STUDIES ON THE ISOTETRACENONE ANTIBIOTICS

IV. HATOMARUBIGINS A, B, C AND D, NEW ISOTETRACENONE ANTIBIOTICS EFFECTIVE AGAINST MULTIDRUG-RESISTANT TUMOR CELLS

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A complex of new isotetracenone group antibiotics was isolated from the cultured broth of *Streptomyces* sp. 2238-SVT4. It consisted of four related compounds, designated hatomarubigins A, B, C and D, whose structures were elucidated by NMR spectral analysis including a variety of 2D techniques. They enhanced the cytotoxicity of colchicine against multidrug-resistant tumor cells.

The isotetracenone antibiotics[1~3] are characterized by containing modified benz[*a*]anthraquinone chromophores and reported to show antimicrobial, antitumor and/or enzyme inhibitory activities. In the course of our screening for isotetracenone antibiotics, *Streptomyces* sp. 2238-SVT4 was found to produce four new members of this family, designated hatomarubigins A, B, C and D.

At lower concentrations than their cytotoxic dose, these compounds inhibited the growth of colchicine-resistant KB(CH^R)[4] cells in the presence of colchicine. Colchicine-resistant tumor cells can be regarded as a model of multidrug-resistance (MDR)[5] because of their simultaneous acquirement of resistance to doxorubicin and vinblastine[6].

In this paper, we report the fermentation, isolation, physico-chemical properties, structural elucidation and biological activities of the hatomarubigins.

Fermentation

A stock culture of *Streptomyces* sp. 2238-SVT4 was maintained on a BENNETT's agar slant at 4°C. The stock culture was inoculated into seed tubes containing 15 ml of a seed medium and incubated by shaking at 27°C for 3 days. The medium consisted of soluble starch 1.0%, molasses 1.0%, Polypepton 1.0% and beef extract 1.0% (pH 7.2 before sterilization). The seed culture was transferred at 2% to 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of soluble starch 2.5%, soybean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4% (pH 6.2 before sterilization). The flasks were incubated on a rotary shaker at 27°C for 3 days. A 600-ml portion of the culture was inoculated into 50-liter jar fermenter containing 30 liters of a production medium having the same composition as the flask fermentation medium. The fermentation was carried out at 27°C for 3 days under agitation of 400 rpm and aeration of 30 liters per minute.

Isolation and Purification

The fermentation broth (60 liters) was centrifuged to give a mycelial cake, which was extracted with

30 liters of acetone. The extract was concentrated to a small volume and then extracted twice with 2 liters of ethyl acetate. After evaporation, the residue was applied to a silica gel column (Wakogel, C-200, 1 liter). The active eluate with chloroform - methanol (100 : 1) was concentrated to dryness and then subjected to preparative silica gel TLC (Merck, Silica gel 60, layer thickness 0.5 mm). Development of the plates with chloroform - methanol (200 : 1) gave three orange fractions. The two fractions with lower Rf values (0.11 and 0.19) were separately chromatographed on Sephadex LH-20 columns (200 ml) with chloroform - methanol (1 : 1). The active fractions were evaporated to dryness to yield orange powders of hatomarubigins C (433 mg) and D (17 mg). The TLC fraction with the highest Rf value (0.24) was further purified by preparative silica gel TLC developed with hexane - chloroform - triethylamine (6 : 3 : 1). Two yellow fractions and one orange fraction thus obtained were separately subjected to Sephadex LH-20 column chromatography (200 ml) with chloroform - methanol (1 : 1). The active fractions were concentrated to dryness to give yellow powders of hatomarubigin A (9 mg) and rubiginone B_2[7,8] (120 mg) and an orange powder of hatomarubigin B (31 mg).

Physico-chemical Properties

The physico-chemical properties of the hatomarubigins are summarized in Table 1. The molecular formulae were determined from HRFAB-MS to be $C_{20}H_{16}O_5$ for hatomarubigins A and B, $C_{20}H_{18}O_5$ for C, and $C_{41}H_{36}O_{10}$ for D. Maximum visible absorption peaks observed at longer than 400 nm indicated the presence of a quinone chromophore in the hatomarubigins. The IR spectra showed absorption bands due to non chelated and chelated quinone carbonyls at 1675 and 1640 cm^{-1} for hatomarubigin A, 1660 and 1640 cm^{-1} for B, 1650 and 1630 cm^{-1} for C, and 1660 and 1620 cm^{-1} for D. Additionally, hatomarubigins A and B exhibited IR absorption bands ascribed to unsaturated ketone carbonyls at 1690 and 1700 cm^{-1}, respectively. The ^1H NMR spectra of hatomarubigins A to D in $CDCl_3$ are shown in Figs. 1~4, respectively.

Structural Elucidation

The structural elucidation of the hatomarubigins was carried out on the basis of spectral analysis of the major component, hatomarubigin C. The ^1H and ^{13}C NMR spectral data for the hatomarubigins are summarized in Tables 2 and 3, respectively. All one-bond ^1H-^{13}C connectivities were established by

Table 1. Physico-chemical properties of hatomarubigins A, B, C and D.

	A	B	C	D
Nature	Yellow powder	Orange powder	Orange powder	Orange powder
MP (°C)	236~238	254~256 (dec)	174~176	286~288 (dec)
$[\alpha]_D^{22}$	+95°	+146°	+514°	+734°
	(*c* 0.1, $CHCl_3$)	(*c* 0.1, $CHCl_3$)	(*c* 0.06, $CHCl_3$)	(*c* 0.17, $CHCl_3$)
Formula	$C_{20}H_{16}O_5$	$C_{20}H_{16}O_5$	$C_{20}H_{18}O_5$	$C_{41}H_{36}O_{10}$
FAB-MS (*m/z*) $(M+H)^+$				
Calcd:	337.1076	337.1076	339.1233	689.2387
Found:	337.1097	337.1105	339.1293	689.2451
UV λ_{max} nm (ε)	224 (21,900),	213 (25,800),	224 (27,200),	260 (42,800),
	260 (24,400),	264 (34,100),	259 (24,200),	292 (13,100),
	413 (7,000)	458 (6,300)	459 (6,600)	342 (5,800),
	in MeOH	in MeOH	in MeOH	473 (11,500)
				in dioxane
IR (KBr) ν cm^{-1}	3440, 1690, 1675,	3450, 1700, 1660,	3440, 1650, 1630	3500, 1660, 1620
	1640	1640		

Fig. 1. ^{1}H NMR spectrum of hatomarubigin A in $CDCl_3$.

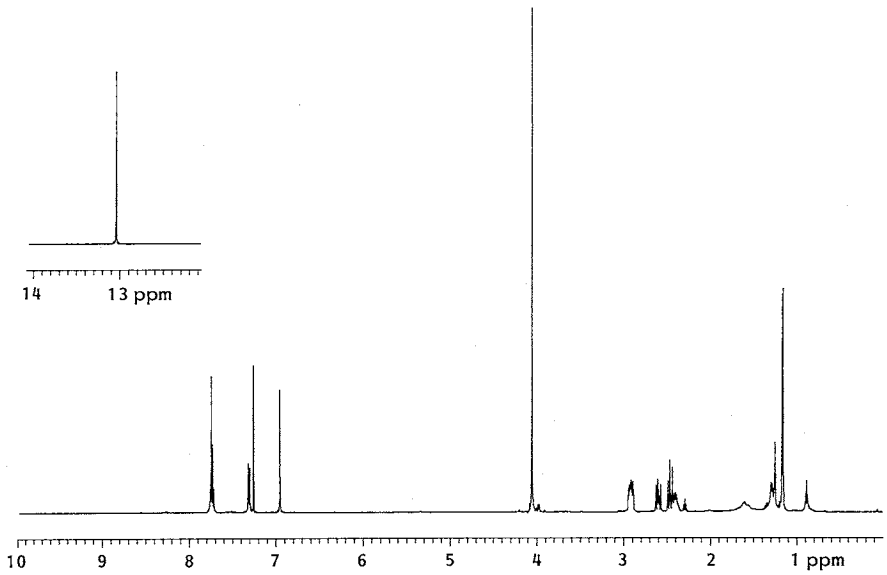
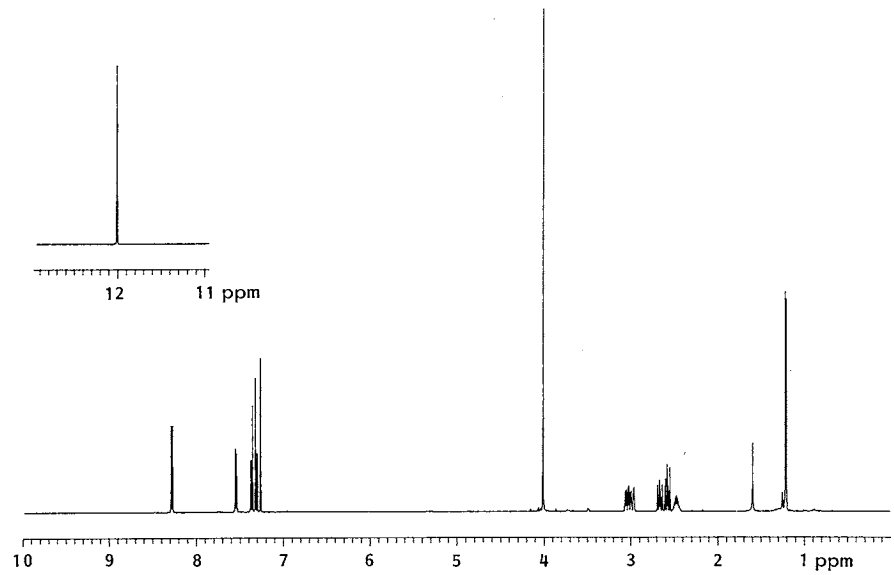


Fig. 2. ^{1}H NMR spectrum of hatomarubigin B in $CDCl_3$.



heteronuclear multiple-quantum coherence (HMQC)[9] experiments.

The structure of the aromatic chromophore in hatomarubigin C was elucidated by a homonuclear multiple-bond correlation (HMBC)[10] experiment as shown in Fig. 5. ^{1}H-^{13}C *meta* couplings were observed between *ortho*-coupled aromatic protons (δ 7.40 and 7.32) and their relevant carbons (δ 157.4 and 118.5; δ 153.8 and 117.1) in a trisubstituted phenol moiety with a methoxy group (δ_H 4.01) at *para* position. The relationship was established by observation of long-range correlations from the phenolic hydroxyl proton

Fig. 3. ^1H NMR spectrum of hatomarubigin C in $CDCl_3$.

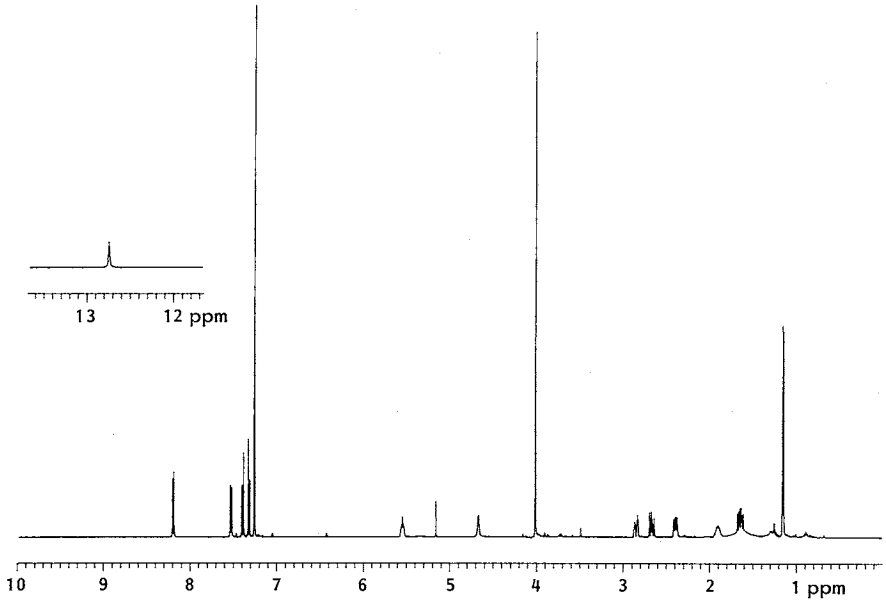
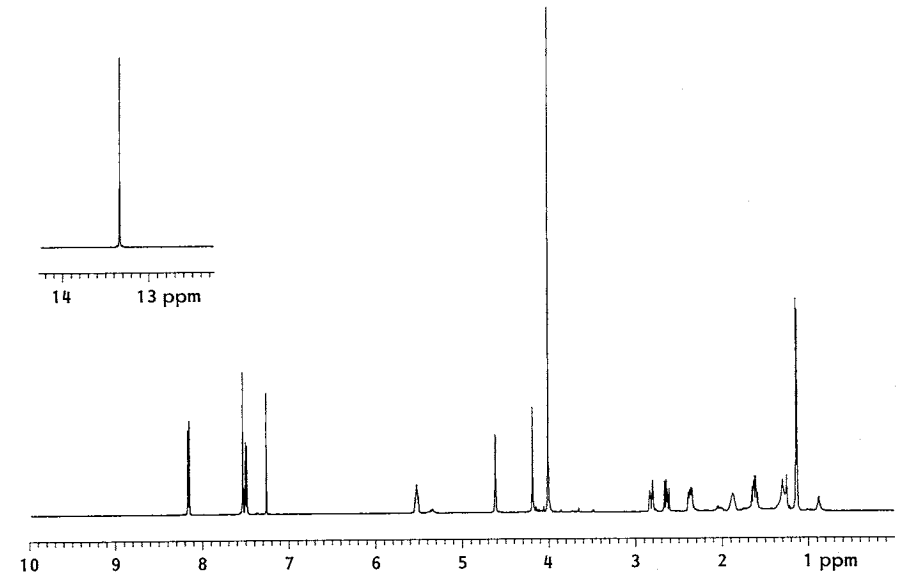


Fig. 4. ^1H NMR spectrum of hatomarubigin D in $CDCl_3$.



(δ_H 12.74) to three aromatic carbons (δ 157.4, 126.6 and 117.1), and from the methoxy proton to one sp^2 carbon (δ 153.8). Another pair of *ortho*-coupled protons at δ 8.19 and 7.53 were coupled to their relevant *meta* carbons (δ 145.4 and 130.9; δ 142.5 and 136.1). In addition, the former proton was coupled to one of the quinone carbonyls (δ 181.8) and the latter to a methylene carbon (δ 40.3). These data revealed the presence of a 1-hydroxy-4-methoxyanthraquinone chromophore in hatomarubigin C. The ring juncture was determined from the lower-field chemical shift (δ 192.8) of the other quinone carbonyl due to

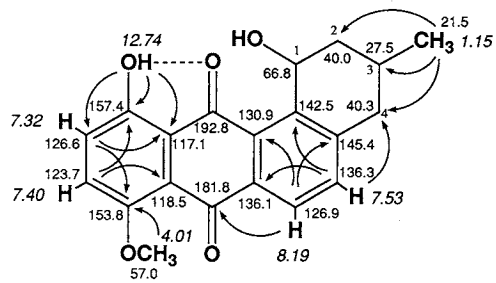
Table 2. ^{1}H NMR data for hatomarubigins A, B, C and D (δ_H, multiplicity (J=Hz)).

No.	A	B	C	D
1			5.54 ddd (9.0, 7.5, 4.0)	5.52 ddd (9.0, 7.0, 4.5)
2	2.92 m,	3.04 ddd (15.5, 5.5, 1.5),	2.39 ddt (13.5, 7.5, 2.5),	2.36 ddt (13.0, 7.0, 2.5),
	2.46 dd (15.0, 11.0)	2.57 dd (15.5, 10.5)	1.64 ddd (13.5, 12.0, 9.0)	1.62 ddd (13.0, 12.0, 9.0)
3	2.40 m	2.47 m	1.90 m	1.87 m
4	2.90 m,	2.98 ddd (16.0, 4.0, 1.5),	2.85 ddd (17.0, 4.0, 2.5),	2.81 ddd (16.5, 3.5, 2.5),
	2.60 dd (16.5, 10.5)	2.66 dd (16.0, 10.5)	2.67 dd (17.0, 11.0)	2.64 dd (16.5, 10.5)
5	6.95 s	7.54 d (8.0)	7.53 d (9.5)	7.49 d (8.0)
6		8.28 d (8.0)	8.19 d (9.5)	8.16 d (8.0)
9	7.31 dd (7.5, 2.0)	7.36 d (9.5)	7.40 d (8.5)	7.53 s
10	7.73 t (7.5)	7.31 d (9.5)	7.32 d (8.5)	
11	7.74 dd (7.5, 2.0)			
3-CH_3	1.16 d (6.5)	1.21 d (6.5)	1.15 d (7.0)	1.14 d (6.5)
8-OCH_3	4.05 s	4.00 s	4.01 s	4.00 s
10-CH_2				4.18 s
1-OH			4.67 d (4.0)	4.61 d (4.5)
6-OH	13.04 s			
11-OH		12.01 s	12.74 s	13.33 s

Table 3. ^{13}C NMR data for hatomarubigins A, B, C and D.

No.	A	B	C	D
1	197.7	199.5	66.8	66.8
2	47.5	47.6	40.0	40.0
3	30.2	29.7	27.5	27.5
4	38.6	38.2	40.3	40.3
4a	152.2	149.1	145.4	145.3
5	120.9	133.7	136.3	136.3
6	163.6	129.4	126.9	126.8
6a	117.7	135.2	136.1	136.1
7	188.4	180.9	181.8	181.4
7a	119.8	118.6	118.5	117.3
8	160.3	154.0	153.8	153.4
9	117.4	122.9	123.7	125.0
10	136.3	126.4	126.6	136.9
11	119.9	156.3	157.4	156.0
11a	137.4	116.7	117.1	116.8
12	184.5	187.8	192.8	193.1
12a	137.8	132.8	130.9	130.9
12b	128.1	135.9	142.5	142.5
3-CH_3	21.3	21.5	21.5	21.5
8-OCH_3	56.6	57.0	57.0	57.0
10-CH_2				31.3

Fig. 5. HMBC data summary for hatomarubigin C.



hydrogen-bonding with the phenolic hydroxyl group.

The COSY spectrum of the aliphatic portion in hatomarubigin C revealed a proton spin network from a hydroxyl (δ 4.67) through a methine (δ 5.54), a methylene (δ 2.39 and 1.64) and a methine (δ 1.90) substituted with a methyl (δ 1.15) to a methylene (δ 2.85 and 2.67). Large vicinal coupling constants observed with the axial methylene protons ($J_{1\text{-}2ax}$= 9.0 Hz, $J_{2ax\text{-}3}$=12.0 Hz, $J_{3\text{-}4ax}$=11.0 Hz) showed equatorial bonds for the hydroxyl and methyl groups. These results established the structure of hatomarubigin C as shown in Fig. 9.

The absolute configuration of hatomarubigin C was determined to be (1*S*,3*S*) from the similarity of its optical rotation value ($[\alpha]_D^{21}$ +514°, *c* 0.64, $CHCl_3$) to that of rubiginone B_1[5,6] (11-deoxyhatomarubigin C; $[\alpha]_D^{25}$ +367°, *c* 0.5, $CHCl_3$) and the co-production of rubiginone B_2[5,6] ($[\alpha]_D^{21}$ +63°, *c* 0.5, $CHCl_3$) by the hatomarubigin producing organism.

The existence of a 1-hydroxy-4-methoxyanthraquinone moiety in hatomarubigin B was required by

Fig. 6. HMBC data summary for hatomarubigin B.

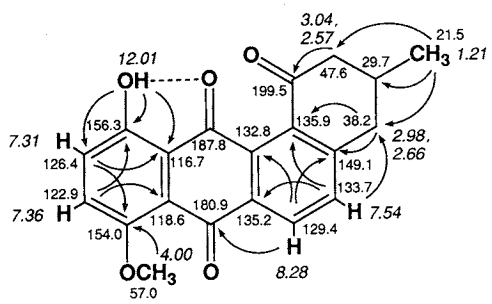


Fig. 7. HMBC data summary for hatomarubigin A.

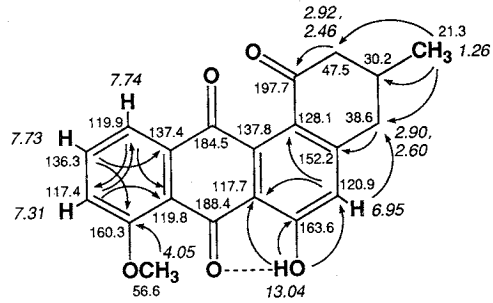
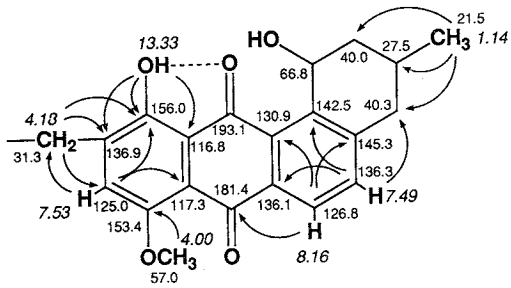


Fig. 8. HMBC data summary for hatomarubigin D.



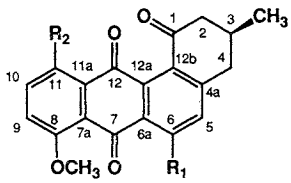
the ^{13}C NMR spectral similarity in the aromatic region between hatomarubigins B and C. Hatomarubigin B, however, contained a conjugated ketone (δ 199.5) in place of the oxymethine in hatomarubigin C resulting in a downfield shift of an adjacent methylene carbon (from δ 40.0 to 47.6). Thus, hatomarubigin B is the 1-dehydro derivative of hatomarubigin C (Fig. 9). The strucrure and ^{13}C chemical-shift assignments of hatomarubigin B were confirmed by an HMBC experiment as shown in Fig. 6.

Hatomarubigin A has the same molecular formula as hatomarubigin B. In the ^{1}H NMR spectrum of hatomarubigin A (Fig. 1), the two pairs of aromatic *ortho* protons in hatomarubigin B were replaced by ABX type three aromatic protons (δ 7.74, 7.73 and 7.31) and a singlet aromatic proton (δ 6.95), which was located next to a hydrogen-bonded phenolic hydroxyl proton observed at δ 13.04. These data indicated transposition of the hydroxyl group from C-11 in hatomarubigin B to C-6 in A. The aliphatic moieties of these two compounds were quite similar in the ^{1}H and ^{13}C NMR spectra, thereby showing that hatomarubigin A is the 11-deoxy-6-hydroxy derivative of hatomarubigin B (Fig. 9). The structure and ^{13}C chemical-shift assignments were established by an HMBC experiment as shown in Fig. 7.

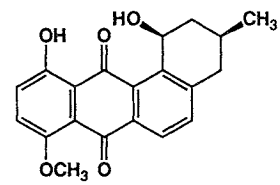
The ^{1}H and ^{13}C NMR spectra of hatomarubigin D were similar to those of hatomarubigin C. Although hatomarubigin D contained 41 carbon atoms, only 21 carbons were observed in the ^{13}C NMR spectra, indicating the presence of a symmetrical portion. The ^{1}H NMR spectrum of hatomarubigin D (Fig. 4) showed almost identical signals to those found in hatomarubigin C. However, one of the pair of aromatic *ortho* protons (9-H and 10-H) in hatomarubigin C was replaced by an aromatic singlet proton (δ 7.53) and a singlet methylene (δ 4.18) with a half integral value as compared with the other signals, suggesting that two completely identical fragments combined to this methylene group. Both the singlet methylene proton and a phenolic hydroxyl proton (δ 13.33) displayed long-range correlations to two aromatic quaternary carbons (δ 156.0 and 136.9) as shown in Fig. 8, revealing an *ortho* relationship of these two substituents on the aromatic ring. Consequently, hatomarubigin D was determined to be a hatomarubigin C dimer with the 10-methylene linkage (Fig. 9).

The structures of the hatomarubigins and rubiginone B_2 are compared in Fig. 9. All these compounds belong to the isotetracenone antibiotics[1]. Rubiginone B_2 differs from hatomarubigins A and B, which are

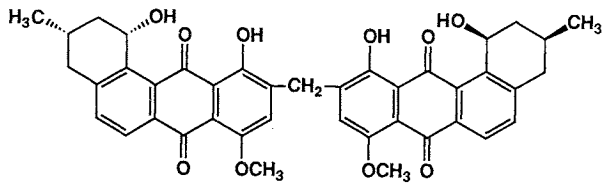
Fig. 9. Structures of hatomarubigins A, B, C and D, and rubiginone B_2.



Hatomarubigin A $R_1=OH$ $R_2=H$
Hatomarubigin B $R_1=H$ $R_2=OH$
Rubiginone B_2 $R_1=R_2=H$



Hatomarubigin C



Hatomarubigin D

regioisomers of the hydroxyl group, in lacking the phenolic-hydroxyl substituent. Hatomarubigin C is a reduced variant of hatomarubigin B at the C-1 carbonyl. Hatomarubigin D is a quite unique dimer of hatomarubigin C with a methylene linkage.

Biological Aactivity

Hatomarubigins A, B, C and D, and rubiginone B_2 inhibited the growth of colchicine-resistant KB(CH^R) cells in the presence of 1.5 μg/ml of colchicine as summarized in Table 4. Hatomarubigin A was ten times more effective than the other compounds, which showed approximately the same IC_{50}s (10~12.5 μg/ml) in this experiment. On the other hand, hatomarubigins A and B were cytotoxic to the cells in a colchicine-free medium with IC_{50}s of 8.5 and 16.0 μg/ml, respectively, while the other compounds showed no cytotoxicity at concentrations less than 50 μg/ml. From the difference in their effective dose with or without colchicine, hatomarubigins A, C, D and rubiginone B_2 appeared to overcome the multidrug-resistance of KB(CH^R) cells, while hatomarubigin B was considered to show an additive effect with colchicine. Further biological studies are in progress.

Table 4. Effects of hatomarubigins A, B, C and D, and rubiginone B_2 on multidrug-resistant tumor cells.

	IC_{50} (μg/ml)	
	Colchicine (1.5 μg/ml)	Colchicine (−)
Hatomarubigin A	0.9	8.5
Hatomarubigin B	10.1	16.0
Hatomarubigin C	10.0	>50
Hatomarubigin D	10.1	>50
Rubiginone B_2	12.5	>50

Cell line: KB(CH^R), colchicine-resistant KB cells; EAGLE'S MEM + 0.1% Bacto-peptone + 10% FCS; 1×10^5 cells/ml, 37°C, 24 hours.

Experimental

General

Specific rotations were obtained on a Jasco DIP-140 spectropolarimeter at 589.6 nm and 22°C. Mass spectra were measured on a Jeol HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol matrix. UV and visible spectra were recorded on a Shimadzu UV-160 spectrophotometer. NMR spectra were obtained on a Jeol JNM-GXS500 spectrometer with ^1H NMR at 500 MHz and ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard.

Microorganism

Streptomyces sp. 2238-SVT4 was isolated from a soil sample collected at Hatoma Island, Okinawa Prefecture. It was deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the accession number FERM BP-3326. Taxonomic studies on the organism will be published elsewhere.

Cells and Cell Culture

KB(CH^R) cells were cultured in EAGLE's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 0.1% Bacto-peptone. The cells at 1×10^5 cells/ml were incubated with testing agents in a humidified atmosphere of 5% CO_2 in air at 37°C for 24 hours. The cytotoxic dose (IC_{50}) of colchicine to KB(CH^R) cells was 8.5 μg/ml, while that to normal KB cell was 0.08 μg/ml. KB(CH^R) cells were about 30 times as resistant to doxorubicin as the parent KB cell.

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