9, 7.9 Hz), 6.92 (2 H, d, *J* = 8.6 Hz), 6.83 (1H, dd, *J* = 7.9, 2.6 Hz), 6.72 (1H, d, *J* = 15.2 Hz), 4.45–4.58 (1H, m), 4.40 (1H, d, *J* = 10.2 Hz), 4.25 (1H, dd, *J* = 9.2, 8.2 Hz), 3.95 (3H, s), 3.84 (3H, s), 3.80 (1H, dd, *J* = 9.6, 2.0 Hz), 3.18 (1H, dd, *J* = 10.2, 9.9 Hz), 2.87 (6H, s), 2.56 (3H, s). IR (KBr): 1734, 1697, 1653, 1603, 1512, 1458, 1406, 1250, 1173, 1093 cm^{-1}. FABMS: *m/z* 648, 646 (M + H) +; FAB-HRMS calcd for $C_{33}H_{33}{}^{79}BrN_3O_6$ $(M+H)^+$ *m/z* 646.1553, found 646.1523. Anal. calcd for $C_{33}H_{32}BrN_3O_6 \cdot 1.0\ H_2O$: C, 59.64; H, 5.16; N, 6.32; found C, 59.68; H, 4.91; N, 6.21.

4′-Methoxycinnamoyl 8-*O*-[4-(4-methyl-1-piperazinylmethyl)benzoyl]-A-ring pyrrole-duocarmycin B2 hydrobromide (13). The free base of **13**: yield 61%; a solution of the free base of **13** (13 mg, 0.018 mmol) in AcOEt (1.2 mL) was treated with 5% HBr in methanol (172 mg) at room temperature for 1 h 30 min. The mixture concentrated under reduced pressure to give 16 mg of **13**: ^1H NMR (270 MHz, DMSO-d_6): δ 12.14 (1H, s), 9.40–9.80 (1H, br), 8.24 (2H, d, *J* = 8.3 Hz), 8.17 (1H, s), 7.76 (2H, d, *J* = 8.6 Hz), 7.67 (2H, d, *J* = 7.3 Hz), 7.59 (1H, d, *J* = 15.5 Hz), 7.08 (1H, d, *J* = 15.2 Hz), 7.00 (2H, d, *J* = 8.6 Hz), 4.44–4.54 (3H, m), 3.86 (3H, s), 3.81 (3H, s), 3.49 (2H, d, *J* = 6.6 Hz), 3.06–3.21 (3H, m), 2.84 (3H, s), 2.63 (3H, s). IR (KBr): 1734, 1697, 1653, 1601, 1512, 1437, 1412, 1252, 1217, 1174, 1093 cm^{-1}. FABMS (the free base): *m/z* 717, 715 $(M+H)^+$; FAB-HRMS (the free base) calcd for $C_{37}H_{40}{}^{79}BrN_4O_6$ $(M+H)^+$ *m/z* 715.2131, found 715.2126. Anal. calcd for $C_{37}H_{39}BrN_4O_6 \cdot HBr \cdot 3.5\ H_2O$: C, 51.70; H, 5.51; N, 6.52; found C, 51.66; H, 5.19; N, 6.32.

β-(4′-Methoxy-3′,5′-pyrimidinyl)acryloyl 8-*O*-[4-(4-methyl-1-piperazinylmethyl)benzoyl]-A-ring pyrrole-duocarmycin B2 hydrobromide (14). Yield 50%; ^1H NMR (270 MHz, DMSO-d_6): δ 12.15 (1H, brs), 9.49 (1H, br), 9.08 (2H, s), 8.22 (2H, d, *J* = 8.3 Hz), 8.16 (1H, s), 7.63 (2H, d, *J* = 8.3 Hz), 7.60 (1H, d, *J* = 15.2 Hz), 7.37 (1H, d, *J* = 15.8 Hz), 4.41–4.50 (3H, m), 3.97 (3H, s), 3.86 (3H, s), 3.82 (1H, brd, *J* = 9.4 Hz), 2.95–3.20 (3H, br), 2.82 (3H, s), 2.63 (3H, s). IR (KBr): 1736, 1697, 1653, 1593, 1475, 1435, 1412, 1338, 1265, 1219, 1092 cm^{-1}. FABMS (the free base): *m/z* 719, 717 $(M+H)^+$; FAB-HRMS (the free base) calcd for $C_{35}H_{38}{}^{79}BrN_6O_6$ $(M+H)^+$ *m/z* 717.2036, found 717.2063. Anal. calcd for $C_{35}H_{37}BrN_6O_6 \cdot 2\ HBr \cdot 1.0\ H_2O$: C, 46.84; H, 4.60; N, 9.36; found C, 47.05; H, 5.10; N, 9.20.

3′-Dimethylamino-4′-methoxycinnamoyl 8-*O*-(pyridine-2-carbonyl)-A-ring pyrrole-duocarmycin B2 hydrobromide (15). Yield 84%; ^1H NMR (270 MHz, DMSO-d_6): δ 12.16 (1H, s), 9.37 (1H, s), 8.96 (1H, d, *J* = 4.3 Hz), 8.57 (1H, dt, *J* = 7.9, 2.0 Hz), 8.23 (1H, brs), 8.12 (1H, brs), 7.93 (1H, d, *J* = 8.3 Hz), 7.73 (1H, dd, *J* = 7.9, 5.0 Hz), 7.65 (1H, d, *J* = 15.2 Hz), 7.35 (1H, d, *J* = 8.9 Hz), 7.22 (1H, d, *J* = 15.2 Hz), 4.47–4.60 (3H, br), 4.02 (3H, s), 3.86 (3H, s), 3.84 (1H, dd, *J* = 10.6, 2.6 Hz), 3.51 (1H, dd, *J* = 9.6, 8.6 Hz), 3.18 (6H, s), 2.64 (3H, s). IR (KBr): 1686, 1647, 1516, 1466, 1458, 1437, 1414, 1279, 1219, 1097 cm^{-1}. FABMS (the free base): *m/z* 649, 647 $(M+H)^+$; FAB-HRMS (the free base) calcd for $C_{32}H_{32}{}^{79}BrN_4O_6$ $(M+H)^+$ *m/z* 647.1505, found 647.1526. Anal. calcd for $C_{32}H_{31}BrN_4O_6 \cdot 2 \cdot HBr \cdot 1.0\ H_2O$: C, 46.46; H, 4.26; N, 6.77; found C, 46.31; H, 4.51; N, 6.58.

3′-Dimethylamino-4′-methoxycinnamoyl 8-*O*-[4-(4-methyl-1-piperazinylmethyl)benzoyl]-A-ring pyrrole-duocarmycin B2 hydrobromide (16). Yield 61%; ^1H NMR (270 MHz, DMSO-d_6): δ 12.14 (1H, s), 9.51 (1H, br), 8.21 (2H, d, *J* = 8.3 Hz), 8.19 (1H, d, *J* = 15.2 Hz), 7.75–7.89 (2H, br), 7.63 (2H, d, *J* = 8.9 Hz), 7.60 (1H, d, *J* = 5.9 Hz), 7.24 (1H, d, *J* = 8.6 Hz), 7.16 (1H, d, *J* = 15.5 Hz), 4.45–4.58 (3H, br), 3.97 (3H, s), 3.86 (3H, s), 3.83 (1H, brd, *J* = 9.7 Hz), 3.06 (8H, br), 2.81 (3H, s), 2.63 (3H, s). IR (KBr): 1734, 1695, 1647, 1616, 1516, 1437, 1412, 1263, 1217, 1092, 1018 cm^{-1}. FABMS (the free base): *m/z* 760, 758 $(M+H)^+$; FAB-HRMS (the free base) calcd for $C_{39}H_{45}{}^{79}BrN_5O_6$ $(M+H)^+$ *m/z* 758.2553, found 758.2527. Anal. calcd for $C_{39}H_{44}BrN_5O_6 \cdot 2\ HBr \cdot 5.5\ H_2O$: C, 45.94; H, 5.63; N, 6.87; found C, 45.92; H, 5.24; N, 6.77.

4′-Methoxycinnamoyl 8-*O*-[4-[(*tert*-butoxycarbonyl)aminomethyl]benzoyl]-A-ring pyrrole-duocarmycin B2 (17). Yield 54%; ^1H NMR (270 MHz, $CDCl_3$): δ 9.44–9.76 (1H, brs), 8.30 (1H, brs), 8.00 (2H, d, *J* = 7.3 Hz), 7.75 (1H, d, *J* = 15.2 Hz), 7.55 (2H, d, *J* = 8.6 Hz), 7.22 (2H, d, *J* = 7.3 Hz), 6.92 (2H, d, *J* = 8.9 Hz), 6.76 (1H, d, *J* = 15.2 Hz), 5.25 (1H, brs), 4.47–4.59 (1H, m), 4.43 (1H, d, *J* = 10.9 Hz), 4.21–4.38 (3H, m), 3.94 (3H, s), 3.84 (3H, s), 3.81 (1H, brd, *J* = 10.9 Hz), 3.20 (1H, dd, *J* = 10.2, 10.2 Hz), 1.45 (9H, s). IR(KBr): 1705, 1695, 1645, 1512, 1410, 1261, 1217, 1173, 1090, 1016 cm^{-1}. FABMS: *m/z* 734, 732 $(M+H)^+$. FAB-HRMS calcd for $C_{37}H_{39}{}^{79}BrN_3O_8$ $(M+H)^+$ *m/z* 732.1921, found, 732.1920. Anal. calcd for $C_{37}H_{39}BrN_3O_8 \cdot 1.5\ H_2O$: C, 58.50; H, 5.44; N, 5.53; found C, 58.34; H, 5.69; N, 4.97.

4′-Methoxycinnamoyl 8-*O*-(4-aminomethylbenzoyl)-A-ring pyrrole-duocarmycin B2 hydrobromide (18). A solution of **17** (65 mg, 0.072 mmol) in $ClCH_2CH_2Cl$ (2 mL) was treated with 5% HBr in methanol (1.45 g) at 60 °C for 5 h 45 min. Aqueous $NaHCO_3$ was added to the reaction mixture. The mixture was extracted with $CHCl_3$, and washed with saturated $NaHCO_3$ and brine. Then, the organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by PTLC ($CHCl_3$:CH_3OH, 4:1) and column chromatography ($CHCl_3$:CH_3OH, 10:1) to give 15 mg (33%) of the free base of **18**. The obtained free base (11 mg) was employed in the same procedure as that of preparation of HBr salt to give 15 mg of **18**: ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.15 (1H, brs), 8.32 (2H, br), 8.27 (2H, d, J = 8.2 Hz), 8.18 (1H, brs), 7.75 (2H, d, J = 8.3 Hz), 7.72 (2H, d, J = 7.6 Hz), 7.58 (1H, d, J = 15.5 Hz), 7.08 (1H, d, J = 15.2 Hz), 7.00 (2H, d, J = 8.6 Hz), 4.50–4.60 (1H, m), 4.41–4.48 (2H, br), 4.21–4.23 (2H, m), 3.86 (3H, s), 3.83 (1H, br), 3.81 (3H, s), 2.63 (3H, s). IR (KBr): 1734, 1697, 1635, 1601, 1514, 1437, 1417, 1259, 1174, 1093 cm^{-1}. FABMS (the free base): m/z 634, 632 $(M+H)^+$. FAB-HRMS (the free base) calcd for $C_{32}H_{31}{}^{79}BrN_3O_6$ $(M+H)^+$ m/z 632.1428, found, 632.1426. Anal. calcd for $C_{32}H_{30}BrN_3O_6$·HBr·7.0 H_2O: C, 45.78; H, 5.40; N, 5.01; found C, 45.81; H, 4.45; N, 4.91.

4′-Methoxycinnamoyl 8-*O*-[(4-methyl-1-piperazinyl)amino]-carbonyl-A-ring pyrrole-duocarmycin B2 hydrobromide (19). Hydrobromic acid (48%, 0.041 mL) was added to a solution of **4a** (30 mg, 0.072 mmol) in CH_3CN (1.7 mL), and the mixture was stirred at room temperature for 2 h. The mixture was poured into 1N HBr, and the whole was extracted with $CHCl_3$. The combined organic extracts were washed with brine and concentrated in vacuo. *p*-Nitrophenyl chloroformate (43 mg, 0.22 mmol) and triethylamine (0.030 mL, 0.22 mmol) were added to a stirred solution of the residue in dry CH_2Cl_2 (1.7 mL) at −78 °C. Then, the resulting mixture was stirred at −78 °C for 40 min. 1-Amino-4-methylpiperazine (0.043 mL, 0.36 mmol) was added to the solution, and the mixture was stirred at 0 °C for 24 h. The mixture was diluted with $CHCl_3$, and washed with aqueous $NaHCO_3$ and brine. The organic extracts were concentrated in vacuo. The residue was purified by PTLC ($CHCl_3$:CH_3OH, 9:1) to give 20.4 mg (44%) of the free base of **19**. The obtained free base (20 mg) was employed in the same procedure as that of preparation of HBr salt to give 23 mg of **19**: ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.07 (1H, brs), 9.49 (2H, brs), 8.04 (1H, brs), 7.76 (2H, d, J = 8.3 Hz), 7.59 (1H, d, J = 15.2 Hz), 7.07 (1H, d, J = 15.5 Hz), 7.00 (2H, d, J = 7.3 Hz), 4.38–4.56 (3H, br), 3.85 (3H, s), 3.82 (3H, s), 3.77 (1H, br), 3.02–3.30 (5H, m), 2.82 (3H, s), 2.66 (3H, s). IR (KBr): 1697, 1647, 1601, 1512, 1437, 1412, 1250, 1217, 1174, 1093 cm^{-1}. FABMS (the free base): m/z 642, 640 $(M+H)^+$. FAB-HRMS (the free base) calcd for $C_{30}H_{35}{}^{79}BrN_5O_6$ $(M+H)^+$ m/z 640.1771, found 640.1769. Anal. calcd for $C_{30}H_{34}BrN_5O_6$·2HBr·1.0 H_2O: C, 43.92; H, 4.67; N, 8.54; found C, 44.01; H, 4.78; N, 8.45.

4′-Methoxycinnamoyl 8-*O*-[1-(4-piperidinopiperidinyl)-amino]carbonyl-A-ring pyrrole-duocarmycin B2 hydrobromide (20). Yield 43%; ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.04 (1H, brs), 9.25 (1H, s), 9.00 (1H, br), 8.02 (1H, s), 7.75 (2H, d, J = 8.6 Hz), 7.59 (1H, d, J = 15.2 Hz), 7.06 (1H, d, J = 15.2 Hz), 7.00 (2H, d, J = 8.9 Hz), 4.36–4.53 (3H, m), 3.85 (3H, s), 3.82 (3H, s), 3.78 (1H, m), 3.17–3.27 (2H, m), 2.86–3.04 (1H, m), 2.69–2.79 (2H, m), 2.65 (3H, s), 1.99–2.07 (2H, br), 1.60–1.91 (9H, m), 1.34–1.49 (2H, m). IR (KBr): 1749, 1695, 1645, 1601, 1512, 1456, 1412, 1250, 1217, 1174 cm^{-1}. FABMS (the free base): m/z 710, 708 $(M+H)^+$. Anal. calcd for $C_{35}H_{42}BrN_5O_6$·2HBr·0.3 H_2O: C, 48.00; H, 5.13; N, 8.00; found C, 48.16; H, 5.83; N, 7.83.

4′-Methoxycinnamoyl 8-*O*-(2-methylcarbazoyl)-A-ring pyrrole-duocarmycin B2 (21). Yield 71%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 10.21 and 9.81 (1H, brs), 8.24 (1H, brs), 7.81 (1H, d, J = 15.2 Hz), 7.57 (2H, d, J = 8.3 Hz), 6.93 (2H, d, J = 8.6 Hz), 6.82 (1H, d, J = 15.5 Hz), 4.47–4.55 (1H, br), 4.46 (1H, d, J = 10.2 Hz), 4.33 (1H, dd, J = 9.2, 8.9 Hz), 3.94 (3H, s), 3.85 (3H, s), 3.77 (1H, dd, J = 9.4, 2.1 Hz), 3.29 (3H, brs), 3.19 (1H, dd, J = 9.9, 9.9 Hz), 2.32 (3H, s). IR (KBr): 1697, 1647, 1599, 1512, 1433, 1410, 1248, 1217, 1173, 1111 cm^{-1}. FABMS (the free base): m/z 573, 571 $(M+H)^+$. FAB-HRMS calcd for $C_{26}H_{28}{}^{79}BrN_4O_6$ $(M+H)^+$ m/z 571.1192, found 571.1192. Anal. calcd for $C_{26}H_{27}BrN_4O_6$·1.0 H_2O: C, 52.98; H, 4.96; N, 9.51; found C, 52.74; H, 4.78; N, 9.33.

4′-Methoxycinnamoyl 8-*O*-(2,3-dimethylcarbazoyl)-A-ring pyrrole-duocarmycin B2 (22). Yield 46%; ^{1}H NMR (270 MHz, $CDCl_3$): δ 9.65 (1H, brs), 9.39 (1H, brs), 8.24 (1H, s), 7.80 (1H, d, J = 15.2 Hz), 7.57 (2H, d, J = 8.9 Hz), 6.94 (2H, d, J = 8.9 Hz), 6.81 (1H, d, J = 15.5 Hz), 4.50–4.57 (1H, m), 4.46 (1H, d, J = 10.2 Hz), 4.31 (1H, dd, J = 9.6, 8.9 Hz), 3.94 (3H, s), 3.86 (3 H, s), 3.78 (1H, dd, J = 9.7, 2.5 Hz), 3.27 (3H, brs), 3.20 (1H, dd, J = 10.2, 9.9 Hz), 2.71 (3 H, brs), 2.44 (3 H, brs). IR (KBr): 1716, 1697, 1686, 1647, 1601, 1512, 1410, 1252, 1219, 1173, 1159, 1109 cm^{-1}. FABMS: m/z 587, 585 $(M+H)^+$. FAB-HRMS calcd for $C_{27}H_{30}{}^{79}BrN_4O_6$ $(M+H)^+$ m/z 585.1349, found 585.1345. Anal. calcd for $C_{27}H_{29}BrN_4O_6$·1.0 H_2O: C, 53.74; H, 5.18; N, 9.28; found C, 53.62; H, 4.90; N, 9.13.

β-(4′-Methoxy-3′,5′-pyrimidinyl)acryloyl 8-*O*-[(4-methyl-1-piperazinyl)amino]carbonyl-A-ring pyrrole-duocarmycin B2 hydrobromide (23). Yield 31%; ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.09 (1H, brs), 9.58 (1H, brs), 9.48 (1H, brs), 9.08 (2H, s), 8.04 (1H, s), 7.60 (1H, d, J = 15.8 Hz), 7.37 (1H, d, J = 15.5 Hz), 4.37–4.57 (2H, m), 4.47 (1H, d, J = 10.2 Hz), 3.97 (3H, s), 3.85 (3H, s), 3.79 (1H, brd, J = 9.6 Hz), 3.00–3.30 (7H, br), 2.80 (3H, s), 2.66 (3H, s). IR (KBr): 1747, 1697, 1653, 1595, 1475, 1437, 1414, 1340, 1219, 1093 cm^{-1}. FABMS (the free base): m/z 644, 642 $(M + H)^+$. FAB-HRMS (the free base) calcd for $C_{28}H_{33}{}^{79}BrN_7O_6$ $(M+H)^+$ m/z 642.1676, found 642.1694. Anal. calcd for $C_{28}H_{32}BrN_7O_6$·HBr·4.0 H_2O: C, 42.28; H, 5.19; N, 12.33; found C, 42.43; H, 5.19; N, 12.11.

3′-Dimethylamino-4′-methoxycinnamoyl 8-*O*-[(4-methyl-1-piperazinyl)amino]carbonyl-A-ring pyrrole-duocarmycin B2 hydrobromide (24). Yield 47%; ^{1}H NMR (270 MHz,

DMSO-d_6): δ 12.09 (1H, s), 9.53 (1H, br), 9.48 (1H, s), 8.03 (2H, brs), 7.85 (1H, d, $J=7.9$ Hz), 7.63 (1H, d, $J=15.2$ Hz), 7.31 (1H, d, $J=8.3$ Hz), 7.18 (1H, d, $J=15.2$ Hz), 4.39–4.58 (3H, m), 4.00 (3H, s), 3.85 (3H, s), 3.80 (1H, dd, $J=9.4$, 2.8 Hz), 3.45 (4H, br), 3.14 (6H, s), 3.08–3.25 (5H, br), 2.82 (3H, d, $J=4.3$ Hz), 2.66 (3H, s). IR (KBr): 1743, 1689, 1649, 1516, 1464, 1435, 1416, 1279, 1219, 1190, 1094 cm^{-1}. FABMS (the free base): m/z 685, 683 $(M+H)^+$. FAB-HRMS (the free base) calcd for $C_{32}H_{40}{}^{79}BrN_6O_6$ $(M+H)^+$ m/z 683.2192, found 683.2219. Anal. calcd for $C_{32}H_{39}BrN_6O_6 \cdot 2$ HBr·3.0 H_2O: C, 42.73; H, 5.27; N, 9.34; found C, 42.53; H, 5.32; N, 9.94.

3′-Dimethylamino-4′-methoxycinnamoyl 8-*O*-(2-methylcarbazoyl)-A-ring pyrrole-duocarmycin B2 hydrobromide (25). Yield 74%; ^1H NMR (270 MHz, DMSO-d_6): δ 12.02 (1H, brs), 8.12 (1H, brs), 7.97 (1H, brs), 7.83 (1H, d, $J=8.2$ Hz), 7.63 (1H, d, $J=14.8$ Hz), 7.29 (1H, d, $J=8.6$ Hz), 7.17 (1H, d, $J=15.2$ Hz), 4.40–4.58 (3H, br), 3.99 (3H, s), 3.85 (3H, s), 3.81 (1H, brd, $J=10.0$ Hz), 3.11 (6H, s), 2.67 (3H, s). IR (KBr): 1650, 1645, 1516, 1464, 1435, 1416, 1279, 1219, 1190, 1159, 1107, 1092 cm^{-1}. FABMS (the free base): m/z 616, 614 $(M+H)^+$; FAB-HRMS (the free base) calcd for $C_{28}H_{33}{}^{79}BrN_5O_6$ $(M+H)^+$ m/z 614.1614, found 614.1596. Anal. calcd for $C_{28}H_{32}BrN_5O_6 \cdot 2HBr \cdot 1.0$ H_2O: C, 42.34; H, 4.57; N, 8.82; found C, 42.23; H, 4.87; N, 8.64.

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 adults male ddY mice. Human xenografts were passaged and used in adult male BALB/c-*nu*/*nu* mice. Murine solid tumor was 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 (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.[59]

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).[50]

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).

Stability of drug in aqueous solution

The stablilty of the 8-substituted derivatives under aqueous conditions was examined by chromatogaphy on a UNISIL pack 5C18 reversed-phase HPLC column (GL Science, Co., Ltd., Tokyo, Japan) or L-column ODS 4.6×150 mm (Chemicals Inspection and Testing Institute, Tokyo, Japan). The compound (1 mg) was dissolved in DMF (10 mL). This solution (0.1 mL) was diluted with aqueous solution (0.1 mL). The aqueous solution was composed of 0.05 M phosphate buffer (pH 7). The resulting solution was incubated at 35 °C. Samples were removed at intervals and injected directly into a HPLC injection port. The compound was eluted with 0.05 M phosphate buffer (pH 5.9): CH_3CN (30:70) and detected by measuring absorbance at 330 nm.

In vitro metabolism of duocarmycin derivatives in serum

Serum was prepared by evacuated serum collection tubes (Venoject II, Terumo Co., Tokyo, Japan) from

the adult male BALB/c mice and the adult male human. Then serum was diluted to 25% with Tris–HCl Buffer (20 mM, pH 7.4). After incubation of 25% serum (198 μL) at 37 °C for 3 min, drugs (2 μL, 200 μM) were added to 25% serum in the dark. After 30 min, acetonitrile was added, and the mixture was centrifuged at 4 °C for 5 min (15000 × *g*) and filtered through an ultrafree-C3-LG (Millipore Co., Bedford, MA, USA). The filtrate (100 μL) was analyzed by HPLC at 300 nm. Contribution of non-enzymatic reaction was examined by performing the same procedure after 25% serum was boiled at 96 °C for 5 min.

Acknowledgements

We express our gratitude to Mikiko Saito, and Etsuko Koshimura for their excellent technical assistance. We are grateful to Dr. Satoshi Kobayashi, Fumiko Oiji for accurate suggestions and valuable discussions. We also thank Mariko Yoshida, Atsuko Kobayashi and Hideko Yokoyama for measuring elemental analyses and MS spectra.

References and Notes

1. Takahashi, I.; Takahashi, K.; Ichimura, M.; Morimoto, M.; Asano, K.; Kawamoto, I.; Tomita, F.; Nakano, H. **1988**, *41*, 1915.
2. Yasuzawa, T.; Iida, T.; Muroi, K.; Ichimura, M.; Takahashi, K.; Sano, H. *Chem. Pharm. Bull.* **1988**, *36*, 3728.
3. Ichimura, M.; Muroi, K.; Asano, K.; Kawamoto, I.; Tomita, F.; Morimoto, M.; Nakano, H. *J. Antibiot.* **1988**, *41*, 1285.
4. Ogawa, T.; Ichimura, M.; Katsumata, S.; Morimoto, M.; Takahashi, K. *J. Antibiot.* **1989**, *42*, 1299.
5. Ichimura, M.; Ogawa, T.; Takahashi, K.; Kobayashi, E.; Kawamoto, I.; Yasuzawa, T.; Takahashi, I.; Nakano, H. *J. Antibiot.* **1990**, *43*, 1037.
6. Ichimura, M.; Ogawa, T.; Katumata, S.; Takahashi, K.; Takahashi, I.; Nakano, H. *J. Antibiot.* **1991**, *44*, 1045.
7. Yasuzawa, T.; Saitoh, Y.; Ichimura, M.; Takahashi, I.; Sano, H. *J. Antibiot.* **1991**, *44*, 445.
8. Yasuzawa, T.; Muroi, K.; Ichimura, M.; Takahashi, I.; Takahashi, K.; Sano, H.; Saitoh, Y. *Chem. Pharm. Bull.* **1995**, *43*, 378.
9. Gomi, K.; Kobayashi, E.; Miyoshi, K.; Ashizawa, T.; Okamoto, A.; Ogawa, T.; Katsumata, S.; Mihara, A.; Okabe, M.; Hirata, T. *Jpn. J. Cancer Res.* **1992**, *83*, 113.
10. A number of reviews on the duocarmycins are available from references.[10–13] Boger, D. L. CHEMTRACTS: *Org. Chem.* **1991**, *4*, 329.
11. Boger, D. L. *Acc. Chem. Res.* **1995**, *28*, 20.
12. Boger, D. L.; Johnson, D. S. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 3642.
13. Boger, D. L.; Johnson, D. S. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1438.
14. Sugiyama, H.; Hosoda, M.; Saito, I.; Asai, A.; Saito, H. *Tetrahedron Lett.* **1990**, *31*, 7197.
15. Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H. *J. Org. Chem.* **1990**, *55*, 4499.
16. Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H.; Munk, S. A.; Kitos, P. A.; Suntornwat, O. *J. Am. Chem. Soc.* **1990**, *112*, 8961.
17. Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H. *J. Am. Chem. Soc.* **1991**, *113*, 6645.
18. Sugiyama, H.; Ohmori, K.; Chan, K. L.; Hosoda, M.; Asai, A.; Saito, H.; Saito, I. *Tetrahedron Lett.* **1993**, *34*, 2179.
19. Boger, D. L.; Johnson, D. S.; Yun, W. *J. Am. Chem. Soc.* **1994**, *116*, 1635.
20. Hanka, L. J.; Dietz, A.; Gerpheide, S. A.; Kuentzel, S. L.; Martin, D. G. *J. Antibiot.* **1978**, *31*, 1211.
21. Martin, D. G.; Chidester, C. G.; Duchamp, D. J.; Mizsak, S. A. *J. Antibiot.* **1980**, *33*, 902.
22. Reynolds, V. L.; McGovren, J. P.; Hurley, L. H. *J. Antibiot.* **1986**, *39*, 319.
23. Hurley, L. H.; Reynolds, V. L.; Swenson, D. H.; Petzold, G. L.; Scahill, T. A. *Science* **1984**, *226*, 843.
24. Reynolds, V. L.; Molineaux, I. J.; Kaplan, D. J.; Swensen, D. H.; Hurley, L. H. *Biochemistry* **1985**, *24*, 6228.
25. Tang, M. S.; Lee, C. S.; Doisy, R.; Ross, L.; Needham-VanDevanter, D. R.; Hurley, L. H. *Biochemistry* **1988**, *27*, 893.
26. Nagamura, S.; Asai, A.; Kanda, Y.; Kobayashi, E.; Gomi, K.; Saito, H. *Chem. Pharm. Bull.* **1996**, *44*, 1723.
27. Kobayashi, E.; Okamoto, A.; Asada, M.; Okabe, M.; Nagamura, S.; Asai, A.; Saito, H.; Gomi, K.; Hirata, T. *Cancer Res.* **1994**, *54*, 2404.
28. Asai, A.; Nagamura, S.; Saito, H. *J. Am. Chem. Soc.* **1994**, *116*, 4171.
29. Nagamura, S.; Kobayashi, E.; Gomi, K.; Saito, H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2147.
30. Nagamura, S.; Kanda, Y.; Kobayashi, E.; Gomi, K.; Saito, H. *Chem. Pharm. Bull.* **1995**, *43*, 1530.
31. A direct relationship between solvolysis stability and in vitro cytotoxic potency with the more sable agents exhibiting the more potent activity is demonstrated.[31–35] Boger, D. L.; Ishizaki, T. *Tetrahedron Lett.* **1990**, *31*, 793.
32. Boger, D. L.; Mesini, P.; Tarby, C. M. *J. Am. Chem. Soc.* **1994**, *116*, 6461.
33. Boger, D. L.; McKie, J. A.; Han, N.; Taby, C. M.; Riggs, H. W.; Kitos, P. A. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 659.
34. Boger, D. L.; Goldberg, J.; McKie, J. A. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1955.
35. Boger, D. L.; Boyce, C.; Johnson, D. S. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 233.
36. Chidester, C. G.; Krueger, W. C.; Mizsak, S. A.; Duchamp, D. J.; Martin, D. G. *J. Am. Chem. Soc.* **1981**, *103*, 7629.
37. Warpehoski, M. A.; Gebnard, I.; Kelly, R. C.; Krueger, W. C.; Li, L. H.; McGovren, J. P.; Prairie, M. D.; Wicnienski, N.; Wierenga, W. *J. Med. Chem.* **1988**, *31*, 590.
38. Boger, D. L.; Tun, W. *J. Am. Chem. Soc.* **1994**, *116*, 5523.
39. Asai, A.; Nagamura, S.; Kobayashi, E.; Gomi, K.; Saito, H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1215.
40. Nagamura, S.; Asai, A.; Amishiro, N.; Kobayashi, E.; Gomi, K.; Saito, H. *J. Med. Chem.* **1997**, *40*, 972.
41. Amishiro, N.; Nagamura, S.; Kobayashi, E.; Gomi, K.; Saito, H. *J. Med. Chem.* **1999**, *42*, 669.
42. Nagamura, S.; Kobayashi, E.; Gomi, K.; Saito, H. *Bioorg. Med. Chem.* **1996**, *4*, 1379.
43. Ogasawara, H.; Nishio, K.; Takeda, Y.; Ohmori, T.; Kubota, N.; Funayama, Y.; Ohira, T.; Kuraishi, Y.; Isogai, Y.; Saijo, N. *Jpn. J. Cancer Res.* **1994**, *85*, 418.
44. Okamoto, A.; Asai, A.; Saito, H.; Okabe, M.; Gomi, K.Jpn. *J. Cancer Res.* **1994**, *85*, 1304.
45. Ogasawara, H.; Nishio, K.; Kanzawa, F.; Lee, Y. S.; Funayama, Y.; Ohmori, T.; Kuraishi, Y.; Isogai, Y.; Saijo, N. *Jpn. J. Cancer Res.* **1995**, *86*, 124.
46. Ogasawara, H.; Nishio, K.; Ishida, T.; Arioka, H.; Fukuoka, K.; Saijo, N. *Jpn. J. Cancer Res.* **1997**, *88*, 1033.

47. Nagamura, S.; Asai, A.; Kanda, Y.; Kobayashi, E; Gomi, K.; Saito, H. *Chem. Pharm. Bull.* **1996**, *44*, 933.
48. Berner, D.; Cox, D. P.; Dahn, H. *J. Am. Chem. Soc.* **1982**, *104*, 2631.
49. Geran, R. I.; Greenberg, N. H.; MacDonald, M. M.; Schumacher, A. M.; Abbott, B. *J. Cancer Chemother. Rep.* **1972**, *3*, 1.
50. Inaba, M.; Kobatashi, T.; Tashiro, T.; Sakurai, Y.; Maruo, K.; Ohnishi, Y.; Ueyama, Y.; Momura, T. *Cancer* **1989**, *64*, 1577.