Halichondrins—antitumor polyether macrolides from a marine sponge

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Abstract – New antitumor polyether macrolides were successfully isolated
from a marine sponge, Halichondria okadai Kadota. One of them, halichondrin
B exhibited remarkable in vivo antitumor activity. Physiological properties
and structures of these compounds are reported herein. The structures have
been characterized by a long-straight carbon chain, a polyether macrolide,
and a novel 2,6,9-trioxatricyclo[3.3.2.0^{3,7}]decane system which is the first
example in natural products as far as we know.

INTRODUCTION

In our continuing search for physiologically active substances from marine sources, we re-
cently found eight antitumor compounds (ref. 1) from Halichondria okadai Kadota which was
a common, widely distributed sponge in the Pacific coast of Japan. Prior studies by Scheuer
and Tsukitani (ref. 2) resulted in the identification of okadaic acid as a cytotoxic con-
stituent of this animal. However, our interest in the same animal focused on the fact that
sponge extracts exhibited remarkable in vivo antitumor activity (ref. 3). Bioassay against
B-16 melanoma cells guided the isolation of extremely bioactive compounds which were named
halichondrins.

The marine sponges live in unique association (ref. 4) with a larger amount of symbionts such
as bacteria than that of their cells, and also they collect micro-organisms by filtration of
sea water. As expected, the unusual metabolites of marine micro-organisms may be concentrated
in the whole body. Although it is reported by Okami (ref. 5) that many of the metabolites of
marine micro-organisms are similar to or identical with those of terrestrial micro-organisms,
it would be necessary to multiply examples because of difficulties in the definition of a
marine micro-organism. In order to find the metabolites of marine micro-organisms which
differ from those of terrestrial micro-organisms, our studies on minor bioactive constituents
screened with the major compounds have been done, on the basis of ecology of the marine sponges.

MATERIALS

Halichondria okadai Kadota was collected on the coast of Aburatsubo in the Miura Peninsula
which is to the south of Tokyo. This black-colored animal is living in the mediolittoral
zone. Since dependence of chemical constituents on the location of collection is well known
(ref. 6), our field collection has been done on the same place from April through September.
However, contents of a series of halichondrins did not depend on this period. Specimens of
600 kg were collected and stored in a chest freezer.

ISOLATION

Our method for the isolation and purification of halichondrins is shown in Fig. 1. Frozen
specimens were crushed in a blender with MeOH. After standing for a period of three days,
the solid residue was removed by filtration. The resulting brownish filtrate was concentrat-
ed carefully under reduced pressure at low temperature. The remaining aqueous solution was
extracted with n-butanol saturated enough with water. The combined organic layers were con-
centrated under reduced pressure. Thus obtained extracts were dissolved with 70% aqueous
MeOH, and then the solution was washed with three portions of n-hexane. The MeOH layer was
concentrated under reduced pressure to give an oily material which was charged on TSK G3000S
polystyrene gel column (ref. 7) well washed with ethanol and then water. The bioactive
fractions against B-16 melanoma cells in vitro were eluted with 50% ethanol and then 60% ethanol.
Each fraction was further separated by the use of LiChroprep RP-8 column and YMC Pack A212
(C-8) column. The repeated purification was done and finally, eight active compounds against
B-16 melanoma cells were obtained. The major component of this series was norhalichondrin A
which was given in 5 X 10^{-6}% yield.

```
Halichondria okadai Kadota (600 kg)
        │   crushed and extracted with MeOH
        │   filtrated and concentrated
Aqueous Extracts
        │   extracted with n-BuOH
n-BuOH Extracts
        │   concentrated
70% Aqueous MeOH Solution
        ┌─────────────────────────────────────────────┐
        │   concentrated                    n-Hexane
Physiologically Active Substances
        │   column-chromatographed on TSK G3000S
┌──────────────┬─────────────────────────────┬──────────────────────────┐
eluted with    │   eluted with 50% EtOH       │   eluted with 60% EtOH
40% EtOH       │   concentrated               │   concentrated
               An Oily Material               An Oily Material
                  │   separated by LC            │   separated by LC
                  │   i) LiChroprep RP-8 column (size C)    i) LiChroprep RP-8 column (size C)
                  │      solvent, 40% MeCN-H2O                 solvent, 40% MeCN-H2O
                  │  ii) LiChroprep RP-8 column (size B)   ii) LiChroprep RP-8 column (size A)
                  │      solvent, 20% MeCN-H2O                 solvent, 30% MeCN-H2O
                  │ iii) LiChroprep RP-8 column (size A)
                  │      solvent, 20% MeCN-H2O
               A Glassy Material               A Glassy Material
                  │   separated by HPLC           │   separated by HPLC
                  │   YMC Pack A212 (C-8) column   YMC Pack A212 (C-8) column
                  │   solvent, 18% MeCN-H2O        solvent, 25% MeCN-H2O
               Norhalichondrin A (1)    35.0 mg    Halichondrin B (4)      12.5 mg
                            B (10)       4.2 mg    Homohalichondrin B (12)  3.1 mg
                            C (11)       2.4 mg
               Homohalichondrin A (6)   17.2 mg
                            C (13)       2.1 mg
               Halichondrin C (8)        7.2 mg
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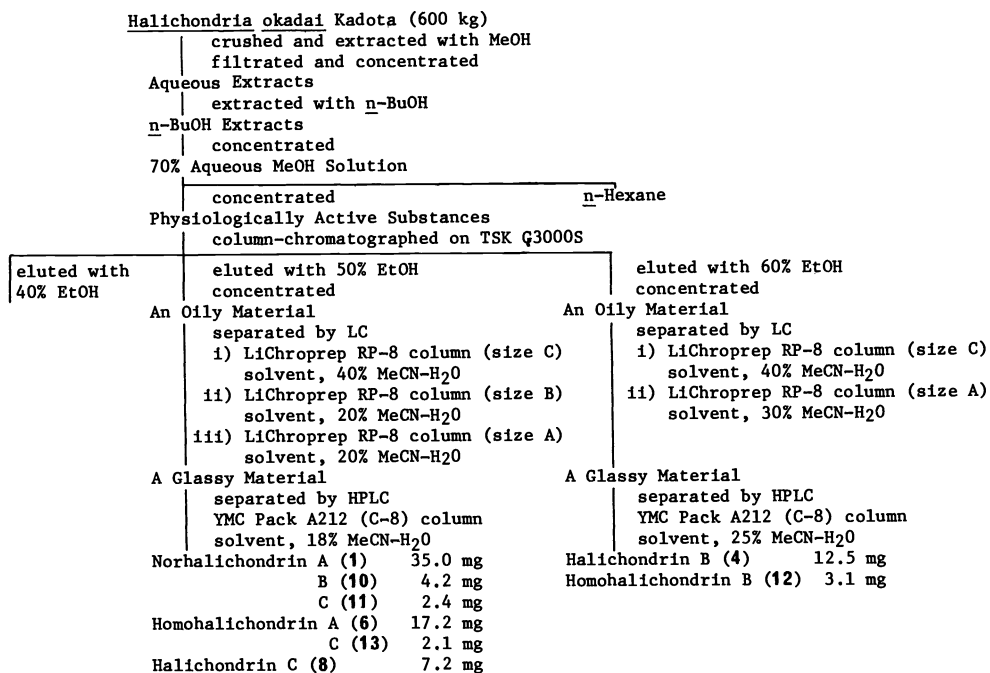
Fig. 1. Scheme for the isolation and purification of a series of halichondrins.

PHYSIOLOGICAL ACTIVITIES

These new compounds were compared by cytotoxicity against B-16 melanoma _in vitro_ as summariz-ed in Table 1. Although data of homohalichondrin C, and norhalichondrins B and C are not shown, the corresponding activities are inferior to that of halichondrin B. Bioactivity of

TABLE 1. Cytotoxicity of halichondrins against B-16 melanoma cells.

Sample	IC_{50}[a] (ng/ml)
Halichondrin B (4)	0.093
Norhalichondrin A (1)	5.2
Homohalichondrin A (6)	0.26
Halichondrin C (8)	0.35
Homohalichondrin B (12)	0.1

[a]Inhibition coefficient

TABLE 2. Antitumor activity against B-16 melanoma _in vivo_.

Sample	Volume (µg/kg)	Dose for mice	M.S.T.[a] (day)	T/C[b] (%)
Halichondrin B (4)	0	day 1-9 i.p.	16	——
	2.5		32.5	203
	5.0		39	244
	0	day 1, 3, 5, 7, 9 i.p.	19	——
	5.0		37.5	197
	10.0		39.5	208
	0	day 1, 5, 9 i.p.	18	——
	10.0		36.5	203
	20.0		39.5	219
	0	day 1, 4, 7, 10 i.v.	17.5	——
	10.0		27.5	157

[a]Median survival time.   [b]Test group/control group.

TABLE 3. Antitumor activity against P-388 leukemia _in vivo_.

Sample	Volume (µg/kg)	Dose for mice	M.S.T. (day)	T/C (%)
Halichondrin B (4)	0	day 1-9 i.p.	11	——
	1.25		15	136
	2.50		16.5	150
	5.00		26	236
	10.0	day 1, 3, 5, 7, 9 i.p.	35.5	323

TABLE 4. Antitumor activity against L-1210 leukemia in vivo.

Sample	Volume (µg/kg)	Dose for mice	M.S.T. (day)	T/C (%)
Halichondrin B (4)	0	day 1-5, 7-12 i.p.	7	——
	30		10	143
	50		14.5	207
	70		14	200
	0	day 1, 3, 5, 7, 9, 11 i.p.	8	——
	50		11	138
	100		30<	375<

TABLE 5. Molecular formula and molecular weight of halichondrins.

	Molecular formula	Molecular weight
Norhalichondrin A (1)	$C_{59}H_{82}O_{21}$	1126
Halichondrin B (4)	$C_{60}H_{86}O_{19}$	1110
Homohalichondrin A (6)	$C_{61}H_{86}O_{21}$	1154
Homohalichondrin C (13)	$C_{61}H_{86}O_{20}$	1138
Norhalichondrin B (10)	$C_{59}H_{82}O_{19}$	1094
Norhalichondrin C (11)	$C_{59}H_{82}O_{20}$	1110
Homohalichondrin B (12)	$C_{61}H_{86}O_{19}$	1122
Halichondrin C (8)	$C_{60}H_{86}O_{20}$	1126

TABLE 6. ^{1}H NMR data of a series of halichondrins (360 MHz in CD_3OD, δ).

	Nor A (1)	Nor B (10)	Nor C (11)	Hali B (4)	Hali C (8)	Homo A (6)	Homo B (12)	Homo C (13)
H2	2.44	2.44	2.44	2.44	2.44	2.44	2.44	2.43
H2	2.58	2.56	2.56	2.57	2.56	2.56	2.56	2.55
H3	3.89	3.88	3.88	3.88	3.88	3.88	3.88	3.88
H6	4.32	4.33	4.30	4.33	4.30	4.31	4.33	4.30
H7	2.93	2.97	2.94	2.98	2.98	2.93	2.98	2.94
H8	4.37	4.30	4.30	4.31	4.30	4.37	4.30	4.30
H9	4.31	4.10	4.10	4.13	4.10	4.31	4.10	4.10
H10	4.21	4.18	4.17	4.18	4.18	4.21	4.18	4.17
H11	4.31	4.60	4.40	4.60	4.40	4.31	4.60	4.40
H12	——	4.70	——	4.71	——	——	4.70	——
H13	3.53	2.00	2.27	1.98	2.27	3.53	2.00	2.27
H13	——	2.08		2.09		——	2.08	
H17	4.09	4.08	4.10	4.08	4.10	4.10	4.10	4.10
H18	2.32	2.32	2.32[a]	2.32	2.32[a]	2.32	2.32	2.32[a]
H18	2.82	2.80	2.80	2.80	2.80	2.82	2.80	2.80
C19=CH$_2$	5.02	5.01	5.01	5.02	5.02	5.02	5.02	5.01
C19=CH$_2$	5.06	5.06	5.06	5.07	5.06	5.07	5.06	5.06
H20	4.44	4.45	4.43	4.46	4.43	4.44	4.45	4.43
H23	3.72	3.71	3.70	3.71	3.70	3.72	3.70	3.72
C25-Me	1.09	1.09	1.10	1.10	1.09	1.09	1.08	1.08
C26=CH$_2$	4.81	4.82	4.82[a]	4.82	4.82[a]	4.80	4.82	4.82[a]
C26=CH$_2$	4.86	4.87	4.87[a]	4.88	4.87[a]	4.85	4.87	4.87[a]
H27	3.61	3.61	3.60	3.62	3.59	3.61	3.61	3.61
H29	4.24	4.24	4.24	4.25	4.25	4.24	4.25	4.24
H30	4.61	4.61	4.61	4.63	4.63	4.62	4.63	4.62
C31-Me	1.06	1.05	1.05	1.07	1.05	1.04	1.05	1.04
H32	3.22	3.21	3.22	3.22	3.22	3.22	3.22	3.22
H33	3.87	3.88	3.88	3.87	3.88	3.88	3.87	3.88
H35	4.11	4.10	4.10	4.12	4.10	4.10	4.10	4.10
H36	4.09	4.10	4.10	4.10	4.10	4.10	4.10	4.10
H40	3.98	3.98	3.96	4.05	4.05	3.96	3.95	3.96
H41	3.69	3.69	3.66	3.69	3.68	3.66	3.66	3.66
C42-Me	0.97	0.98	0.98	0.94	0.96	0.94	0.94	0.96
C46-Me	0.96	0.95	0.96	1.01	1.00	0.93	0.93	0.95
H47	3.30	3.30	3.30[a]	3.56	3.56	3.12	3.12	3.12
H48	3.77	3.78	3.78	4.10	4.10	3.58	3.58	3.58
H49	1.94	1.95		1.83		1.91	1.90	
H49	2.09	2.11		2.27		2.16	2.16	
H50	3.61	3.61	3.60	4.00	3.99	3.90	3.90	3.90
H51	3.78	3.78	3.78	3.78	3.78	4.02	4.02	4.02
H52	2.47	2.47	2.47	1.61	1.61[a]	2.00	2.00	2.00[a]
H52	2.47	2.47	2.47	1.75	1.75[a]	2.00	2.00	2.00[a]
H53	——	——	——	3.87	3.88	4.23	4.23	4.23
H54	——	——	——	3.46	3.47	3.50	3.50	3.50
H54	——	——	——	3.53	3.53	——	——	——
H55	——	——	——	——	——	3.58	3.58	3.58
H55	——	——	——	——	——	3.59	3.59	3.59

[a]Assignments are tentative.

TABLE 7. ^{13}C NMR data of halichondrins (75.4 MHz in CD_3OD, δ).

	Nor A	Nor B	Hali B	Hali C	Homo A		Nor A	Nor B	Hali B	Hali C	Homo A
C1	172.8	173.6	172.8	172.8	172.8	C30	77.3	77.3	77.3	77.3	77.3
C2	41.1	41.2	41.2	41.2	41.1	C31	37.5	37.4	37.5	37.5	37.4
C3	75.1	75.0	74.9	74.7	75.1	C31-Me	15.9	15.8	15.9	15.9	15.8
C6	69.6	69.6	69.6	69.5	69.6	C32	77.9	78.0	78.0	78.0	77.9
C7	79.0	79.1	79.1	79.0	79.0	C33	65.6	65.6	65.5	65.5	65.6
C8	75.8	75.8	75.8	76.1	75.9	C35	77.6	77.3	77.3	77.3	77.6
C9	73.8	74.9	73.3	73.3	73.8	C36	78.0	78.0	78.0	78.0	78.0
C10	85.5	77.9	78.0	73.0	85.5	C37	45.6	45.5	45.6	45.5	45.6
C11	75.5	83.8	83.8	86.2	75.5	C38	114.9	114.8	114.8	114.9	114.8
C12	113.4	82.4	82.5	114.2	113.3	C39	44.9	44.9	45.0	45.0	44.9
C13	82.4	49.4[a]	49.4	49.3	82.3	C40	72.7	72.6	73.0	73.0	72.3
C14	112.9	111.2	111.3	110.4	112.8	C41	80.7	80.6	80.8	80.8	81.0
C17	76.3	76.3	76.3	76.3	76.3	C42	27.3	27.3	27.1	27.1	27.1
C18	39.8	39.7	39.7	39.7	39.8	C42-Me	18.1	18.1	18.2	18.1	18.2
C19	153.2	154.0	153.2	153.2	153.0[a]	C44	98.5	98.5	98.4	98.4	98.1
C19=CH2	105.8	105.7	105.8	105.8	105.8	C46	30.1	30.1	27.1	27.1	30.1
C20	76.0	76.1	76.1	76.3	75.9	C46-Me	17.5	17.4	18.3	18.3	17.6
C23	75.3	75.3	75.3	75.3	75.2	C47	77.2	77.2	81.3	81.3	74.5
C25	37.2	37.1	37.5	37.5	37.2	C48	68.2	68.2	75.1	74.8	65.2
C25-Me	18.4	18.4	18.4	18.4	18.4	C50	68.1	68.1	81.3	81.3	75.4
C26	153.2	154.0	153.2	153.2	153.2[a]	C51	79.1	79.1	73.1	73.2	78.4
C26=CH2	104.8	104.8	104.8	104.8	104.8	C53	172.8	173.6	71.6	71.6	79.8
C27	75.1	75.0	75.1	74.8	75.0	C54	——	——	67.1	67.1	75.0
C29	73.8	73.8	73.8	73.8	73.8	C55	——	——	——	——	65.1

[a]Assignments are tentative.

halichondrin B is about 50 times that of norhalichondrin A whereas the acute toxicity of nor-halichondrin A (LD_{50}, approximately 50 μg/kg for mice) is the highest among those. Antitumor activity of halichondrin B was investigated by the use of in vivo system. Tables 2, 3 and 4 show results against B-16 melanoma, and P-388, L-1210 leukemia, respectively. Dose of halichondrin B for mice in low concentration resulted in the high T/C% and also dose by intravenous injection was effective. Homohalichondrin B is also bioactive comparably with halichondrin B in these in vivo system. Further studies on activities of halichondrin B and homohalichondrin B are currently under way.

PROPERTIES OF A SERIES OF HALICHONDRINS

The molecular formulae and molecular weights of this series were summarized in Table 5. These compounds were not labile in basic media whereas acid solution caused obvious decomposition. Halichondrins B and C were obtained as a crystalline form: halichondrin B, m.p. 164-166°C; halichondrin C, m.p. 169-172°C. Optical rotations were measured in MeOH: halichondrin B, $[\alpha]_D$ -58.9° (c, 0.94); halichondrin C, $[\alpha]_D$ -41.6° (c, 0.49); norhalichondrin A, $[\alpha]_D$ -47.8° (c, 1.13); homohalichondrin A, $[\alpha]_D$ -97.1°(c, 1.23). The ^1H and ^{13}C NMR data of halichondrins are shown in Tables 6 and 7.

STRUCTURE OF NORHALICHONDRIN A

At first we began to determine the structure of halichondrin B, the most effective antitumor substance among those. However, X-ray crystallographic studies of a crystal of halichondrin B unfortunately failed because of its relatively large molecular weight, 1110, and then the absence of any heavy atoms such as bromine or iodine atoms. Therefore, structural elucidation of norhalichondrin A, the major component of this series, has been done (ref. 8).
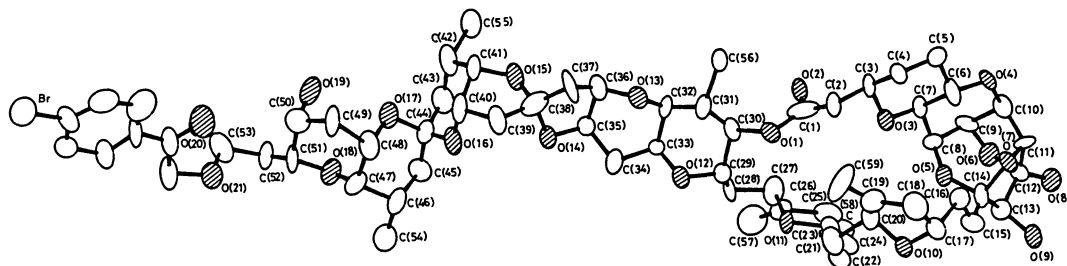

1   R^1, R^2, R^3, R^4 = H
2   R^1 = $CH_2COC_6H_4Br$ R^2, R^3, R^4 = H
3   R^1, R^2: R^3, R^4 = COC_6H_4Br
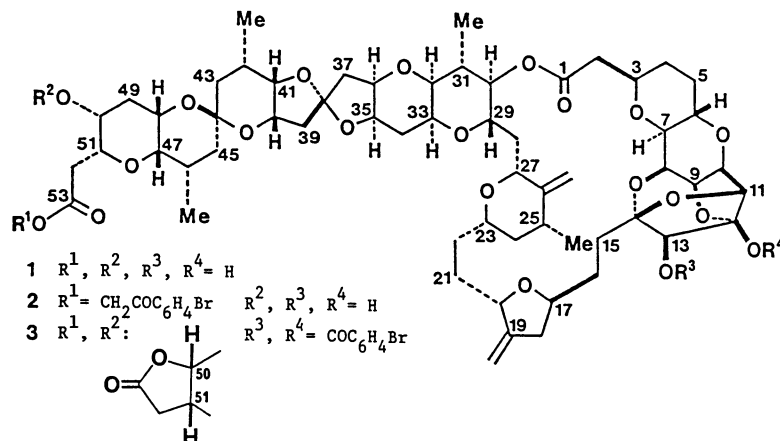
Fig. 2. Computer-generated perspective drawing of the final X-ray model of the p-bromophenacyl ester **2**.

R= COC$_6$H$_4$Br

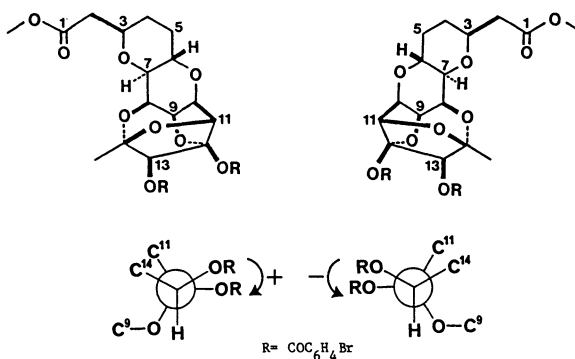Fig. 3. Exciton chirality of the 12,13-bis(p-bromobenzoate) system in **3**.

The SIMS spectrum showed the highest mass peak at m/z 1127 (M + 1) corresponding to the molecular formula C$_{59}$H$_{82}$O$_{21}$. The IR spectrum (KBr) indicated the presence of hydroxyls (3450 cm^{-1}), lactone larger than five-membered ring or ester (1740 cm^{-1}) and carboxylate (1590 cm^{-1}). The ^{1}H and ^{13}C NMR spectra were not fruitful for structural elucidation because of their complexity. However, two sets of exomethylene (^{1}H NMR δ 4.81, 4.86, 5.02, 5.06, 1H each; ^{13}C NMR δ 104.81, 105.76, 153.17, 153.32), four carbon atoms bearing two oxygen atoms (^{13}C NMR δ 98.51, 112.86, 113.38, 114.87), four secondary methyl groups (^{1}H NMR δ 0.96, 0.97, 1.06, 1.09, 3H each as a doublet; ^{13}C NMR δ 15.88, 17.47, 18.13, 18.42), and then carbonyl group(s) (^{13}C NMR δ 172.81) were recognized by the NMR spectra in CD$_3$OD.

Norhalichondrin A (**1**) was treated with p-bromophenacyl bromide and triethylamine in DMF at 50°C. After separation by preparative TLC, the desired crystalline p-bromophenacyl ester **2** [IR (CHCl$_3$) 1735, 1705, 1590 (weak) cm^{-1}] was recrystallized from acetone-methanol, furnishing well formed, monoclinic crystals: m.p. 173.5-175.0°C. Its structure has been unambiguously determined by X-ray crystallographic analysis. The computer-generated perspective drawing of a molecule of **2** is shown in Fig. 2, including its absolute configuration. Application of the nonempirical dibenzoate chirality method (ref. 9) was also consistent with the X-ray diffraction result. Treatment of **1** with p-bromobenzoyl chloride in pyridine at 80°C yielded product **3** with the γ-lactone [IR (CHCl$_3$) 1775 cm^{-1}]. Compound **3** contains two p-bromobenzoyl groups judging from the ^{1}H NMR spectrum [C$_6$D$_6$, δ 6.9-7.8 (8H, m), 5.89 (1H, s, H13)]. This reaction seems to proceed the reasonable C13→C12 acyl migration (ref. 10), followed by ordinary acylation of the secondary alcohol. The bis(p-bromobenzoate) **3** possessing the 1,2-dibenzoate system showed a typical positive split CD; EtOH 241 (Δε -19.3) and 257 nm (Δε 22.1) (ref. 11). This observation confirmed the chirality of the C13-OCOC$_6$H$_4$Br/C12-OCOC$_6$H$_4$Br as shown in Fig. 3. Unique, novel structure **1** of norhalichondrin A is characterized by a long-straight C$_{53}$ chain, a polyether macrolide, cis-fused and boat-shaped pyranose rings, and a 2,6,9-trioxatricyclo[3.3.2.0^{3,7}]decane system in particular.

STRUCTURE OF HALICHONDRIN B

The molecular weight of halichondrin B was secured to be 1110 by the FDMS spectrum [m/z, 1133 (M + Na)$^+$]. The molecular formula of halichondrin B is C$_{60}$H$_{86}$O$_{19}$. Its IR spectrum indicated the presence of the macrocyclic lactone (1735 cm^{-1}) and hydroxyls (3400 cm^{-1}). Acetylation of halichondrin B (**4**) with acetic anhydride and pyridine gave triacetate **5**, the IR spectrum of which indicated disappearance of all hydroxyls. The ^{1}H NMR spectrum of **5** in C$_6$D$_6$ revealed the presence of the following grouping, -O-CH-CHOCOCH$_3$-CH$_2$-CHOCOCH$_3$-CH$_2$OCOCH$_3$. By acetylation

the signals at δ 3.46, 3.53, 3.87 and 3.78 in the [1]H NMR spectrum (Table 6) shifted to downfield, δ 4.11, 4.40, 5.38 and 5.45, respectively.
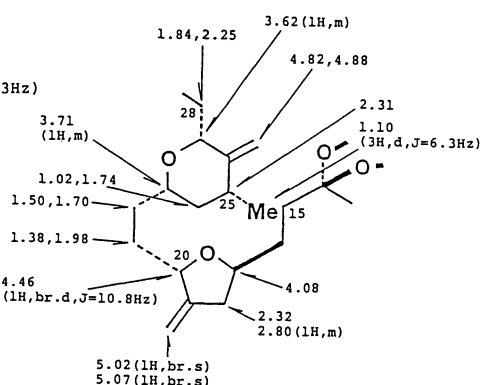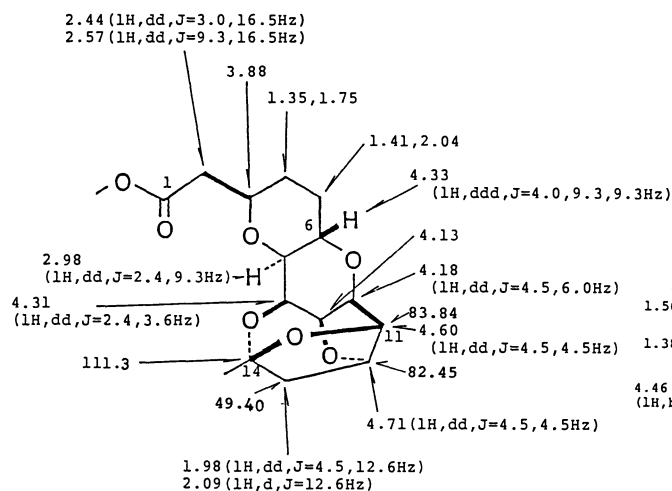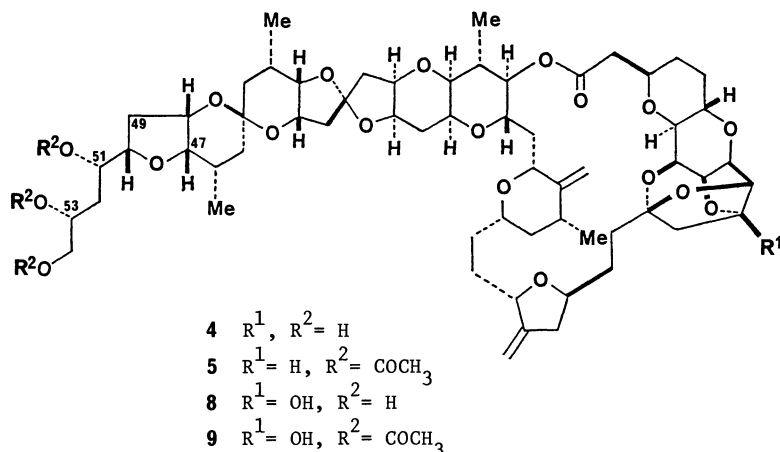

4 R^1, R^2= H

5 R^1= H, R^2= COCH$_3$

8 R^1= OH, R^2= H

9 R^1= OH, R^2= COCH$_3$

Fig. 4. Structure of the C1-C14 moiety of 4.

Fig. 5. Structure of the C15-C28 moiety of halichondrin B (4).

A comparison of the COSY spectrum (ref. 12) of halichondrin B with that of norhalichondrin A (1) has been carefully performed. The C1-C14 partial structure and the corresponding assignment of each proton signal in the [1]H NMR spectrum are shown in Fig. 4. The tricyclo structure was confirmed by observation of long-range couplings between H8 and H10, and between H9 and H11. The absence of the coupling between H13α and H12 in the tricyclo system suggests that the corresponding dihedral angle is 90°. Experiment by the aid of a molecular model was also consistent with the presence of the tricyclo ring. The [1]H-[13]C shift correlation spectrum of halichondrin B (4) indicated that C13 was assigned to the signal at δ 49.4, which was lower field than that of methylene carbon in a furanose ring in the ketal functionality such as dianemycin (ref. 13). As Fig. 5 and Fig. 6 show, the C15-C44 structure was also secured by the detailed analysis of each proton in the [1]H NMR spectrum. The chemical shifts and coupling constants of each proton on the C15-C44 of halichondrin B (4) were relatively consistent with those of norhalichondrin A (1). The [13]C NMR data (Table 7) also confirmed this conclusion. Based on the detailed analysis of the [1]H and [13]C NMR spectra, the C45-C54 of halichondrin B was assigned as shown in Fig. 7. The signals assigned to H47 and H48 were observed at lower field than those of 1. It means that the six-membered ring including C47-C51 in 1 fused to the six-membered ring (C44-C48) has disappeared. Since one ether ring is remaining from consideration of the molecular formula, it is deduced that halichondrin B possesses alternative five-membered ring (C47-C50) fused to the six-membered ring (C44-C48). The sequence of each proton was revealed by the COSY spectrum and also by decoupling experiments. Relationship among C50, C51, C52, C53 and C54 is consistent with partial structure described before.
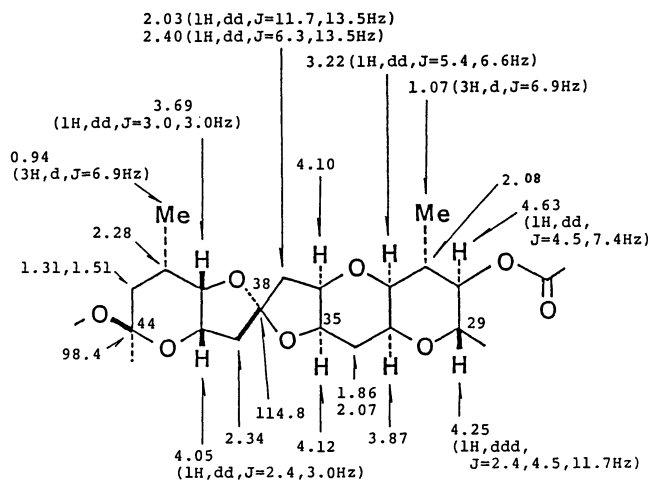
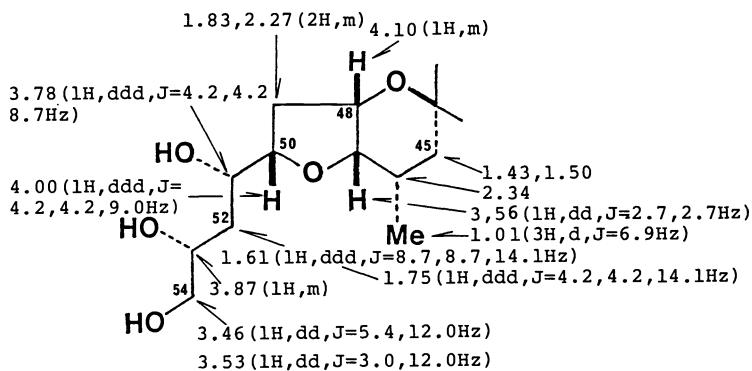Fig. 6. Structure of the C29-C44 moiety of halichondrin B (4).

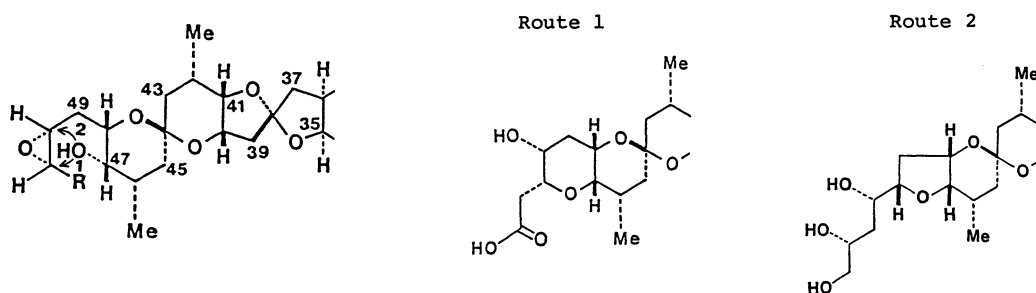Fig. 7. Structure of the terminal moiety of halichondrin B.

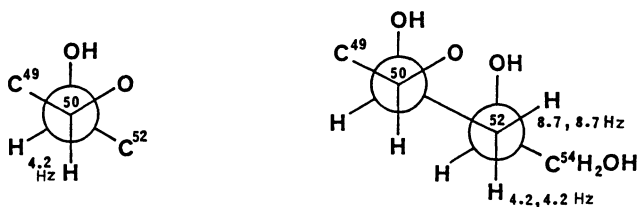Fig. 8. The plausible pathway in biosynthesis of norhalichondrin A (1) and halichondrin B (4).

Fig. 9. The predominant conformers around C51 and C53 of 4.

Stereochemistry of halichondrin B (4) was also proposed by analysis of coupling constants of each proton and by biogenetic consideration. The asymmetric centers at C3, C6, C7, C8, C9, C10, C11, C12, C14, C17, C20, C23, C25, C27, C29, C30, C31, C32, C33, C35, C36, C38, C40, C41, C42, C44 and C46 are consistent with those of norhalichondrin A (1), based on comparison of chemical shifts and coupling constants. Stereochemistry of C47 and C48 was suggested by the following observation. Coupling constants between H46 and H47, and between H47 and H48 were 2.7 Hz each. These values were also observed among H40, H41 and H42 in both of 1 and 4: 1, 2.5 Hz; 4, 3.0 Hz. Stereochemical assignment at C50 and C51 has been performed by bio-genetic consideration. Cyclization of norhalichondrin A (1) at the final stage of its biosynthesis seems to proceed via Route 1 (Fig. 8) involving ring opening of the cis-epoxide by an attack of hydroxyl. Fig. 8 visualizes the hypothetical step producing the final ether linkage in biosynthesis. The important role of cis-epoxide in biosynthesis of this series is speculated by the presence of several cis-fused ether rings. Route 2 leads to production of halichondrin B (4). Actually, relationship between each proton at C50 and C51 was recognized to be gauche in the predominant conformer (Fig. 9) because coupling constant between H50 and H51 was 4.2 Hz (ref. 14). Furthermore, the final C53 stereochemistry was revealed by analysis of coupling constants among H51, H52 and H53 as shown in Fig. 9. Based on the aforementioned results, it is concluded that the proposed structure of halichondrin B is 4.

## STRUCTURE OF HOMOHALICHONDRIN A

The spectral data (Tables 6 and 7) of homohalichondrin A (6) suggested that the C1-C48 structure was consistent with that of norhalichondrin A (1). The remaining C49-C55 part of 6 was revealed by the molecular formula (Table 5) and the following data. Acetylation of 6 with acetic anhydride and pyridine has furnished triacetate 7, which has partial structures: -O-CH-CHOCOCH₃-CH₂OCOCH₃ [^{1}H NMR δ 4.42 (1H, m), 5.31 (1H, ddd, J= 3.3, 4.6, 7.3 Hz), 4.41 (1H, dd, J= 3.3, 12.1 Hz), 4.18 (1H, dd, J= 7.3, 12.1 Hz)], and •CHOCOCH₃• [^{1}H NMR δ 5.60 (1H, s)] in the tricyclo system. The sequence from H47 to H55 was refined by the COSY spectrum and decoupling experiments. The chemical shifts of the corresponding protons were also reasonable for full structure 6. However, stereochemistry at C50, C51, C53 and C54 is not settled yet.

6   R^1, R^2= OH, R^3= H

7   R^1= OCOCH₃, R^2= OH, R^3= COCH₃

12   R^1, R^2, R^3 = H

13   R^1, R^3= H, R^2= OH
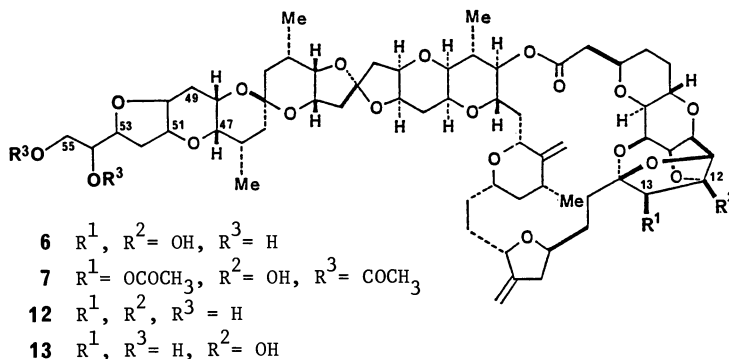
STRUCTURES OF NORHALICHONDRINS B AND C, HALICONDRIN C, AND HOMOHALICHONDRINS B AND C

The structures of the remaining halichondrins were proposed by the spectral data (Tables 6 and 7) and the molecular formulae (Table 5). Especially, it is suggested by the ^{13}C NMR spectrum that halichondrin C has one hydroxyl in the tricyclo ring as a hemiketal functionality. The signal assigned to C12 appears at δ 114.2 as quaternary carbon atom which corresponds to C12 of norhalichondrin A (1). The methylene protons at C13 were assigned to the typical AB signals centered at δ 2.27 in the ^{1}H NMR spectrum of 8. Therefore, we conclude that hali-chondrin C should be drawn by structure 8. As expected, acetyaltion of 8 with acetic

10   R= H

11   R= OH
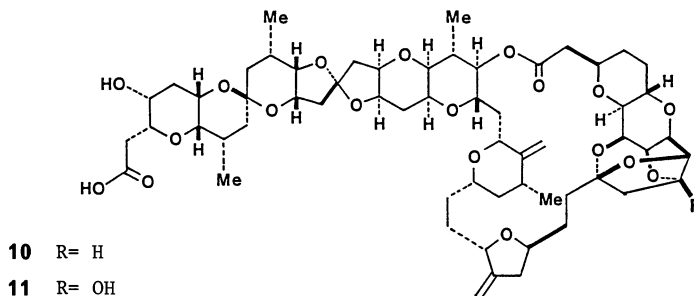
anhydride and pyridine gave triacetate **9**. Norhalichondrins B and C are carboxylic acids because the Rf values on the HPTLC NH2 plates (E. Merck, No. 15647) are zero with 10% MeOH–CHCl3 as solvent. Spectral analysis of norhalichondrins B and C led us to structure **10** for norhalichondrin B, and to structure **11** for norhalichondrin C. Also, the terminal moiety (-O-CH-CHOH-CH2OH) of homohalichondrins B and C corresponds to homohalichondrin A (**6**). On the other hand, the tricyclo parts of homohalichondrins B and C are consistent with those of halichondrins B (**4**) and C (**8**), respectively. Therefore, it is deduced that the structure of homohalichondrin B is **12**, and that of homohalichondrin C is **13**.

STRUCTURE-ACTIVITY RELATIONSHIP

Although we have not enough data concerning antitumor activities, we would like to discuss the structure-activity relationship of this series. Both of halichondrin B (**4**) and homohalichondrin B (**12**) show comparable activity, but that of norhalichondrin A (**1**) is very weak. It is to be desired that the terminal moiety is halichondrin-type or homohalichondrin-type. Alternatively, the desirable structure of the 2,6,9-trioxatricyclo[3.3.2.0^{3,7}]decane system is B-type. By the way, the length of a molecule of halichondrins, 30-35 Å (Fig. 2), corresponds to half of the lipid bilayer of biomembrane. This may be associated with the following facts. Structural variations of this series depend on "head and tail" of a long molecule, which are assigned to the tricyclo system and the terminal moiety. It is very important for antitumor activity that the tricyclo ring is relatively lipophilic and then the terminal moiety contains two or three hydroxyls but not a carboxylate.

Studies on the antitumor constituents of Halichondria okadai Kadota have been done as described before. The reasons why this animal was selected in our investigation were as follows: (1) since the marine sponges belong to unique animals in their ecology exemplified by symbiosis (ref. 4) and(or) food chain, it is conceivable to isolate the favorable metabolites of marine micro-organisms; (2) in order to find the minor bioactive principles screened by the major substances, specimens can be easily collected on a large scale. Since the extracts fortunately exhibited antitumor activity, we succeeded in the isolation of active principles. Especially, halichondrin B (**4**) reveals potent antitumor activity as described before. Bioactivity of **4** was characterized by effects caused by injection in low concentration and by intravenous injection. Occurrence of activity depends on the structural variations in the long molecule. It is of interest that the lipophilic head, the tricyclo ring, of these molecules may get into the lipid bilayer of biomembrane.

The structure of norhalichondrin A was unambiguously determined by X-ray analysis. Based on spectral analysis, halichondrin B was assigned to proposed structure **4**, including its stereochemistry. Other halichondrins were also structurally identified. Interestingly, a variation of the structures exists in only "head and tail" of the molecule. The important features of these molecules are as follows: (1) a long-straight carbon chain such as palytoxin (ref. 15) and brevetoxin (ref. 16); (2) a novel 2,6,9-trioxatricyclo[3.3.2.0^{3,7}]decane system, the first example naturally found as far as we know; (3) a polyether macrolide such as pectenotoxins (ref. 17); (4) two spiro systems, involving a 1,6-dioxaspiro[4.4]nonane system; (5) two boat-shaped pyranose rings; (6) two cis-fused pyranose rings. Subsequently, our interests concentrated on the biological origins of these molecules as well as tedanolide (ref. 18). However, it is known that okadaic acid (ref. 2) is also isolated from a dinoflagellate such as Prorocentrum lima (ref. 19). We also attempted to isolate halichondrins from various dinoflagellates. Unfortunately, we were unable to identify halichondrins. Therefore, it is proposed that halichondrins may be produced by symbiotic bacteria such as blue-green algae. It should be conceivable by culture of these marine micro-organisms to elucidate the biosynthetic pathway of halichondrins, which involves a general concept in biosynthesis of long-straight carbon chains such as palytoxin. Furthermore, we are able to obtain a large amount of **4** which needs to investigate the detailed pharmacological effects. Further studies on the biological origins are currently under way. Finally, it should be noted that molecular weight, spectral data and chromatographic behavior of halichondrin B (**4**) resemble those of ciguatoxin(s) reported by Scheuer (ref. 20).

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