Studies on Anti-cancerous Substances from Higher Plants in East Asian Region

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Summary

To date many kinds of compounds have been obtained from plants kingdom as antineoplastic and anti-cancerous agents. However, there is no special type of compounds for cancer therapy. Various types of substances are effective for various types of cancers and tumors: for instance, alkaloids, lignans, terpenes and steroids etc. Curcumol obtained from *Curcuma aromatica* was tested and noticed to be effective against cancer of the uterine cervix clinically. Oridonin isolated from *Rabdosia* ssp.is now investigated for clinical trials in China. Moreover, camptothecine isolated from *Camptotheca acuminata* is also antineoplastic alkaloid, but is very toxic. Chemical modification has been tried to decrease its toxicity. This compound is now using as clinical agent. Harringtonin was investigated as an anticancerous drug in China. Taxol, a compound with a taxane ring isolated from the bark of *Taxus brevifolia*, has been demonstrated to have substantial anticancer activity in patients with solid tumors refractory standard chemotherapy. Supply of this drug has severely limited full exploration of its antineoplastic potential. Some efforts are continued in National Cancer Institute (NCI) Washington for surveying various *Taxus* species for optimal taxol content, improvement in semi-synthesis from baccatin III, improvement in method of extraction, and development of alternative renewable resources. Further, there are many compounds which have been reported as antineoplastic agents.

On the other hand, we have screened on higher plants collected in Japan, China, Korea, Southeast Asia and South America for antineoplastic activity, which has been done using Sarcoma 180 ascites in mice, P388 lymphocytic leukemia in mice, Chinese hamster lung V-79 cells, P388 cells and nasopharynx carcinoma (KB) cells in our laboratory, as primary screening. In this meeting, I will present on antitumor and cytotoxic substances of the higher plants (*Rubia cordifolia*, *Ailanthus vilmoriniana*, *Aster tataricus*, *Taxus cuspidata* var. *nana*, *Cephalotaxus harringtonia* var. *drupacea*, etc.) selected from above screening tests.

I. Introduction

A lot of anticancerous agents have been isolated from natural sources; especially from microorganisms and plants. However, there is no one special type of compound for cancer therapy: various types of substances are effective for various types of cancers and tumors, for instance, alkaloids, lignans, terpenes and steroids[1] In this report, concentrates on those antitumor compounds isolated from higher plants.

The most important components obtained from higher plants are *Vinca* alkaloids and *Podophyllum* lignans. *Vinca rosea (=Catharanthus roseus)* has been used as an inhibiting agent for milk secretion, hypotensor, astringent and emetic as folks medicines in Madagascar. Moreover, native people in the West Indian Islands have been using *Vinca* spp. as depression agent of blood sugar levels. When the extract of this plant was given non-orally, leucopenie and indirect inhibition of the nuclear division of cells were observed. More than 60 kinds of alkaloids have been isolated from *Vinca* spp. Of these vinblastine (**1**) and vincristine (**2**) are the most active: the former is effective against Hodgkin disease and the latter against leukemia. Podophyllotoxin (**3**) is a representative lignan isolated from the rhizome of *Podophyllum peltatum.* Podophyllum rhizome has traditionally been used as an emetic and an anthelmintic by American Indians. Because podophyllotoxin inhibits cell-division, it was tested for and found to have antineoplastic activity.

Curcumol (**4**) obtained from *Curcuma aromatica* was tested and found to be effective against cancer of the uterine cervix. Oridonin (**5**) isolated from *Rabdosia* spp. is now undergoing clinical trials in China. Camptothecine isolated from *Camptotheca acuminata* is also an antineoplastic alkaloid, but is very toxic. Chemical modification have decreased its toxicity, and the compound (**6**) is now in use as a clinical agent.[2] Colchicine (**7**) derivatives are said to inhibit cell-division. Demecolcine (**8**) and colchicine (**9**)act against mammary cancer. Harringtonin (**10**) has been studied as an anticancerous drug in China. Taxol (**11**), a compound with a taxane ring isolated from the bark of *Taxus brevifolia*, has been demonstrated to have substantial anticancer activity in patients with solid tumors refractory to standard chemotherapy. However, supply of this drug has severely limited full exploration of its antineoplastic potential. Efforts are continuing in NCI Washington to survey various *Taxus* species for optimal taxol content, to improve semi-synthesis from baccatin III, to improve methods of extraction, and to develop alternative renewable resources.[3] Further, several other compounds have been reported as antineoplastic agents.

Table I Screening Systems for Antitumor Activity

Tumor	Host animal	Inoculation size	Inoculation site	Administration*[1] route	Administration*[1] period	Criteria of activity*[2]
Leukemia and ascites tumor						
Sarcoma 180A	ICR	10^6	i.p.	i.p.	1 - 5	TPCV
Ehrlich carcinoma	ICR	5×10^6	i.p.	i.p.	1 - 5	MST
P388 leukemia	CDF1	10^6	i.p.	i.p.	1,5,9	MST
L1210 leukemia	CDF1	10^5	i.p.	i.p.	1 - 5	MST
MM2 mammary carcinoma	C3H/He	106	i.p.	i.p.	1 - 9	MST
B-16 melanoma	BDF1	homogenate	i.p.	i.p.	1 - 9	MST
Meth-A	BALB/C	2×10^5	i.p.	i.p.	1 - 5	MST
Colon26 adenocarcinoma	CDF1	homogenate	i.p.	i.p.	1 - 9	MST
Yoshida Sarcoma	Donryu rat	10^6	i.p.	i.p.	1 - 9	MST
AH-13	Donryu rat	10^6	i.p.	i.p.	1 - 9	MST
Solid tumor						
Lewis lung carcinoma	C57BL/6	5×10^5	s.c.	i.p.	1 - 11	TWD17 or MST
Colon38 adenocarcinoma	BDF1	homogenate	s.c.	i.p.	1 - 11	TWD17 or MST
Meth-A	BALB/C	10^6	s.c.	i.p.	1 - 11	TWD14
Ehrlich carcinoma	ICR	5×10^6	s.c.	i.p.	1 - 11	TWD14
B-16-BL6	C57BL/6	2.5×10^5	s.c.	i.p.	1 - 11	*[3]

*[1] Drugs were administered on the days indicated.
*[2] TPCV; total packed cell volume, MST; mean survival time, TWD; tumor weight on the day (Tumor weight was determined with calipers $LXW^2/2$).
*[3] Tumor size on day 18, numbers of pulmonary nodules and weight of lymph node on day 35.

Table II Antitumor Activity of Crude Drugs and Plants Collected in East Asia

original plant	part used	extract solvent	BWC (g)	PCV /TV	GR (%)	assessment
Fomes spp.	fruit body	W	-2.1	0.15	22	++
Cyperus rotundus	rhizome	W	-4.6	0.15	28	++
Areca catechu	seed	E	-1.8	0.07	9	+++
		W	-0.9	0.04	6	+++
Pinellia ternata	tuber	W	-0.9	0.13	31	++
Amomum xanthioides	seed	W	-3.3	0.23	14	++
Amomum cardamon	fruit	W	-3.5	0.06	11	++
Curcuma longa	rhizome	W	-2.2	0.14	38	++
Curcuma zedoaria	rhizome	W	-2.5	0.15	20	++
Zigiber officinale	rhizome	W	-2.0	0.09	18	++
Kaemferia galanga	rhizome	W	-4.3	0.08	9	+++
Alpinia oxyphylla	fruit	W	-0.1	0.15	25	++
Piper nigrum	fruit	W	-3.0	0.12	19	++
Reynoutria japonica var. *typica*	root	W	-0.5	0.13	32	++
Kochia scoparia	seed	W	-0.5	0.13	30	++
Wisteria floribunda	gall	W	-0.9	0.22	16	++
Tribulus terrestris	fruit	W	-2.0	0.18	36	++
Euphorbia lathyris	seed	E	-1.9	0.14	33	++
		W	-1.7	0.05	8	+++
Hovenia dulcis	fruit	W	-2.8	0.14	29	++
Hypericum erectum	herb	W	-4.2	0.10	32	++
Aralia elata	bark	W	-2.6	0.28	39	++
Nepeta japonica	herb	W	-1.1	0.11	21	++
Isodon japonica	herb	W	-0.3	0.46	9	+++
Trapa quadrispinosa	fruit	W	-0.1	0.24	40	++
Torilis japonica	fruit	W	-0.3	0.09	12	++
Eupatorium fortunei	herb	E	+1.3	0.25	18	++
Periploca sepium	root bark	E	-5.9	0.13	31	++
Benincasa cerifera	seed	W	-1.1	0.07	11	++
Stellaria media	herb	W	+0.1	0.31	39	++
Rubia cordifolia	root	M	-3.4	0.27	20	++
Musa basjoo	rhizome	W	-1.4	0.06	11	++
Selaginella tamariscina	herb	W	-0.5	0.14	38	++

E: ethanol, W: water, M: methanol, dose: 100 mg/kg/day

Table III Antitumor Activity of Crude Drugs and Plants Collected in East Asia

Indonesian name	original plant	part used	extract solvent	BWC (g)	PCV /TV	GR (%)	assessment
Pinang	*Areca catechu*	seed	E	+0.5	0.09	30	++
	(Palmae)		W	-0.7	0.12	48	+
Akar Usar	*Andropogon zizanioides*	rhizome	E	+3.0	0.40	105	-
(from Jawa 1)	(Graminaceae)		W	+2.6	0.33	83	-
Akar Usar	*Andropogon zizanioides*	rhizome	E	-2.2	0.24	42	+
(from Jawa 2)	(Graminaceae)		W	-3.1	0.28	27	++
Lengkuas	*Alpinia galanga*	rhizome	E	+0.4	0.35	2	+++
	(Zingiberaceae)		W	+1.6	0.20	69	-
Kapol	*Amomum compactum*	fruit	E	+2.8	0.29	102	-
	(Zingiberaceae)		W	-2.2	0.14	35	++
Temu Lawak	*Curcuma xanthorrhiza*	rhizome	E	+2.8	0.02	1	+++
	(Zingiberaceae)		W	+1.3	0.22	50	+
Temu Mangga	*Curcuma mangga*	rhizome	E	-0.4	0.29	4	+++
	(Zingiberaceae)						
Temu Itam	*Curcuma phaeocaulis*	rhizome	E	+1.7	0.26	45	+
	(Zingiberaceae)		W	-0.7	0.20	31	++
Kunyit	*Curcuma longa*	rhizome	E	+2.1	0.29	87	-
	(Zingiberaceae)		W	-2.0	0.10	10	++
Lempuyang	*Zingiber aromaticum*	rhizome	E	-0.6	0.39	97	-
	(Zingiberaceae)		W	-2.6	0.19	24	++
Lempuyang Pait	*Zingiber amaricans*	rhizome	E	+2.9	0.17	9	+++
	(Zingiberaceae)						
Kumis Kucing	*Orthosiphone aristatus*	leaf	E	+2.0	0.17	41	+
	(Labiatae)		W	+0.7	0.39	89	-
Pabia (=Kedawung)	*Parkia roxburgii*	seed	E	+2.0	0.30	52	+
	(Mimosaceae)		**W(50)	-2.3	0.32	38	++
Cincau Hitam	*Mesona palustris*	leaf	E	+3.9	0.05	11	++
	(Labiatae)	& Stem					
Cabe Jawa	*Piper retrofractum*	fruit	**E(50)	+1.2	0.36	53	+
	(Piperaceae)		W	+2.1	0.23	71	-
Sinkong Racum	*Manihot utilissima*	root	E	+4.9	0.42	104	-
	(Euphorbiaceae)		W	+2.2	0.56	25	++
Kayu Secang	*Caesalpinia sappan*	wood	E	+1.6	0.42	87	-
	(Leguminosae)		W	+3.0	0.31	33	++
Kayu Manis	*Glycyrrhiza glabra*	root	E	+3.7	0.37	92	-
	(Leguminosae)		W	-1.2	0.20	43	+
Sri Kaya Laut	*Sterculia lychophora*	fruit	E	+0.9	0.29	75	-
	(Sterculiaceae)		W	-2.2	0.19	65	+
Kenanga	*Canangium odoratum*	*wood	E	+4.3	0.36	81	-
	(Annonaceae)		W	+2.6	0.32	64	+
Pasak Bumi	*Eurycoma longifolia*	root	**E(30)	+1.2	0.21	34	++
	(Simaroubaceae)		**W(30)	-1.9	0.10	13	++
Kulit Lawang	*Cinnamomum* sp.	bark	E	+3.2	0.38	75	-
	(Lauraceae)		W	+1.3	0.22	19	++
Daun Perawas	*Litsea odorifera*	leaf	E	-2.1	0.12	26	++
	(Lauraceae)		W	-2.1	0.21	40	++
Sambilata	*Andrographis paniculata*	aerial	E	+2.3	0.19	41	+
	(Acanthaceae)	part	W	+1.7	0.33	77	-
Kayu Rapet	*Parameria laevigata*	bark	E	-0.3	0.41	105	-
	(Apocynaceae)		W	-2.2	0.11	14	++

E: ethanol extract, W: water extract, dose: 100 mg/kg/day

* Identifications are still uncertain.

** Extracts were given at the indicated doses (mg/kg/day).

The development of clinically useful anticancer agents is dependent on screening system, and sample sources for the bioassay. The search for potential anticancer agents from natural sources has mainly utilized bioassays confirmed by the National Cancer Institute (NCI),[4-10] because the large number of natural products screened by the NCI program has enabled assesment of the relationship between experimental animals and clinical patients for drug development, and screening protocols for each tumor system have been well-established. Part of the antitumor *in vivo* screening system used in our laboratory is shown in Table I. It is considered to be a "compound-oriented" *in vivo* screening. Such screenings did not lead us to develop new drugs for solid cancers.[11-14]

Recently, NCI has established a "disease-oriented" approach to antitumor activity screening[5,15,16], the biological response modifiers (BRM)[17,18] program, which takes into account the diversity and specificity of tumor, and the requirements of novel structure types and novel action-mechanistic types of anticancer agents.

This screening system has led to the isolation of many antineoplastic compounds from plants[19-22], microorganisms[23,24] and marine metabolites[4,12] *etc.* We have screened higher plants collected in Japan, China, Korea, Southeast Asia and South America[25-27] for antineoplastic activity, using Sarcoma 180 ascites in mice, P388 lymphocytic leukemia in mice, Chinese hamster V-79 cells, P388 cells and nasopharynx carcinoma (KB) cells, as a primary screening. Some of the of these studies results are shown in Tables II and III. In this review, we will describe antitumor and cytotoxic substances of higher plants selected using the above screening tests. In 1982, a definition for the expression of activity was established, that is, the word cytotoxicity is to be used only for *in vitro* activity, while antineoplastic and antitumor are to be used only for *in vivo* tests using animal. Finally, activity in clinical trials of humans is to be described as anticancerous.[12]

II. Terpenes

A. Taxan-Type Compounds

There have been numerous reports and reviews on the constituents of *Taxus* spp., especially, taxol.[28] Recently, J. Kobayashi and K.Fuji reported on the constituents of *Taxus* spp.[29,30] This paper will review the work of these authors.

The authors have isolated several kinds of taxan-type compounds from *Taxus cuspidata* Sieb. et. Zucc. var. *nana* Rehder.[31,32,33] For instance, taxuspinananes A, B and C were isolated from the stems of this plant. Taxuspinanane B showed moderate cytotoxic activity against P388 cells (IC_{50} 10 μg/ml). Taxuspinanane C and 7,9,10-deacetylbaccatin VI also showed moderate cell growth inhibitory activity against these cells (IC_{50} 10 μg/ml, 35 μg/ml, respectively). More than 40 taxoids have been isolated from this plant including 13 new taxoids as shown in Fig. 2.

A series of taxoids, isolated from the *T. cuspidata* var. *nana*, showing cell growth inhibitory activity, were investigated for 3D QSAR by comparative molecular field (CoMFA)

analysis. The results indicated a strong correlation between the P388 cell growth inhibitory activity of these taxoids and the steric and electrostatic factors which modulate their biological activity, and accounted for the potent activities of the taxoids with the N-acylphenylisoserine derivatives at C-13 and the weak activities of those without this kind of ester group. It was also suggested that the substituent at C-2 is not a requisite for the biological activity, smaller substituent seemed to favor the biological activity. 34)

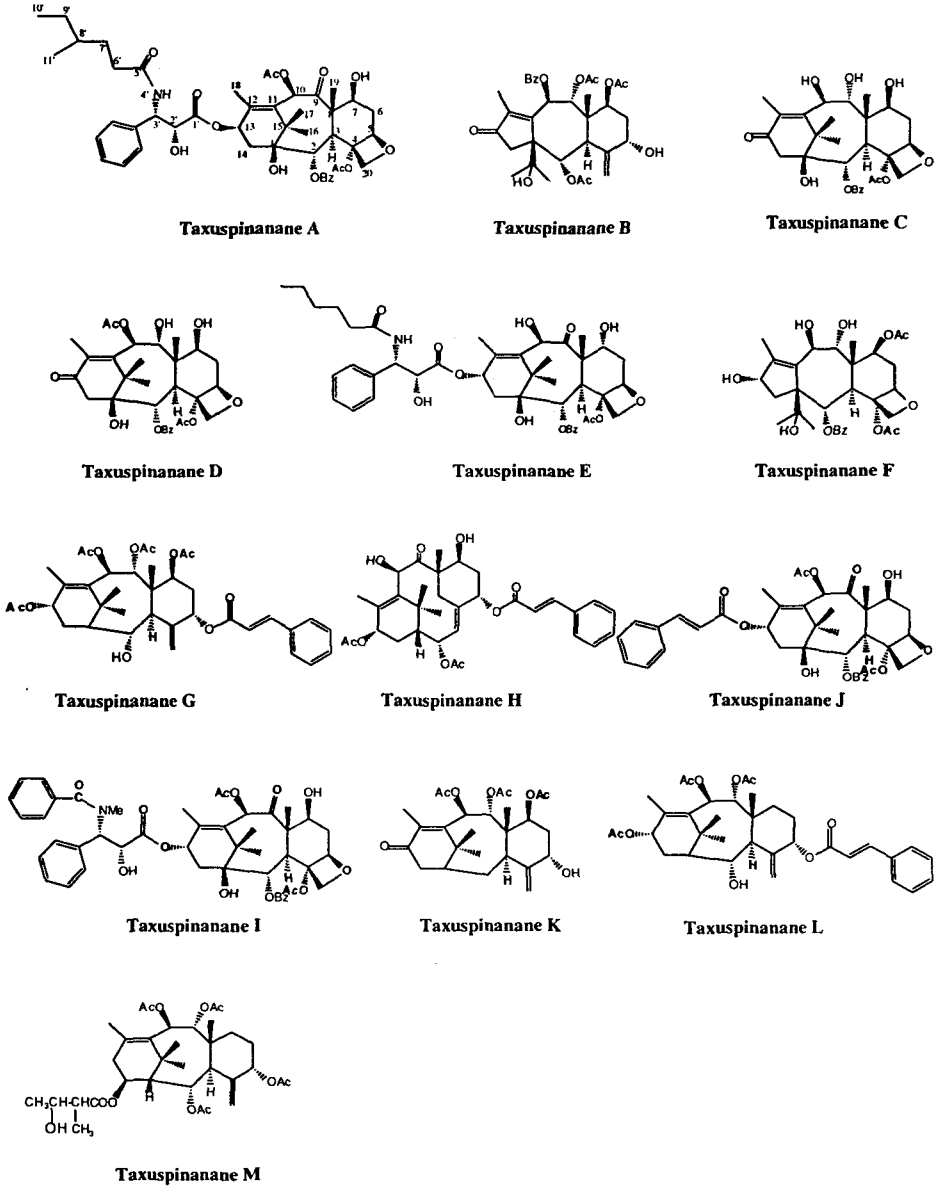


Fig. 2 New Taxoids, Taxuspinananes A - M from *Taxus cuspidata* var. *nana*

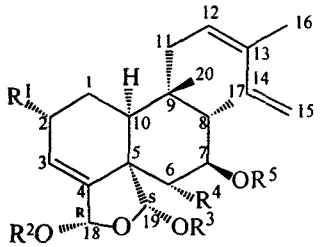
B. Clerodane Diterpenes from *Casearia sylvestris*

Antitumor Clerodane Diterpenes from *Casearia sylvestris* 35-37)

Casearia sylvestris Sw. (Flacourtiaceae), Paraguayan name "Burro-Kaa" and Brazilian name "Guassatonga", is a medicinal plant from South America used as a tonic and an

antispasmodic by native people. The ethanolic extract prepared from the leaves of *C. sylvestris* showed antitumor activity against Sarcoma 180A in mice (100 mg/kg/day dose, for 5 consecutive days, growth ratio (GR): 13% (++), as assessed according to the total packed cell volume (TPCV) method). When an aqueous solution of the ethanolic extract was partitioned successively with *n*-hexane, chloroform and ethyl acetate, the antitumor activity was concentrated in the *n*-hexane extract (100 mg/kg/day dose, GR: 2% (+++)). Its chromatographic purification with the guidance of bioassay against Sarcoma 180A in mice led us to isolate new antitumor clerodane diterpenes, named casearins A - F (**12 - 17**). Further investigation of the active fractions 5 and 6 by high performance liquid chromatography (HPLC) led to the isolation of minor clerodane diterpenes, named casearins G - R (**18 - 29**).

Fig. 4 Structures of Casearins and Cytotoxic Activities against V-79 Cells



Casearins	R^1	R^2	R^3	R^4	R^5	IC_{50} (μmol/l)*
A (12)	OMe	Ac	Ac	OH	Bu	1.0
B (13)	OMe	Ac	Ac	O Ac	Bu	8.5
C (14)	OH	Ac	Ac	O Ac	Dc	0.77
D (15)	OH	Bu	Ac	OH	Bu	1.8
E (16)	OH	Et	Ac	OH	Dc	4.7
F (17)	OH	Et	Ac	OH	Bu	29
G (18)	OMe	Ac	Ac	H	Bu	0.17
H (19)	OH	Ac	Ac	H	Bu	0.37
I (20)	OH	Ac	Bu	H	Bu	0.51
J (21)	OMe	Bu	Ac	OH	Bu	1.1
K (22)	OAc	Ac	Ac	OH	Bu	0.52
L (23)	OMe	Bu	Ac	O Ac	H	1.6
M (24)	OH	Bu	Bu	O Ac	H	1.8
N (25)	OMe	Ac	Bu	O Ac	Bu	5.9
O (26)	OMe	Bu	Ac	O Ac	Bu	6.0
P (27)	OMe	Ac	Ac	O Ac	Ac	7.8
Q (28)	OH	Ac	Ac	O Ac	Bu	4.3
R (29)	=O	Ac	Ac	OH	Bu	5.4
Aa (30)	OMe	Ac	Ac	=O	Bu	0.55
Ab (31)	OMe	Ac	Ac	OPr	Bu	17
Ac (32)	OMe	Ac	Ac	OBu	Bu	38
Da (33)	=O	Bu	Ac	=O	Bu	19

* Cytotoxic activities against V-79 cells

Me: CH_3, Et: CH_2CH_3, Ac: $COCH_3$, Bu: $CO(CH_2)_2CH_3$, Pr: $COCH_2CH_3$, Dc: $CO(CH_2)_8CH_3$

C. Quassinoids, and Linear Triterpene from *Eurycoma longifolia*

Cytotoxic Quassinoids, Linear Triterpenes and Canthin Alkaloids from *Eurycoma longifolia*[38,39]

Eurycoma longifolia Jack (Simaroubaceae), a famous folk medicine known as "Pasak Bumi" in Southeast Asia, has been used as an antimalaria and tonic *etc.* The roots of *E. longifolia* collected in Indonesia were treated with 50% aqueous methanol. The extract was partitioned between water and ether, then *n*-butanol successively. The chromatographic purification of ether and *n*-butanol soluble fractions furnished canthin alkaloids (**34** - **36**) and quassinoids (**37** - **42**), respectively. Their structures were confirmed as shown in Fig. 5 by various spectral data or comparison with various data in the literatures. These compounds exhibited cytotoxic activities as is evident from Fig. 5.

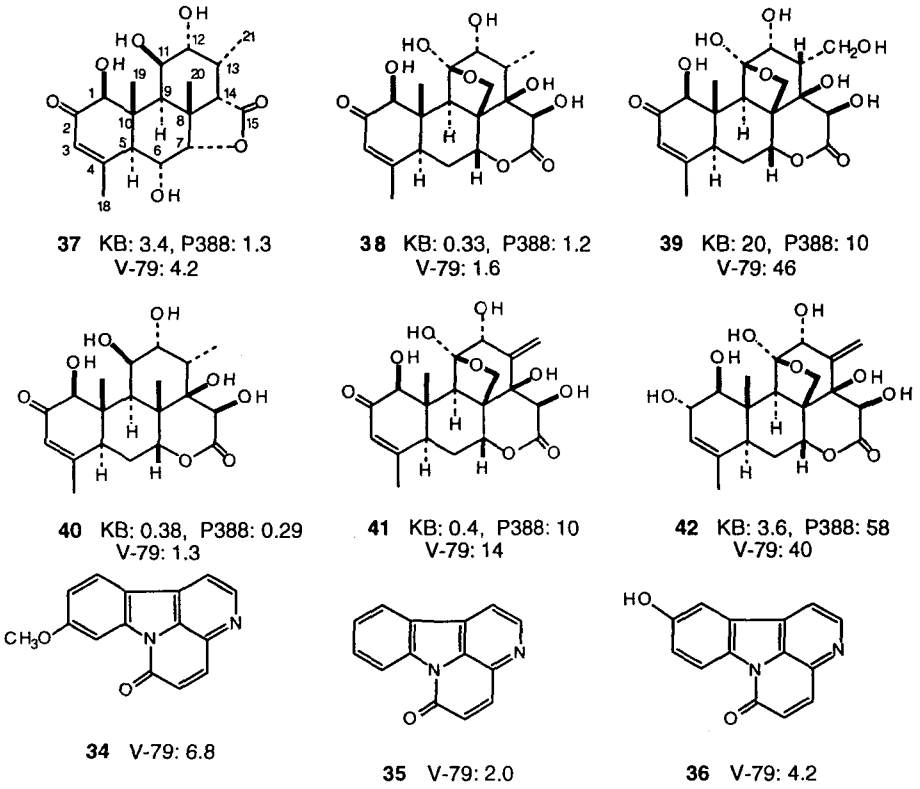
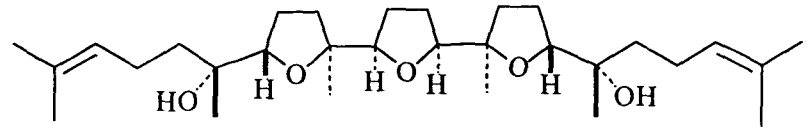


Fig. 5 IC_{50} Values (μg/ml) of Canthins and Quassinoids against KB, P388 and V-79 Cells

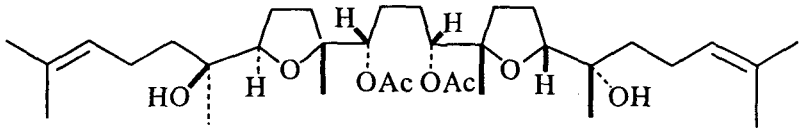
From the structure-activity relationships discussed among quassinoids, it has been reported that the partial structures of the C1-OH, C12-OH, 2-keto-3-ene and oxide-bridge are essential features for antileukemic activity.

In this continuing series of studies on cytotoxic compounds of *E. longifolia*, two unique squalene-type triterpenes, characterized by eight asymmetric carbons and two or three tetrahydrofuran rings, were isolated from the woods of *E. longifolia*. While one of them was identified as the marine meso-triterpene ether, teurilene (**43**), the other was found to be a new compound, named eurylene (**44**), whose relative structure was established by spectroscopic data and X-ray analysis. The absolute stereostructure was determined by an advance Moshor's method.

The structures and cytotoxic activities of **43** and **44** are shown in Fig. 6. The activities of **43** against V-79, P388 and KB cells were stronger than those of **44**. The perspective views of both compounds from their X-ray analyses gave a curvature form in **43** and a linear one in **44**. These molecular forms are presumed to be correlated with the cytotoxic activities from the related compounds.



teurilene (**43**), KB: 27.5, P388 2.3, V-79: 4.8



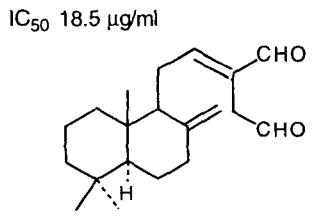
eurylene (**44**), KB: >30, P388: 55, V-79: >100

Fig. 6 IC_{50} Values (μg / ml) of Teurilene and Eurylene

D. Diterpene from *Hedychium coronarium*

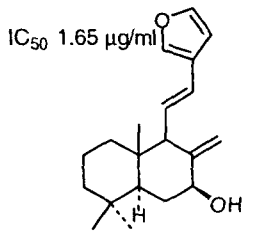
Cytotoxic Diterpenes from *Hedychium coronarium* [40,41]

The chloroform extract prepared from the rhizomes of *Hedychium coronarium* Koeng (Zingiberaceae, Brazilian name "Lirio-do-brejo"), which is used for rheumatism in Brazil, showed a significant effect against V-79 cells and Sarcoma180A in mice. Fractionation of the chloroform extract was made with the guidance of bioassay against V-79 cells. The extract was subjected to silica gel column chromatography and separated to seven fractions A - G. Significant cytotoxic activity of the fractions D, E, F and G against V-79 cells led us to isolate



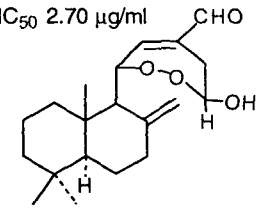
IC_{50} 18.5 μg/ml

(E)-labda-8(17),12-diene-15,16-dial (**45**)



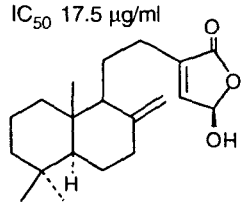
IC_{50} 1.65 μg/ml

Coronarin A (**46**)



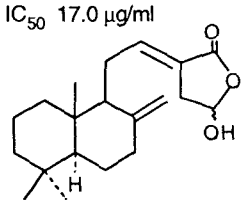
IC_{50} 2.70 μg/ml

Coronarin B (**47**)



IC_{50} 17.5 μg/ml

Coronarin C (**48**)



IC_{50} 17.0 μg/ml

Coronarin D (**49**)

Fig. 7 Diterpenes from *Hedychium coronarium* and Their Cytotoxity against V-79 Cells

the known (E)-labda-8(17),12-diene-15,16-dial (**45**), and six new labdane-type diterpenes, named coronarins A (**46**), B (**47**), C (**48**), D (**49**), E (**50**) and F (**51**) by means of repeated chromatography of each fraction. The structures and IC_{50} values of their labdane-type diterpenes against V-79 cells are presented in Fig. 7. Coronarins A (**46**) and B (**47**) exhibited particularly significant cytotoxic activity.

E. Triterpenes from *Maytenus ilicifolia*

Cytotoxic Triterpenes from *Maytenus ilicifolia*[42,43]

Maytenus ilicifolia Mart. ex Reiss. (Celastraceae) is used as an analgesic, antipyretic, antiseptic and anticancer agent. in South America, and in Paraguay, where it is known as "Cangorosa", it is employed for birth control.

The methanolic extract of *M. ilicifolia* was fractionated by partitioning between chloroform and water, and then *n*-butanol and water. Purification of each extract by the guidance of cytotoxic activity led us to isolate friedelane- and pristimerin-type triterpenes, D:A-friedoolean-24-al-3-en-3-ol-2-on-29-oic acid (**52**), D:A-friedoolean-1-en-29-ol-3-one (**53**), maytenoic acid (**54**), D:B-friedoolean-5-en-3β,29-diol (**55**), D:A-friedoolean-29-ol-3-one (**56**), pristimerin (**57**) and salaspermic acid (**58**), and triterpene dimers named as cangorosin A (**59**) atropcangorosin A (**60**), dihydroatropcangorosin A (**61**) and cangorosin B (**62**) from the chloroform soluble extract, and isopristimerin III (**63**) and isotingenone III (**64**) from the *n*-butanol soluble extract.

Table V Cytotoxic Activity of Triterpenes from *Maytenus ilicifolia*

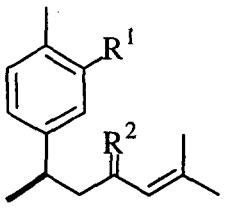
	IC_{50} values (μg/ml)		
	V-79 cells	KB cells	P388 cells
D:A-friedoolean-1-en-29-ol-3-one (**53**)	> 100	> 100	> 100
D:A-friedooleanan-3-on-29-oic acid (**54**)	38	12	23
D:B-friedoolean-5-en-3β,29-diol (**55**)	> 100	1.1×10^{2}	95
D:A-friedooleanan-29-ol-3-one (**56**)	1.1×10^{2}	1.0×10^{2}	98
pristimerin (**57**)	1.3×10^{-1}	2.3×10^{-1}	5.2×10^{-2}
isopristimerin III (**63**)	9.4	1.7	2.0
isotingenone III (**64**)	1.4	1.1	1.8×10^{-1}

F. Bisabolane-Type Sesquiterpenes from *Curcuma xanthorrhiza*

***Curcuma xanthorrhiza* (Zingiberaceae, known as Temu Lawak in Indonesia)**

The root has been utilized as a tonic in southeast Asia and as a choleretic drug in Europe. The active *n*-hexane extract against Sarcoma 180A in mice was fractionationed by repeated column chromatography to give the antitumor bisabolane sesquiterpenes,[44] α-curcumene (**65**), ar-turmerone (**66**) and xanthorrhizol (**67**) and a minor related one.[45,46] The structures and antitumor activity against Sarcoma 180A in mice are shown in Fig. 8. α-Curcumene exhibited a dose-dependent effect: (-) at 10 mg/kg, (++) at 20 mg/kg and (+++) at 50 mg/kg. ar-Turmerone and xanthorrhizol showed lower activity (++) at 50 mg/kg than α-curcumene. On the other hand,

α-curcumene showed no significant activity against P388 lymphocytic leukemia in mice, in the dose range of 50 to 200 mg/kg.



65: R^1=H, R^2=H_2
66: R^1=H, R^2=O
67: R^1=OH, R^2=H_2

Compound	Dose (mg/kg)	BWC	PCV / TV	GR (%)	Assessment
65	10	+4.2	0.31	97	-
	20	+2.1	0.16	31	++
	50	+2.4	0.00	0	+++
66	25	+3.1	0.39	77	-
	50	+2.7	0.12	22	++
67	50	+3.6	0.13	18	++

Fig. 8 Antitumor Activity of **65** - **67** against Sarcoma 180A in Mice

G. Ingenol Type Diterpenes from *Euphorbia lathyris*

The extract of seeds of *Euphorbia lathyris* (Euphorbiaceae) showed antitumor activity against Sarcoma 180A in mice.[47] Systematic fractionation of the extract led to the characterization of ingenol-3-hexadecanoate as an active principle, together with inactive diterpenes ingenol-20-hexadecanoate and lathyrane diterpenes. Though ingenol-3-hexadecanoate is well known as a tumor-promoting agent, it showed antitumor activity against Sarcoma 180A in mice. This result indicates a paradoxical action, cocarcinogenic and antitumor, for the diterpene esters of the Euphorbiaceae.

H. Cardenolides and Pregnanes from *Periploca sepium*

The crude drug "beiwujiapi", from the root bark of *Periploca sepium* (Asclepiadaceae), is one of the famous "wujiapi" in Chinese literature and has been widely used as a tonic. When the chloroform extract obtained by partitioning the methanolic extract of *P. sepium* between water and chloroform was subjected to column chromatography on silica gel using a solvent system of benzene, benzene-$CHCl_3$, $CHCl_3$, $CHCl_3$-MeOH (10:1) and (1:1) successively, the antitumor activity against Sarcoma 180A in mice was concentrated in the fraction eluted with $CHCl_3$-MeOH (10:1). This fraction exhibited powerful antineoplastic activity (growth ratio: 4.6%, +++) at a dose of 10 mg/kg/day given for 5 consecutive days and was a mixture of pregnanes, cardenolides and their glycosides.[48-53] The antitumor activity of each compound was weaker than that of the $CHCl_3$-MeOH (10:1) fraction.

III. Alkaloids

A. *Cephalotaxus* Alkaloids[54-60]

Cephalotaxus harringtonia (Knight) Koch. f. *drupacea* (Sieb. & Zucc.) Kitamura (= *C. harringtonia var. drupacea* (Sieb. & Zucc.) = *C. drupacea* Sieb. & Zucc.) growing in Japan, was reported to contain characteristic *Cephalotaxus* alkaloids; cephalotaxine (**68**), harringtonine (**69**), homoharringtonine (**70**), isoharringtonin (**71**), deoxyharringtonin (**72**) and drupacine (**73**).

Homoharringtonin (**70**) has undergone Phase II clinical trials as an anticancerous agent at N.C.I. in U.S.A. However, there still remains the issue of its side effects.

The authors have continued to study new components from this plant and to find new analogues based on the relationship between the structure and the activity of the alkaloids.

Isolation of New *Cephalotaxus* Alkaloids from *C. harringtonia* f. *drupacea*

The new *Cephalotaxus* alkaloids isolated by us can be classified into three types of compounds based on their side chain structures. The first type have a carboxyl group at the end of the side chain, i.e. 5'-des-O-methylharringtonine (**74**), 5'-des-O-methylhomoharringtonine (**75**), 5'-des-O-methylisoharringtonin (**76**) and 3'*S*-hydroxy-5'-des-O-methylharringtonine (**77**).

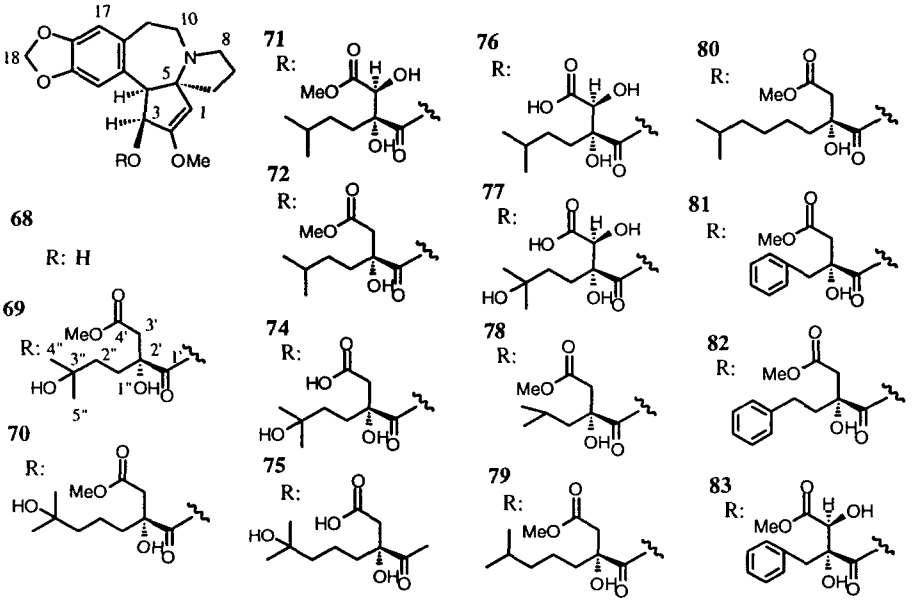


Fig. 9 *Cephalotaxus* alkaloids having various side chains

The second have side chains, which differ in the number of methylene groups, nordeoxyharringtonine (**78**), homodeoxyharringtonine (**79**) and bishomodeoxyharringtonine (**80**). The third type of compound have aromatic rings at the end of the side chains, and include neoharringtonine (**81**), homoneoharringtonine (**82**) and 3'*S*-hydroxyneoharringtonine (**83**).

Also, there are two types of new compounds: those with hydroxyl groups at carbon 11, such as 11α-hydroxyhomodeoxyharringtonine (**84**), 11β-hydroxyhomodeoxyharringtonine (**85**) and 11β-hydroxydeoxyharringtonine (**86**), and those with an ester alkaloid, drupangtonine (**87**), which has a drupacine skeleton. Moreover, a new dimer alkaloid, cephalotaxidine (**88**) was obtained from *C. harringtonia* f. *drupacea*.

Modification of the Skeleton of Homoharringtonine (70) through Unusual Rearrangements

Previous efforts to modify *Cephalotaxus* alkaloids addressed the substitution of the ester moiety by various acyl groups, and a number of minor alkaloids possessing a different ester group have been isolated from *Cephalotaxus* plants. The evaluation indicated that the acyl

moiety is very important for expression of the activity. To examine the influence of the cephalotaxine skeleton upon the activity, retention of this acyl moiety in the skeleton-modified analogues is requisite. Thus, we devised a method to produce analogues from homoharringtonine (**70**), the most abundant ester-type *Cephalotaxus* alkaloid showing potent antitumor activity. Since we suspected that the nitrogen lone pair may play an important role in expression of the activity, modification was done around this region.

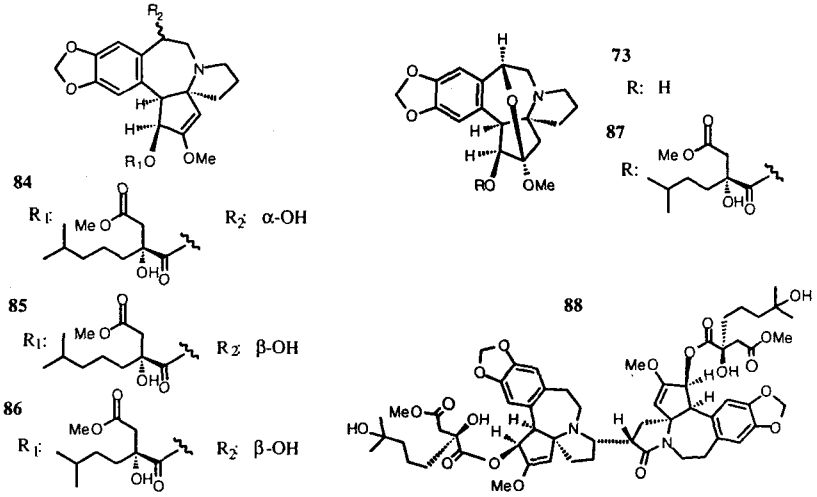


Fig. 10 *Cephalotaxus* alkaloids having an oxygen function at C-11 and A Dimer Alkaloid

Oxidation of **70** with hydrogen peroxide in methanol gave β-*N*-oxide **89** and α-*N*-oxide **90** in 26% and 36% yields, respectively (Fig. 11). When the 1,2-dimethoxyethane solution of β-*N*-oxide **89** was heated in a sealed tube at 105 °C for 2 h, compound **91** and unexpected compounds **92** and **93** were obtained in yields of 37%, 44% and 7.7%, respectively. Heating of α-*N*-oxide **90** under same conditions also gave compound **91** (32%), **92** (36%) and **93** (7.6%).

Zinc and acetic acid reduction of **92** and **93** gave ring-contracted homoharringtonine analogues **94** and **95** in yields of 96% and 67%, respectively. Their stereostructures were confirmed by the NOESY spectra.

Cytotoxicity of *Cephalotaxus* Alkaloids

The cytotoxic activity of *Cephalotaxus* alkaloids **68** - **95** against P388 leukemia cells *in vitro* has been examined and the results are shown in Table VII. As can be seen from the Table VII, these alkaloids generally have strong cytotoxicity, especially compounds **72** and **87** (IC_{50} values: 0.0075 and 0.0070 μg/ml). In terms of the relationship between structure and activity, 1) harringtonine acids (**74** - **77**) having a carboxylic acid at the side chain showed weak activity compared with the carbomethoxyl groups (**69** - **72**), 2) hydroxylation to the C-11 (**84** - **86**) reduced the activity, 3) difference of length (**78** - **80**) and functional groups (**81** - **83**) at the side chains influenced the activity, 4) the nitrogen lone pair on the cephalotaxine skeleton appears to be essential for the activity by comparing the cytotoxicity of *N*-oxides **89** and **90** with that of **70** 5) the weaker activity of analogues **91** - **95** compared with that of **70** is accounted for by the changes in the topology of the cephalotaxine skeleton through chemical modification.

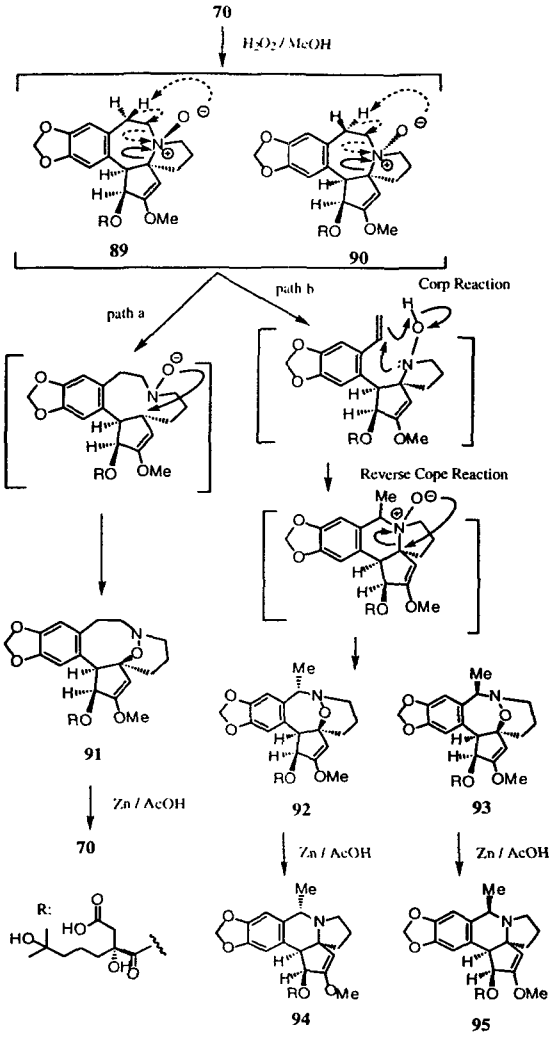


Fig. 11

Table VI Cytotoxic Activity of **68** - **95** against P388 Cells *In Vitro*

compd.	IC_{50} (μg/ml)	compd.	IC_{50} (μg/ml)	compd.	IC_{50} (μg/ml)
68	0.10	**78**	0.027	**88**	1.8
69	0.032	**79**	0.017	**89**	0.92
70	0.017	**80**	0.024	**90**	1.9
71	0.018	**81**	0.012	**91**	4.0
72	0.0075	**82**	0.28	**92**	15
73	1.4	**83**	0.19	**93**	6.0
74	1.2	**84**	0.38	**94**	6.2
75	4.6	**85**	0.33	**95**	1.5
76	0.41	**86**	0.17		
77	65	**87**	0.0070		

B. Morphinane Alkaloid from *Cocculus trilobus*

An Antitumor Morphinane Alkaloid, Sinococuline, from *Cocculus trilobus*, and related compounds[61-63]

Cocculus trilobus DC. (Menispermaceae, found in the mountainous areas of East Asia, has been used in folk medicine as a diuretic, analgesic and antiinflamatory crude drug. When an

aqueous solution of the methanolic extract prepared from the stems and rhizomes of *C. trilobus* was partitioned successively with *n*-hexane and ethyl acetate, the antitumor activity against Sarcoma 180 ascites in mice was concentrated in the aqueous-layer residue. The residue was subjected to Amberlite XAD-2 column chromatography eluting with an H_2O-MeOH gradient system (10:0, 4:1, 1:1 and 0:10 successively). In the bioassay of each fraction against Sarcoma 180A, the fraction eluted with H_2O exhibited significant antitumor activity. This active fraction was purified by preparative TLC on silica gel using a $CHCl_3$-Et_2NH (3:2) solvent system to give an antitumor alkaloid, named sinococuline (**96**). The relative structure was established by various spectroscopic methods and the C_9 configuration was assumed to be *S* based on chemotaxonomy, and this was confirmed by measuring the CD spectrum (positive maximum at 238 nm). Further, in order to determine the absolute structure using the exciton chirality rule, sinococuline (**96**) was converted to the 6,7-dibenzoate derivative

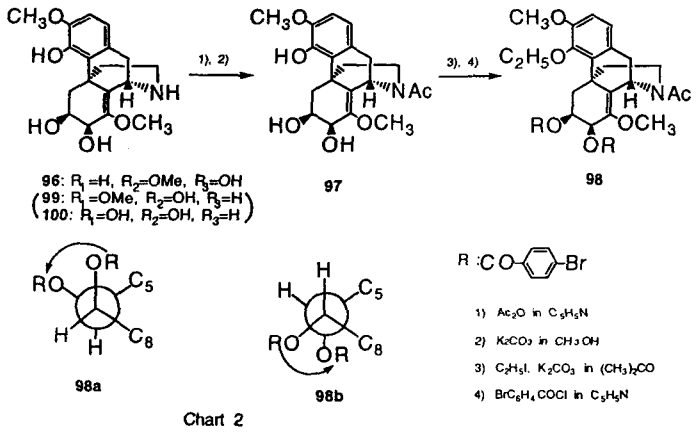


Chart 2

as shown in Chart 2. Its CD spectrum showed a negative Cotton effect at 252 nm and the coupling constant between H_6 and H_7 was 3.5Hz. This is suggestive of both structures **98a** (6*S*, 7*S*) and **98b** (6*R*, 7*R*) in Chart 2. However, the NOE between H_6 and H_{15} supported structure **98a** only. Further research into antitumor substances from *Cocculus* plants led us to isolate sinococulin (**96**) and the related compounds **99** and **100** from *C. sarmentosus*. These compounds had antitumor activity against Sarcoma 180A (40 mg/kg/d dose for 5 consecutive days, GR (growth ratio = T/C): 56% (+) in **96**) and P388 leukemia in mice shown in Table VII. Also, various derivatives were prepared and applied to P388 *in vivo* test, however, none were more effective than sinococulin (**96**).

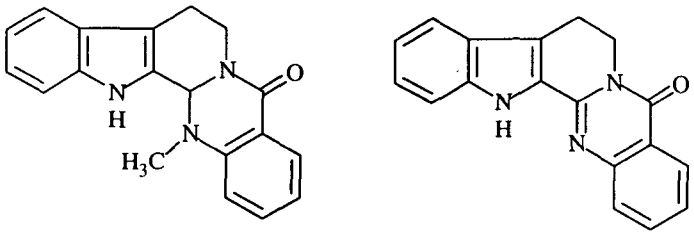
Table VII Antitumor Activity of Sinococuline (**96**) and Its Related Compound **99** against P388 Leukemia in Mice

compound	dose (mg/kg)	survival time (d, mean ± S.E.)	T/C (%)	BWC (g)
	10	12.5 ± 0.48	154.6	+0.9
96	25	13.5 ± 0.34	167.0	+0.6
	50	14.3 ± 0.49	177.0	-0.6
	100	16.2 ± 1.92	200.0	-4.7
99	10	11.3 ± 0.21	140.2	+0.9
	25	12.7 ± 0.42	156.7	-1.1

P388: 10^6 cells / 0.1 ml, *i.p.*, CDF1 mice (n=6)
Drug: *i.p.*, d 1 - 5.

C. *Evodia* Alkaloid

The fruits of *Evodia rutaecarpa* (Rutaceae) are a crude drugs in Chinese medicine. The alcoholic extract exhibited a significant effect against V-79 cells (IC_{50}=5.2 μg/ml).[64] The cytotoxic activity on V-79 cells was concentrated in the chloroform subextract (IC_{50}=5.6 μg/ml) by partitioning between aqueous solution of the alcoholic extract and each organic solvent. The subextract was fractionated with the guidance of bioassay to give (+)-evodiamine (**101**) and rutaecarpine (**102**) from the cytotoxic fraction. In the cytotoxic test using V-79, KB and P388 cells, it is worth noting that **101** showed effective activity, while **102** did not in spite of the similarity of the two structures as shown in Fig. 12



evodiamine (**101**) V-79: 0.19 KB: 0.98 P388: 0.43

rutaecarpine (**102**) V-79: >100 KB: >100 P388: >100

Fig. 12 IC_{50} Values (μg / ml) of **101** and **102** against V-79, KB and P388 Cells

IV. Phenols

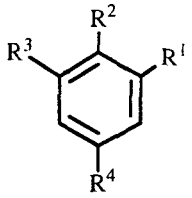
A. Antitumor Long-Chain Phenols from *Ginkgo biloba*[65,66]

Ginkgo biloba L. (Ginkgoaceae) is a tree that grows to 30 to 40 m native to China. Its seeds are used for allaying coughing and tonic. The methanolic extract from the sarcotesta of *G. biloba* L. showed remarkable antitumor activity against Sarcoma 180A in mice. When an aqueous solution of the extract was partitioned successively with chloroform, ethyl acetate, and *n*-butanol, the antitumor activity was concentrated in the chloroform extract.

The extract was subjected to silica gel and alumina column chromatography to give fractions containing anacardic acid, bilobol and cardanol. Further purification with an ODS column furnished anacardic acid (**103a, b, c**),bilobol (**104a, b**) and cardanol (**105a, b**) as shown in Fig. 13. The antitumor activity of these compounds is summarized in Table VIII. It is speculated that the antitumor activity of long-chain phenols against Sarcoma 180A in mice appears not to require the carboxyl group.

Further, a bioassay based on the cytotoxic activity against Chinese hamster lung V-79 cells instead of the antitumor activity against Sarcoma 180A in mice was employed in a search for antitumor principles by means of quantitative structure-activity relationship (QSAR) analysis, because there was a good correlation between the results of the biological tests of long-chain phenols using V-79 cells and Sarcoma 180A in mice. We considered that the activities of antitumor long-chain phenols were controlled by both hydrophobic and electronic parameters based on the alkyl side chain moiety and the aromatic rings contribution to hydroxyl function,

respectively, because acetates and methyl esters of the long-chain phenols did not show antitumor activity against Sarcoma 180A in mice as can be seen from Table IX.



103a:	$R^1=(CH_2)_{12}CH_3$	R^2=COOH	R^3=OH	R^4=H
103b:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$	R^2=COOH	R^3=OH	R^4=H
103b':	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$	$R^2=COOCH_3$	R^3=OH	R^4=H
103c:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$	R^2=COOH	R^3=OH	R^4=H
104a:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$	R^2=H	$R^3=R^4$=OH	
104a':	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$	R^2=H	$R^3=R^4=OCOCH_3$	
104b:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$	R^2=H	$R^3=R^4$=OH	
105a:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$	$R^2=R^4$=H	R^3=OH	
105a:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$	$R^2=R^4$=H	$R^3=OCOCH_3$	
105b:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$	$R^2=R^4$=H	R^3=OH	

Fig. 13 Structures of Compounds **103a - 105b**

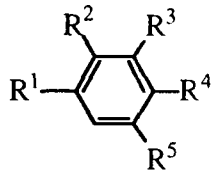
Table VIII Antitumor Activity on Sarcoma 180 Ascites in Mice

Compound	Dose (mg/kg)	GR (%)	Assessment
103b	40	17.4	++
104a	40	0.4	+++
105a	40	0.0	+++
103b'	60	110.4	-
104a'	40	81.9	-
105a'	40	105.7	-

Thirty long-chain phenol derivatives, which were divided into six groups consisting of five compounds having the same aromatic ring contribution and a different alkyl side chain moiety, were synthesized by Grignard reaction of alkyl bromide and hydroxybenzaldehyde in the usual way. Each compound was tested for cytotoxic activity against V-79 cells and its IC_{50} value determined. Also, for all synthesized compounds (**105 - 135**), the log P values (P stands for the *n*-octanol - water partition coefficient) were measured by the HPLC method as the hydrophobic parameter. As the electronic parameter, the energy of the lowest unoccupied molecular orbital (E_{LUMO}) was calculated by using the modified neglect of diatomic differential overlap (MNDO) method, because Hammett's substituent constants were not suitable for both *ortho*- and di-substituted aromatic rings.[67]

Among the synthesized long-chain phenols (**106 - 135**), the activity of **134** was about 10 times stronger than that of the others against V-79 cells, and **134** also showed antitumor activity against Sarcoma 180A in mice at a low dose, 10 mg/kg/d. Natural compounds (**136 - 138**) from *G biloba* did not show activity at the same dose, as shown in Table X. Furthermore, **134** exhibited significant activity against P388 lymphocytic leukemia in mice at 100 mg/kg.

Table IX Structures and Parameters for Multiple Regression Analysis



Compound	R^1	R^2	R^3	R^4	R^5	Yield	mp (°C)	MS (M^+)	-log ED_{50}	log P	E_{LUMO}	E_{HOMO}
A-7 (106)	C_7H_{15}	OH	H	H	H	63.5	-	192	1.16	4.45	0.142	-8.86
A-9 (107)	C_9H_{19}	OH	H	H	H	59.0	-	220	1.38	5.76	0.142	-8.86
A-11 (108)	$C_{11}H_{23}$	OH	H	H	H	75.4	32.0-33.0	248	1.39	7.17	0.142	-8.86
A-13 (109)	$C_{13}H_{27}$	OH	H	H	H	70.4	42.5-43.5	276	1.42	8.61	0.142	-8.86
A-15 (110)	$C_{15}H_{31}$	OH	H	H	H	29.3	53.0-54.0	304	1.43	10.07	0.142	-8.86
B-5 (111)	C_5H_{11}	H	OH	H	H	50.0	-	164	-	3.13	0.135	-8.88
B-9 (112)	C_9H_{19}	H	OH	H	H	56.4	-	220	1.16	5.61	0.135	-8.88
B-11 (113)	$C_{11}H_{23}$	H	OH	H	H	57.6	-	248	1.14	6.98	0.135	-8.88
B-13 (114)	$C_{13}H_{27}$	H	OH	H	H	31.7	41.0-42.0	276	1.34	8.84	0.135	-8.88
B-15 (115)	$C_{15}H_{31}$	H	OH	H	H	11.2	50.0-51.0	304	1.33	10.11	0.135	-8.88
C-7 (116)	C_7H_{15}	H	H	OH	H	82.4	-	192	1.43	4.15	0.179	-8.82
C-9 (117)	C_9H_{19}	H	H	OH	H	35.7	41.0-42.5	220	1.29	5.76	0.179	-8.82
C-11 (118)	$C_{11}H_{23}$	H	H	OH	H	86.6	56.5-57.0	248	1.14	7.18	0.179	-8.82
C-12 (119)	$C_{12}H_{25}$	H	H	OH	H	79.8	67.5-68.0	262	1.14	7.91	0.179	-8.82
C-13 (120)	$C_{13}H_{27}$	H	H	OH	H	84.5	68.0-69.0	276	1.39	8.20	0.179	-8.82
D-7 (121)	C_7H_{15}	OH	OH	H	H	48.8	-	208	1.76	3.59	0.020	-8.60
D-9 (122)	C_9H_{19}	OH	OH	H	H	46.6	-	236	2.04	4.86	0.020	-8.60
D-11 (123)	$C_{11}H_{23}$	OH	OH	H	H	63.5	51.8-52.5	264	2.34	6.30	0.020	-8.60
D-13 (124)	$C_{13}H_{27}$	OH	OH	H	H	43.0	56.0-56.5	292	2.08	7.75	0.020	-8.60
D-15 (125)	$C_{15}H_{31}$	OH	OH	H	H	41.0	60.5-61.0	320	2.01	9.27	0.020	-8.60
E-7 (126)	C_7H_{15}	OH	H	OH	H	3.9	70.8-71.3	208	1.10	2.90	0.098	-8.75
E-9 (127)	C_9H_{19}	OH	H	OH	H	7.6	72.0-72.7	236	1.11	4.10	0.098	-8.75
E-11 (128)	$C_{11}H_{23}$	OH	H	OH	H	2.9	72.5-73.0	264	1.43	5.43	0.098	-8.75
E-13 (129)	$C_{13}H_{27}$	OH	H	OH	H	6.2	72.3-73.0	292	1.36	6.84	0.098	-8.75
E-15 (130)	$C_{15}H_{31}$	OH	H	OH	H	2.2	84.5-85.1	320	1.68	8.29	0.098	-8.75
F-5 (131)	C_5H_{11}	H	OH	OH	H	37.5	-	180	1.57	3.13	0.098	-9.06
F-9 (132)	C_9H_{19}	H	OH	OH	H	35.6	75.5-77.0	236	1.82	5.54	0.098	-9.06
F-11 (133)	$C_{11}H_{23}$	H	OH	OH	H	73.6	84.0-85.0	264	2.28	6.94	0.098	-9.06
F-13 (134)	$C_{13}H_{27}$	H	OH	OH	H	80.0	90.0-91.5	292	2.33	8.42	0.098	-9.06
F-15 (135)	$C_{15}H_{31}$	H	OH	OH	H	40.6	88.5-91.0	320	2.11	9.90	0.098	-9.06
136	$C_{15}H_{29}$	COOH	OH	H	H		40.0-41.0	346				
137	$C_{15}H_{29}$	H	OH	H	OH		30.0-31.0	318				
138	$C_{15}H_{29}$	H	OH	H	H		-	302				

ED_{50} in mM, E_{LUMO} and E_{HOMO} in eV.

Table X Antitumor Activity against Sarcoma 180 Ascites in Mice

Compound	Dose (mg/kg)	BWC (g)	PCV/TV	GR (%)	Assessment
123	10.0	+6.1	0.43	65	-
134	10.0	+5.8	0.42	38	+++
138	10.0	+4.8	0.37	101	-

The effectiveness was evaluated by means of the total packed cell volume method. BWC: body weight change = (day 7 weight - TV) / day 0 weight. PCV: packed cell volume. TV: total volume. GR: growth ratio = PCV (test groups) / PCV (control groups) x 100.

B. Phenylpropanoids from *Alpinia galanga*[68]

Alpinia galanga Wild. (=*Languas galanga* Stuntz, Zingiberaceae) grows in southeast Asia,where it is widely cultivated. The rhizomes of this plant are used for flavouring foods in the preparation of meat dishes and curries[69,70] and showed antiulcer[71], antifungal[72] and

xanthine oxidase inhibitor activities[73]. The alcoholic extract prepared from the rhizomes of *A. galanga* (Indonesian name "Lengkuwas") showed a significant effect against Sarcoma 180A in mice. Fractionation of the extract was made with the guidance of the above bioassay as shown in Table XII. Repeated column chromatography of the active *n*-hexane extract gave 1'-acetoxychavicol acetate (**139**) as a potent antitumor substance.

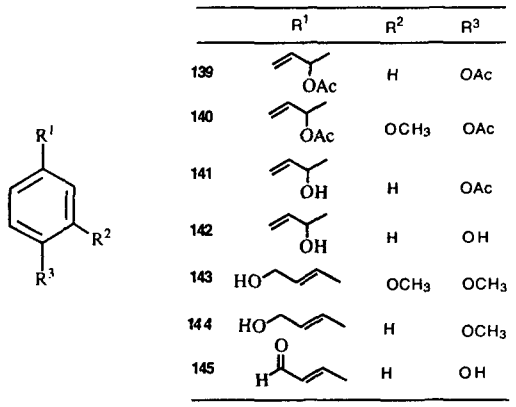


Fig. 14 Structures of Phenylpropanoids from *Alpinia galanga*

Table XII Antitumor Activity against Sarcoma 180 Ascites in Mice

	dose (mg/kg/day)	deaths due to toxicity	administration schedule	BWC (g)	PCV /TV	GR (%)	assessment
MeOH ext.	100	0	1 - 5	+0.4	0.35	1.8	+++
n-Hex. ext.	5	0	1 - 5	+2.4	0.44	122.9	-
(Y: 17)	10	3	1 - 3	-0.3	0.30	26.3	++
$CHCl_3$ ext.	20	0	1 - 5	+1.2	0.32	103.7	-
(Y: 5)							
residue ext.	80	0	1 - 5	+1.7	0.36	102.2	-
(Y: 78)							
comp. **139**	5	0	1 - 5	+3.4	0.31	79.3	-
	7	3	1 - 3	+0.1	0.31	54.6	+
	7	0	1 - 2	+0.7	0.52	36.4	++
	10	3	1	+2.4	0.22	26.7	++
	10	3	1 - 2	-1.8	0.40	1.0	+++
comp. **140**	10	1	1 - 5	+0.2	0.32	10.0	+++
comp. **141**	10	0	1 - 5	+3.4	0.36	92.5	-
comp. **142**	10	0	1 - 5	+2.4	0.37	76.3	-
comp. **143**	10	0	1 - 5	+4.1	0.37	94.7	-
comp. **144**	10	0	1 - 5	+3.6	0.34	95.7	-
comp. **145**	10	0	1 - 5	+2.7	0.37	92.6	-

The effectiveness was evaluated by means of the total packed cell volume method. BWC: body weight change = (day 7 weight - TV) / day 0 weight. PCV: packed cell volume. TV: total volume. GR: growth ratio = PCV (test groups) / PCV (control groups) x 100. Y means yield (%) from the MeOH extract.

So, with the aim of obtaining analogs of **139**, the MeOH extract from the fruits of *A. galanga* was fractionated in a similar manner as described above to furnish 1'-acetoxyeugenol acetate (**140**), *trans*-3,4-dimethoxycinnamyl alcohol (**143**), *trans*-4-methoxycinnamyl alcohol (**144**) and *trans*-4-hydroxycinnamaldehyde (**145**). Also, compounds **139**, **140**, 1'-hydroxychavicol acetate (**141**) and 1'-hydroxychavicol (**142**) were synthesized according to the previously outlined procedure.[71]

The antitumor activity of compounds **139** - **145** against Sarcoma 180A in mice is summarized in Table XII. As can be seen from the Table, **140** was a more useful agent than **139** from the

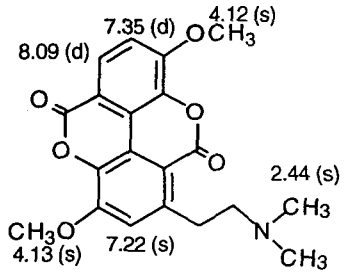
viewpoint of antitumor activity and toxicity. The results for **139** - **142** suggested that a 1'-acetoxyl group in the chavicol and eugenol analogs was required for the appearance of the antitumor activity. Therefore, the action mechanism appears to be a nucleophilic reaction, caused by transfer of the double bond resulting from elimination of the 1'-acetoxyl group. The elimination seems to be regulated through variation of the functional groups attached to the benzene ring.

C. Phenolic Compounds from *Croton palanostigma*

Sangre de Grado is a red viscous sap produced by several *Croton* species (Euphorbiaceae) growing in the upper Amazon basin in Peru. This sap has been extensively used by Peruvian natives for several medicinal purposes, including wound healing and cancer treatment. Sangre de Grade obtained from *C. palanostigma* was found to be cytotoxic to V-79 cells (IC_{50} value=3.7 μg/ml) in vitro[74]. Bioassay-directed purification guided by cytotoxicity against V-79 cells led to the isolation of taspine (**146**), which showed strong cytotoxicity against V-79 (IC_{50}=0.17 μg/ml) and KB cells (IC_{50}=0.39 μg/ml) as shown in Fig. 15.

Fig. 15 Cytotoxic Activity of Taspine

	IC_{50} (μg/ml)
KB cells	0.39
V-79 cells	0.17



V. Cyclic Oligopeptides

A. RAs Compounds from *Rubia* spp.

1. Structures of RAs

Rubiae Radix is common Rubiaceous plant; *Rubia akane* in Japan, *R. cordifolia* in China and *R. tinctorum* in Europe. The former two show antineoplastic activity, but the latter does not. Because the extract of Rubiae Radix showed antineoplastic activity against Sarcoma 180A, the compounds were pursued as active principles. After repeated fractionation and purification of the extract, oligopeptides were obtained as active principles against P388 leukemia. The extract was partitioned with water and benzene, and water and ethyl acetate. From both fractions, seven components were isolated as crystal, and named as RA-I - VIII (**147** - **154**) after *Rubia akane*.[75-79]

The physical data for the RA components are shown in Table XIII. These compounds were assumed to be small peptides from the IR values showing 3390, 1640 cm^{-1} due to amide bonding. It was determined from the ^{13}C-NMR data of RA-VII (**153**) that there were three C-Me, three CH_2-, Three *N*-Me, two O-Me, six CH, eighteen aromatic carbons, eleven tertiary carbons, seven quaternary carbons (three C-C bonds and four C-O bonds), and six carbonyl carbon groups.

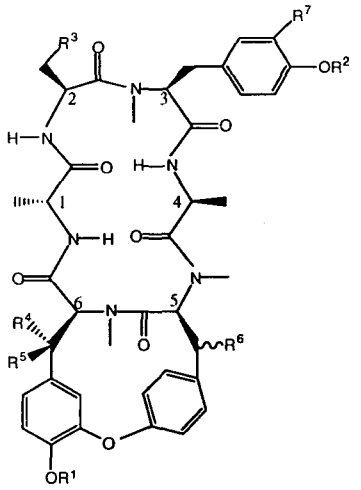
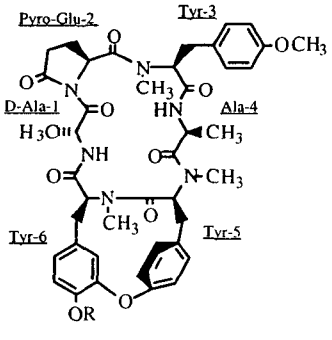


Fig. 16

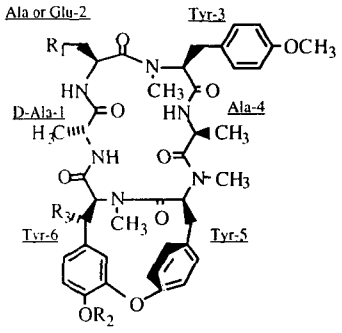
	R^1	R^2	R^3	R^4	R^5	R^6	R^7
Rubia cordifolia							
RA-I (**147**)	H	Me	OH	H	H	H	H
RA-II (**148**)	Me	H	H	H	H	H	H
RA-III (**149**)	Me	Me	OH	H	H	H	H
RA-IV (**150**)	Me	Me	H	OH	H	H	H
RA-V (**151**)	H	Me	H	H	H	H	H
RA-VI (**152**)	Me	Me	OH	H	H	H	H
RA-VII (**153**)	Me	Me	H	H	H	H	H
RA-VIII (**154**)	Me	Me	Me,OH	H	H	H	H
Bouvardia ternifolia							
bouvardin (**165**)	H	Me	H	H	H	β-OH	H
deoxybouvardin (RA-V) (**151**)	H	Me	H	H	H	H	H
microbial transformation products							
O-desmethyl-bouvardin	H	H	H	H	H	β-OH	H
bouvardin-catechol	H	H	H	H	H	β-OH	OH

By hydrolysis of RA-VII (**153**), one D-alanine, two molecules of L-alanine, *N*-methyl-4-methoxy-L-phenylalanine, and *N*-methyltyrosine dimer having ether linkage were obtained. RA-VII was assumed to be cyclic hexapeptides consisting of three alanines and three molecules of tyrosine derivatives. From these findings, the structure of RA-VII was assumed to be a bicyclic hexapeptide having ether linkage. However, it was difficult to decide the sequence of amino acids and the configuration stereochemically. Lastly, X-ray analysis was applied to p-bromobenzoate of RA-V (**151**).

From various reactions and instrumental analysis, structural relationships and the structures of RA-I (**147**) - RA-VIII (**154**) were determined as illustrated in Fig. 16. RA-VI (**152**) was elucidated as the configurational isomer of RA-III (**149**) at the moiety of D-O-methyl-tyrosine; RA-VIII (**154**) has L-threonine instead of the L-serine in RA-III molecule. However,



RA-IX (**155**): R=CH_3
RA-XIV (**160**): R=β-D-glucose

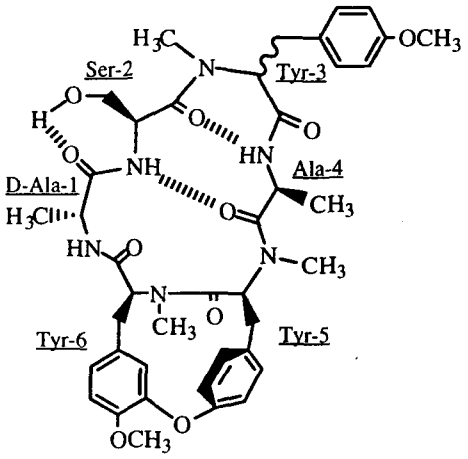


RA-X (**156**): R_1=CH_2COOH; R_2=CH_3; R_3=H
RA-XI (**157**): R_1=CH_2COOH; R_2=H; R_3=H
RA-XII (**158**): R_1=H; R_2=βD-glucose; R_3=H
RA-XIII (**159**): R_1=CH_2COOH; R_2=β-D-glucose; R_3=H
RA-XV (**161**): R_1=H; R_2=6'-Ac-β-D-glucose; R_3=H
RA-XVI (**162**): R_1=H; R_2=β-D-glucose; R_3=OAc

Fig. 17

it was observed that RA-VII and RA-V were the main components in these oligopeptides.

Moreover, RA-IX (**155**) and RA-X (**156**) were also included in this RA-series. Their structures were determined by spectroscopic and chemical methods. RA-IX contained a pyroglutamic acid instead of the Ala-2 found in RA-VII, and RA-X had glutamic acid instead of Ala-2. RA-XI (**157**) was similar to RA-X having a glutamic acid moiety. Recently, RA-XII (**158**) - XVI (**162**) have been isolated as glucosides from the same plant (Fig. 17).[80-83]



RAI-III (**163**) : L-Tyr-3
RAI-VI (**164**) : D-Tyr-3

Fig. 18 Structures of RAI-III and VI.

As minor constituents, RAI-III (**163**) and RAI-VI (**164**) were isolated from the same plants. These compounds had γ-turn structures at residues 2, 3 and 4, which were stabilized by a hydrogen bond between Ser-2-OH and D-Ala-1-CO (Fig. 17).[84] Conformations regarding RA compounds are discussed in Conformational Analysis (A-4).

Bouvardin (**165**) as the first compound of this type of cyclic hexapeptide to be isolated from *Bouvardia ternifolia* belonging to Rubiaceae, was isolated by Cole *et al.*[85].

2. Cytotoxic Activity and Antineoplastic Activity

Cell growth and inhibitory effects were examined against KB cells, P388 lymphocytic leukemia cells, and MM2 mammary carcinoma cells by using the lead compound RA-V and *n*-hexyl ether derivative, which had shown the strongest antitumor activity in the *in vivo* assay. The results are shown in Fig. 19. The *n*-hexyl ether showed clear growth-inhibitory effects at concentrations higher than $5x10^{-2}$ μg/ml and $5x10^{-2}$ μg/ml, respectively, in KB cells, and 1x10 μg/ml and $1x10^{-1}$ μg/ml in MM2 cells. Thus the growth inhibitory effect of the *n*-hexyl ether derivative was stronger than that of RA-V in each cell line and showed dose-dependency.[86-87]

Microscopically, mitomycin C-treated KB cells showed deformation, and enlargement and abnormality of nuclei, whereas KB cells treated with RA-V and its *n*-hexyl ether derivatives showed globulization as compared with control cells.

RA-IV was considered to have an additional alcoholic hydroxyl group as compared with RA-VII. It was concluded that the hydroxyl group in RA-IV is linked to the β-carbon (β) of Tyr-6 by comparing the ^{13}C chemical shift values of RA-IV with those of RA-VII; Cβ signal at δ 35.56 (t) due to Tyr-6 of RA-VII was shifted down field to 73.49 (d) in RA-IV, while other carbon signals in both peptides were similar. Next, in order to introduce an oxygen functional group into the benzyl position of Tyr-6, RA-V was oxidized with 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) as shown in Chart 4. This reaction gave selectively compound E in methanol and compound A in 90% aqueous tert-BuOH solution. Compound A was methylated with diazomethane to yield RA-IV. In addition, to confirm the configuration of the hydroxyl

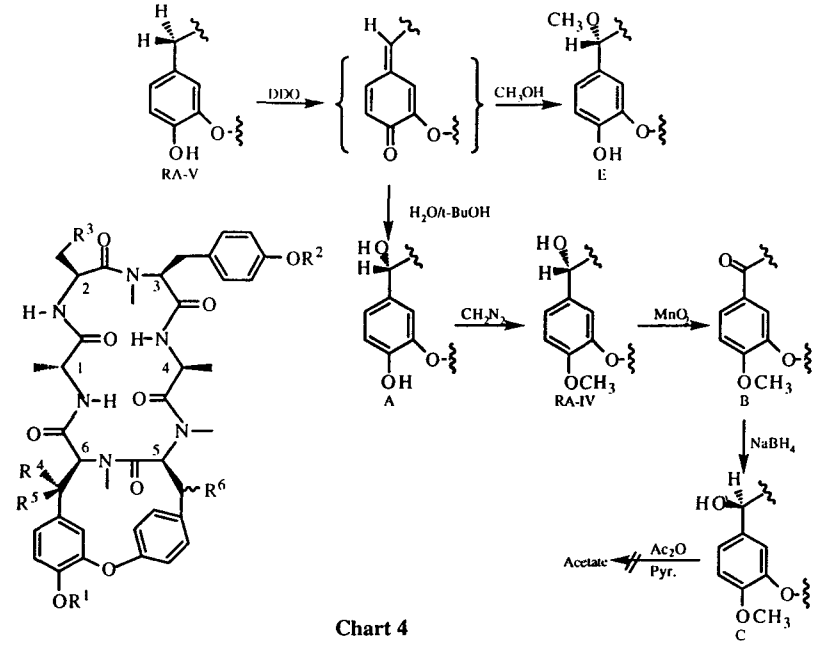
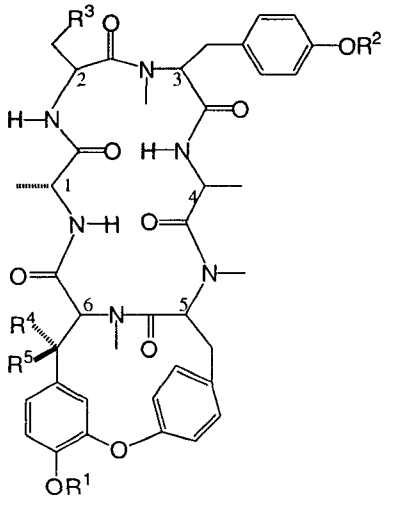


Chart 4

group in RA-IV, its epimer (C) was synthesized. This epimer could not be acetylated with anhydrous acetic acid pyridine at room temperature. These findings can be reasonably explained by the following stereochemical considerations: the reagent in this series of reactions can approach only from the α-side, because the β-side at the benzyl location of Tyr-6 is strongly blocked by the *N*-methyl group of this tyrosine moiety, as noted from the X-ray conformation. Consequently, the hydroxyl group of RA-IV was determined to have an *S* configuration.



	R^1	R^2	R^3	R^4	R^5	Antitumor activity[a] T/C (%)
RA-I	H	Me	OH	H	H	169.3
RA-I-diAc	Ac	Me	OAc	H	H	182.8
RA-II	Me	H	H	H	H	142.2
RA-III	Me	Me	OH	H	H	179.4[b]
RA-VI	Me	Me	H	OH	H	149.0
RA-V	H	Me	H	H	H	187.4
RA-VII	Me	Me	H	H	H	173.6[c]
A	H	Me	H	OH	H	126.3
A-diAc	Ac	Me	H	OAc	H	98.2
B	Me	Me	H	=O	=O	171.9
C	Me	Me	H	H	OH	160.0
E	H	Me	H	OMe	H	118.5
E-Me	Me	Me	H	OMe	H	132.0
E-Ac	Ac	Me	H	OMe	H	116.9

a) P388 : 10^6 cells/0.1 ml, i. p., CDF1 mice (n = 6).
Dose : 10.0 mg/kg., i. p., day 1-5.
b) Dose : 2.0 mg/kg.
c) Dose : 4.0 mg/kg.

Fig. 20 Structures and Antitumor Activity of Native Cyclic Hexapeptides and Related Compounds

We also examined the antineoplastic activity of six native cyclic hexapeptides (RA-I, II, III, IV, V and VII) and seven related compounds against *P388 lymphocytic leukemia in mice*. The mice received 10 mg/kg/day (except for RA-VII and RA-III: 4.0 and 2.0 mg/kg/day) *i.p.* for 5 consecutive days. The result is shown in Fig. 20. The small differences of antitumor activity among these compounds could be explained to some extent by the molecular hydrophobicities as previously mentioned, but a remarkable decrease of antitumor activity was observed in RA-IV compounds A, A-diAc, E, E-Me and E-Ac, whose α-proton at the Cβ-position of Tyr-6 was replaced with bulky substituent groups. In spite of a similar replacement at Cβ, the activity of compounds B and C did not decrease. From these findings, it may be concluded that the introduction of large substituent groups at the α-side of the RA-series results in a decrease of antitumor activity. This seems to have an important effect on the mechanism of antitumor activity. The antitumor activity decrease of RA-II is better explained in term of molecular hydrophobicity from the α-block hypothesis.

In order to obtain RA-analogs with higher pharmacological and lower toxicological activities, several derivatives were synthesized by substituting the phenol moiety of RA-V, and their quantitative structure-activity relationships (QSAR) were investigated from the viewpoint of molecular hydrophobicities. The activity values (log 1/IC 50) of ether derivatives of RA-V gave an upward parabolic or bilinear relationship when plotted against log P (P: partition coefficient determined with the 1-octanol/water system) as the carbon number of the side chain at the phenol moiety of RA-V was increased. The optimum log P values were in the range of 3.5 to 4.9. The ester derivatives showed a similar relationship, the optimum log P values being 6.3 -6.7, which was higher than that of the ether derivatives. The relationship among the ILS (150 and 160%), the minimum lethal dose (MLD) and the hydrophilic coefficient of the ether series of RA-V were analyzed according to both the Hansch-Fujita and the bilinear models of Kubinyi. When the parabolic model obtained from the Hansch-Fujita equation was applied to the ILS and MLD, no significant results were obtained.

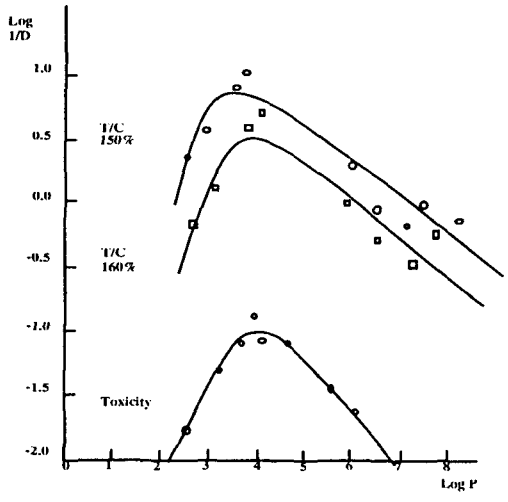


Fig. 21 Structure-Antitumor Activity and Toxicity Relationships of Alkyl Ethers of RA-V on P-388 Leukemia in Mice

However, since the optimum log P values of ILS 150 and 160% differed from that of MLD, it was considered that the most suitable ether derivatives of RA-V for antitumor activity might be selected from the region away from the optimum log P of MLD and approximating the log 1/D value in the optimum log p of ILS. Thus, RA-VII and the *n*-hexyl ether of RA-V should be useful compounds on this basis.

The therapeutic ratio of RA-VII was 400, compared with 10 for MMC (Table XV). The mechanism of action of RA-VII was also investigated and was assumed to inhibit protein biosynthesis, since ^{3}H-leucine was not taken in. The lethal effect of RA-V on KB cells was clearly different from that of MMC, and RA-VII was concluded to be a "time-dependent drug" like vincristine. Further, RA-VII was effective against Colon 38 (s.c.-i.p., s.c.-i.v.), P388 (i.p.-i.v.), L1210 (i.p.-i.v.), Meth A (s.c.-i.p.), and M5076 (i.p.-i.p.). Inhibition was also found against pulmonary metastasis of B16-BL-6 (s.c.-i.v.).

Moreover recently, mechanistic studies using purified elongation factors and ribosomes identified RA-VII as a peptidyl transferase inhibitor. Thus similar to the related natural products bouvardin (**165**) and RA-XII (**158**), RA-VII (**153**) appears to function by binding to eukaryotic ribosomes.[88]

Table XV Therapeutic Effects of RA-VII on P388 Leukemia

Group	Dose (mg/kg)	Route	Survival effects: Survival time (d, mean±S. E.)	T/C (%)	B. W. (g)	
Control	10 ml	i. p.	10.1 ± 0.18	100.0	+5.0	
RA-VII	0.005	i. p.	11.0 ± 0.26	109.2	+3.8	
	0.01	i. p.	13.3 ± 1.15	132.3	+2.6	
	0.05	i. p.	15.5 ± 1.12	153.8	+2.1	
	0.5	i. p.	16.7 ± 0.33	165.4	+1.3	T. R. = 400
	2.0	i. p.	18.6 ± 1.21	184.3	+0.4	
	4.0	i. p.	23.6 ± 2.62[a)]	234.2	-0.6	
	6.0	i. p.	6.0 ± 2.61	62.7		
MMC	0.005	i. p.	10.8 ± 0.31	107.5	+4.8	
	0.01	i. p.	10.5 ± 0.22	104.2	+5.2	
	0.1	i. p.	13.7 ± 0.67	135.6	+2.8	T. R. = 10
	0.5	i. p.	15.8 ± 0.31	157.1	+0.8	
	1.0	i. p.	18.0 ± 0.68	178.1	-0.3	
	2.0	i. p.	12.7 ± 0.33	125.7	-1.8	
Control	10 ml	i. v.	9.5 ± 0.15	100.0	+4.0	
RA-VII	0.25	i. v.	10.0 ± 0.27	105.3	+3.2	
	1.0	i. v.	11.0 ± 0.19	115.8	+1.9	
	2.5	i. v.	13.4 ± 0.18	140.8	-0.3	
	4.0	i. v.	14.5 ± 1.25	152.6	-2.0	
	6.0	i. v.	15.9 ± 0.23	167.1	-4.7	
MMC	0.1	i. v.	10.5 ± 0.19	110.5	+4.4	
	0.5	i. v.	12.5 ± 0.19	131.6	+2.3	
	1.0	i. v.	13.6 ± 0.18	143.4	+0.9	
	2.0	i. v.	12.1 ± 0.13	127.6	-3.6	

a) 1/6 animal survived 60 d. P388 was implanted i. p. (1 x 10^{6} cells/0.1 ml) in CDF1 mice at day 0. Drugs were given daily at indicated doses for a consecutive 9 d from day 1 to 9.

RA-V is the same compound as deoxybouvardin (**151**). Bouvardin (**165**) has been investigated for development as an antitumor drug at the U.S. NCI. Adryamycin has CH_2OH instead of CH_3 as in daunomycin. With such minor chemical differences, adryamycin was revealed to have a much stronger activity and less toxicity than daunomycin. Therefore, it is expected that RA-VII will show different activity from that of bouvardin. RA-VII (RA-700) is now under investigation for phase I clinical trials at the NCI in Japan.

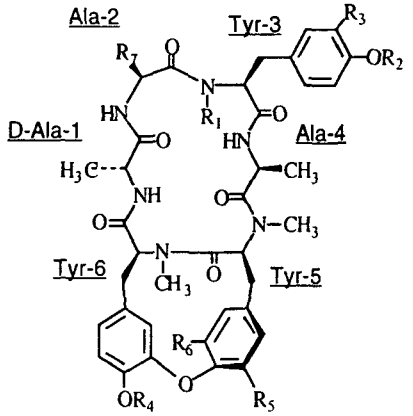


Fig. 22

	R_1	R_2	R_3	R_4	R_5	R_6	R_7
165	H	Me	H	Me	H	H	Me
166	Me	Me	H	Me	OH	H	Me
167	Me	Me	H	Me	H	OH	Me
168	Me	Me	OH	Me	H	H	Me
169	Me	Me	OH	Me	OH	H	Me
170	Me	H	H	Me	H	OH	Me
171	Me	Me	OH	H	H	H	Me
172	Me	H	H	H	H	H	Me
173	Me	H	H	Me	OH	H	Me
RA-VII (**153**)	Me	Me	H	Me	H	H	Me
RA-II (**148**)	Me	H	H	Me	H	H	Me
RA-V (**151**)	Me	Me	H	H	H	H	Me
RA-X (**156**)	Me	Me	H	Me	H	H	
CH_2CH_2COOH							

Structures of RA-VII and Its Metabolites

The *in vitro* phase II trial of RA-700 employed human tumor clonogenic assay. From the results using a human tumor cell line of lung cancer (PC-6), RA-700 appears to possess time-dependent antitumor activity. The chemosensitivity rate of RA-700 was 67%, 22%, 17% and 10% for ovarian cancer, non-small cell lung cancer, breast cancer and colorectal cancer, respectively. RA-700 showed almost the same chemosensitivity as that of five standard anticancer drugs (adryamycin, mitomycin C, cisplatin, vinbrastine and 5-FU), but the spectrum of RA-700 activity appeared to be different. Furthermore, the antitumor activity of RA-700 had no relationship with prior chemotherapy. These results indicated that RA-700 is a candidate for phase I study.[89]

3. Metabolites of RA-VII and RA-X [90-92]

The biotransformation was examined by using rat hepatic microsomes and bile juice of rabbit. RA-VII was incubated aerobically with rat liver microsomes in the presence of an NADPH-generating system. In the course of researching the metabolites from the bile juice in rabbits, eleven metabolites (**165** - **173**), RA-II and RA-V were isolated (Fig. 22).

RA-V and compound **165** were isolated as two main metabolites from the chloroform extract of the incubation mixture. The structure of **165** was determined to be [*N*-demethyl-Tyr-3]RA-VII. Compound **166** and **167** were characterized as [ε1-hydroxyl-Tyr-5]RA-VII and [ε2-hydroxyl-Tyr-5]RA-VII, respectively. When RA-X was administrated in rabbits, the total recovery of RA-X-Na was 75%. RA-X-Na did not show antitumor activity against P388

leukemia in mice by *i.v.* administration. This was also presumed to be attributable to the fast metabolic turnover indicated in this experiment.

4. Conformational Analysis

The therapeutic potential of many biologically active cyclic peptides is intimately related to their conformation. Structural analogues of many of these biologically active RAs have been synthesized to study the biological systems with which they interact. The relationship between conformation and biological function of RAs is a topic of great interest and importance. Much work is being done in the field of peptide structural and conformational analysis, using X-ray crystallography, circular dichroism, NMR spectroscopy, and computational methods. These methods provide information about the conformation of RAs in the solid state and in solution.

a. Crystal Conformation

Bouvardin (**165**), originally isolated in 1977 from *Bouvardia ternifolia* (Rubiaceae), was the first member of the RA family and its solid state conformation was reported.[93] The molecule consists of four L-amino acids and two D-amino acids joined by peptide linkages. A characteristic feature of this molecule is that it has, in addition to an 18-membered peptide ring, a 14-membered ring formed by the oxidative coupling of the phenolic oxygen of one tyrosine with a carbon *ortho* to the phenolic OH group of an adjacent tyrosine. The *N*-methyltyrosine residue on the 18-membered peptide ring extends outward. Five of the peptide bonds are in *trans* conformation while the sixth, between Tyr^5 and Tyr^6, is in *cis* conformation (type VI β-turn) which serves to fold the peptide chain to form a cyclic structure. Another turn (type II β-turn) was found between Ala^2 and Tyr^3. It is an unusual cyclic hexapeptide because there is an absence of any hydrogen bonding within the 18-membered ring. Itokawa *et al.* reported the X-ray structure of RA-V p-bromobenzoate in 1990.[94]: the backbone conformation possesses almost the same characteristics as that of bouvardin.

Later, the crystal structures of RA-VI (**152**)[95] and isomerized RA-VII (**174**),[96] neither showing antitumor activity, were reported. The backbone conformation of these molecules differ from that of the 18-membered ring of RA-VII and bouvardin.

b. Solution Conformation

The presence of two conformers in chloroform solution was suggested by HPLC analysis[97]. However, separation on a preparative scale was not achieved because of fast exchange at room temperature. Conformational analysis of the two states in chloroform solution was performed using NMR and computational methods. R. B. Bates *et al.* indicated that the predominant stereoisomer and conformer in solution for deoxybouvardin and bouvardin is the same as that found in the solid state by X-ray diffraction.[98] A minor stereoisomer (ca. 15%) separated by a 20.6 kcal/mol barrier was detected and is believed to contain a rotation about the Tyr^5 and/or Tyr^3 amide bond, however its precise conformation could not be deduced.

A combination of different homo- and heteronuclear 2D NMR techniques at 500 MHz

have enabled complete assignment of the ^{1}H and ^{13}C signals of the two conformers of RA-VII in $CDCl_3$ (major conformer: conformer A; minor one: conformer B).[94] The structures of the two conformers (A and B) in $CDCl_3$ were elucidated based on temperature effects on NH protons, deuterium exchange rate, vicinal coupling constants and NOE experiments (Fig. 27). These conformational analyses showed that the structure of these conformers is a result of geometrical isomerization and that the predominant conformer A exhibits a typical type II β-turn between Ala^2 and Tyr^3, similar to the crystal structure as analyzed by X-ray diffraction. The minor conformer B exhibits a type VI β-turn between Ala^2 and Tyr^3, showing a *cis* amide bond. In the 18-membered ring, the presence of two intramolecular hydrogen bonds between Ala^4-NH and D-Ala^1-CO, and between D-Ala^1-NH and Ala^4-CO was suggested. This differs between solid and solution conformations. The reduced biological activity of the *N*-methyl derivative of RA-VII in comparison with RA-VII may be responsible for the small population of conformer A molecules in solution. Further, the presence of a highly strained 14-membered ring is necessary to maintain the typical type II β-turn structure of conformer A. The ring system and structure are considered to play an important role in antitumor activity.[94]

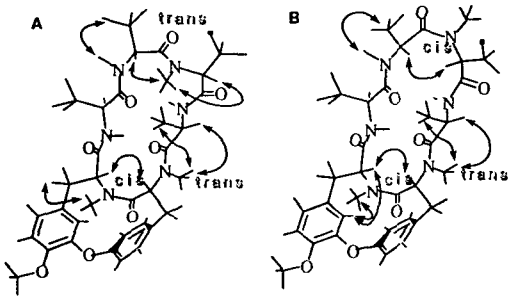
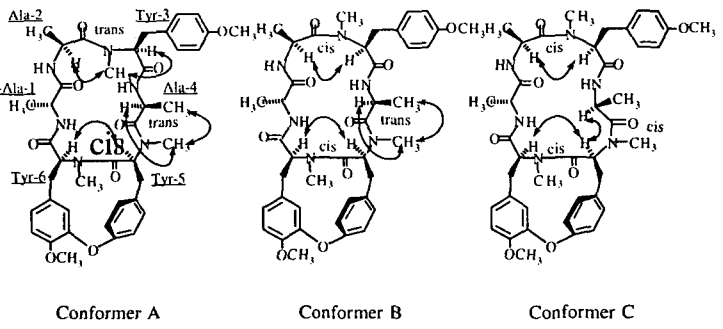


Fig. 27 NOE Enhancements in Conformers A and B of RA-VII
The arrows show the NOE relationships confirmed by 1D-NOE and NOESY experiments in $CDCl_3$ at 303K.

The conformation of RA-VII in THF-*d*8 was found to be similar to that observed in $CDCl_3$.[99] The addition of LiCl caused no conformational change and resulted in the adoption of a single dominant solution form (conformer A, 94%).[99]



Conformer A Conformer B Conformer C

Fig. 28 Molecular Structures of Three Different Conformers (A, B and C) of RA-VII in DMSO-d_6
The arrows show the NOE relationships confirmed by NOESYPH experiments.

Three discernible isomers observed in DMSO-d_6 were assigned for RA-VII (Fig. 28).[100] The largest isomer, conformer A, accounting for 64% of all isomers has a cis configuration between Tyr5 and Tyr6 only. The second configurational isomer, conformer B, 32%, has adopted cis configurations between both Tyr5 and Tyr6 and between Ala2 and Tyr3. The third isomer, conformer C, 4%, has cis configurations for all of the three N-methyl amide bonds.

A molecular design was carried out to lock the type II β-turn conformation of RA-VII, by removing the N-methyl group of the Tyr3 residue. Conformational analysis of [N-demethyl-Tyr(OCH3) -3]RA-VII (165), produced by hepatic microsomal biotransformation, was based on 2D NMR techniques, temperature effect on NH protons, and NOE experiments.[101] It showed a restricted conformational state with a typical type II β-turn between Ala2 and Tyr3 in DMSO-d_6. The relationship of NOE enhancement (all negative NOEs) is shown in Fig. 29. This analogue was recently synthesized by D. L. Boger *et al.* who showed that *N*-methyl removal does not alter its conformational or biological properties.[102] The disfavored *cis* amide bond between Tyr^5 and Tyr^6 is the predominant conformation observed with [desmethyl-Tyr^6]RA-VII. In contrast, [desmethyl-Tyr^5]RA-VII in solution possesses a *trans* amide bond between Tyr^5 and Tyr^6, resulting in a loss of biological activity. Thus, the *N*-methyl amide group between Ala^4 and Tyr^5 is essential for maintenance of the conformational and biological properties of RA-VII.[102]

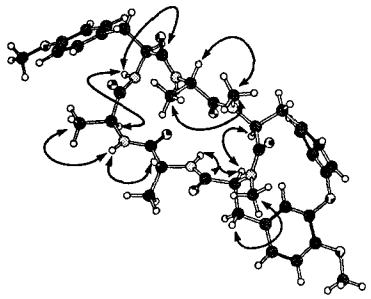


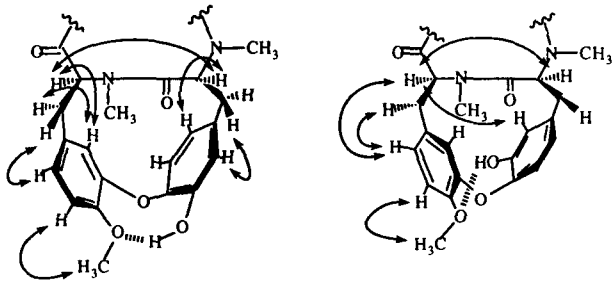
Fig. 29 NOE Relationship of [N-demethyl-Tyr(OCH_3) -3]RA-VII
The arrows show the negative NOE relationships by 1D-NOE and NOESYPH in DMSO-d_6 at 303K.

Furthermore, conformational change in the 14-membered ring system of [ε1-hydroxyl-Tyr^5]RA-VII (**166**) and [ε2-hydroxyl-Tyr^5]RA-VII (**167**) was observed in the metabolites of RA-VII and RA-X, produced by using rat hepatic microsomes and rabbit biliary juice (Fig. 30).[103]

By conformational analysis using spectroscopic and computational chemical methods, RAI-III (**163**) and VI (**164**), which were isolated from *R. akane* as minor constituents, were shown to have γ-turns at residues 2, 3 and 4, which were stabilized by a hydrogen bond between Ser^2-OH and D-Ala^1-CO.[104]

In related work, the glycopeptide RA-XII also has been shown to bind to 80S ribosomes.[105] In NMR titration experiments, line-broadening in the ^1H NMR spectrum for

RA-XII was observed upon addition of a small quantity of 80S ribosomes to the NMR sample. Selective binding of the major conformer of the peptide was also observed.



[ε1-hydroxyl-Tyr^5]RA-VII [ε2-hydroxyl-Tyr^5]RA-VII

Fig. 30 NOE Relationships of the 14-Membered Rings of [ε1-hydroxyl-Tyr^5]RA-VII and [ε2-hydroxyl-Tyr^5]RA-VII

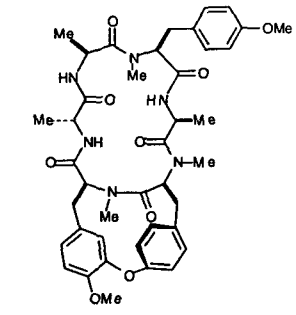
The arrows show the NOE relationships confirmed by NOESYPH experiments in $CDCl_3$ at 303K.

c. Molecular modeling

In order to obtain details of structure and conformation which agree more closely with the NMR data, calculations of molecular dynamics, starting with X-ray structure and applying the distance constraints obtained from the NOE experiments, were performed[94]. The final structures obtained after several such calculations were examined for their overall energetic favorability, compared with the structure derived from the NMR data, and classified into two conformers; corresponding to conformers A and B deduced by the NMR study. Conformer A contains Ala^2 in the I + 1 and Tyr^3 in the i + 2 positions with stabilization of the transannular H-bridge between Ala^4-NH and Ala^1-CO. The solution structure of the minor conformer B of RA-VII has not been determined, because no sufficient NOE values are available. This simulation indicated that the type II β-turn involving Ala^2 and Tyr^3 is converted to a type VI β-turn, by the NOE enhancements between Ala^2-Hα and Tyr^3-Hα, suggesting a *cis* peptide bond. A conformational search of RA-VII using Monte Carlo simulation was conducted by D. L. Boger *et al.*[99] Using the OPLSA force field, the three lowest energy conformations corresponded to conformer A, conformer B (ΔE=1.3 kcal/mol), and conformer C (ΔE=2.4 kcal/mol) detected by ^{1}H NMR and paralleled the relative stabilities of the three conformational isomers detected in DMSO-d_6.[100] The conformation of a cyclic bouvardin analogue, *cyclo*(-D-Ala-Pro-MeTyr-Ala-MeTyr-MeTyr-) (**175**) has been determined by distance geometry calculation and restrained energy minimization from NMR data.[106] Calculations done on the major conformer revealed a unique backbone conformation, consisting of two β-turns, a type II β-turn at Pro-MeTyr and a type VI β-turn at MeTyr^4-MeTyr^5. In this analogue, with a proline residue at position 2, the lack of a 14-membered ring formed by the oxidative coupling of the phenolic oxygen did not affect backbone conformation in the 18-membered ring, however, an analogue with an alanine residue at position 2 and lacking the 14-membered ring strongly influenced the population in solution.[94]

5. Correlation between Structures and Biological Activities

Recently a number of analogs of these peptides has been synthesized and biologically evaluated. The results suggest some insights into the structure–activity relationships and identification of the pharmacophore for these peptides.



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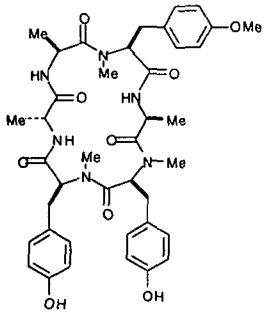

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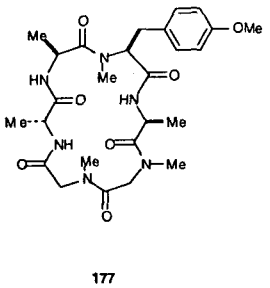

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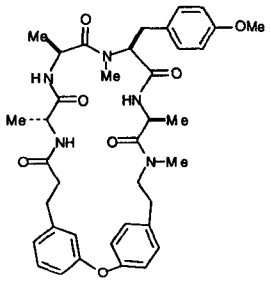

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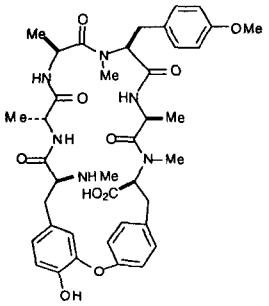

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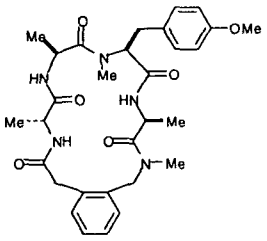

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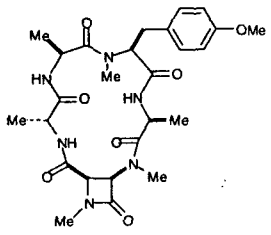

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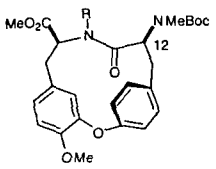
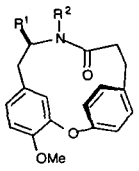


182 R = H
183 R = Me



184 $R^1 = R^2 = H$
185 $R^1 = H$, $R^2 = Me$
186 $R^1 = CO_2Me$, $R^2 = H$

The analogs **176** – **181** in which the 14-membered cycloisodityrosine moiety was modified were designed and synthesized.[107-112] These analogs showed little or no cytotoxicity (Table XVI). Since even analog **181** possessing conformational property similar to the most stable conformation of the natural peptides showed very weak cytotoxicity, the tetrapeptide moiety (D-Ala-1–Ala-2–Tyr-3–Ala-4) appeared not to be essential for the activity. Based on these observations and in conjunction with recent reports regarding cyclo-isodityrosine analogs **182** and **183** showing fairly potent cytotoxicity, the 14-membered cyclo-isodityrosine moiety is currently considered to be the pharmacophore unit for this class of natural peptides.[111] However, more simplified analogs **184** – **186** showed no activity, which defined the C-12 amine substituent playing the essential role for the activity.[113]

Peptide **153** possesses two methoxy groups on the aromatic ring of Tyr-3 and Tyr-6 residues. Substitution of the methoxy group at the Tyr-6 residue by a hydrogen atom (**190**) or a hydroxyl group (**151**) causes little effects on the cytotoxicity (Table XVI).[114] However,

Table XVI Cytotoxicity of RA Analogs against L1210 cells

Compound	IC_{50} (μg/mL)
153 (RA-VII)	0.002
176	>100
177	>100
178	20
179	>10
180	25
181	50
182	0.06
183	0.03
184	>100
185	>100
186	>100

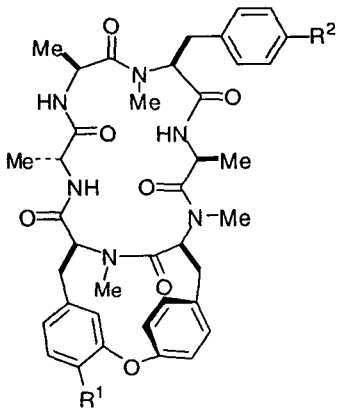
when the methoxy at the Tyr-3 residue is substituted by a hydrogen atom (**188** and **189**) or a hydroxy group (**187** and **191**), cytotoxicity is reduced 100 to 1000 - fold. Thus, this methoxy group appears to be essential for the activity. However, since C-alkyl analogs **194** – **197** were rather weaker than **153**, but still showed significant activity both *in vitro* (Table XVII) and *in vivo* (Table XVIII), it can be concluded that this appendage moiety is important for the activity although the ether linkage is not essential.[115]

Since RA-V (**151**) possesses a reactive phenolic hydroxyl group on the Tyr-6 residue, its derivatization has been extensively studied. A number of alkyl and acyl groups were introduced into this position. Some examples of their antitumor activities are listed in Table XIX.[116] Interestingly, analogs possessing a rather long alkyl or an acyl group retained potent antitumor activity, which suggests that modification at this position may lead to analogs more biologically promising than peptide RA-VII (**153**).

Table XVII Cytotoxicity of RA Analogs against P388 and KB Cells

Compound	IC_{50} (μg/mL) P388	KB
153 (RA-VII)	0.0013	0.0023
187	>10	7.8
188	0.37	0.84
189	0.031	0.36
151 (RA-V)	*0.012*	*0.0019*
190	0.0025	0.0063
191	>10	>10
192	0.22	0.42
193	0.018	n.t.
194	0.0072	n.t.
195	0.020	n.t.
196	0.013	n.t.
197	0.0039	n.t.

n.t.: not tested.



153 $R^1 = R^2 = OMe$
187 $R^1 = R^2 = OH$
188 $R^1 = R^2 = H$
189 $R^1 = OH$, $R^2 = H$
151 $R^1 = OH$, $R^2 = OMe$
190 $R^1 = H$, $R^2 = OMe$
191 $R^1 = H$, $R^2 = OH$
192 $R^1 = OMe$, $R^2 = H$
193 $R^1 = OMe$, $R^2 = Me$
194 $R^1 = OMe$, $R^2 = Et$
195 $R^1 = OMe$, $R^2 = Pr^n$
196 $R^1 = OMe$, $R^2 = vinyl$
197 $R^1 = OMe$, $R^2 = allyl$

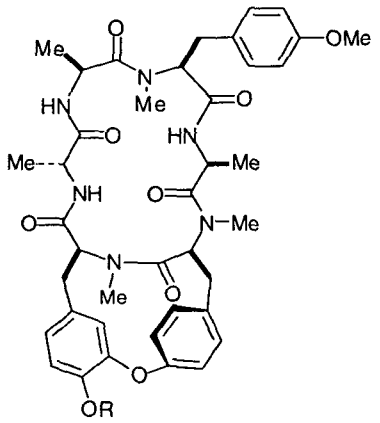
Various Ala-2 modified analogs of **153** have been synthesized from RA-III (**149**) and RA-X methyl ester (**209**).[117,118] Comparison of their cytotoxicity reveals some tendencies between the side chain structure and the activity (Table XX). Compounds which possess a polar functionality at the side chain showed reduced activity, especially in the case of ornithine

(**220**) and aspartic acid (**217**) derivatives. In this regard, it is noteworthy that homoserine (**210**) and ε-hydroxynorleucine (**212**) derivatives are less toxic than norvaline (**215**) and methionine (**218**) derivatives, respectively, having a non-polar residue with similar length. Another clear relationship exists between the length of the residue and cytotoxicity. In a series of compounds having a hydroxyl group at the end of the side chain, cytotoxicities decreased with length of the carbon chain (**149** > **210** > **211** > **212**). The same tendencies are also observed among other homologues (*e.g.* **216** > **209**; **153** > **215**). The observation, however, that the azido intermediate **221** having a rather long residue shows effective cytotoxicity suggests that a lengthy side chain can be compatible with the activity in the case of a less polar functionality.

Table XVIII Antitumor activity of RA-VII Analogs against P-388 Leukemia in mice

# / Dose[a]	T/C (%) 0.4	0.8	1.6	3.13	6.25
153 (RA-VII)	144	144	152	163	toxic
192	92		100		101
193	105		121		149
194	108		120		151
195	109		121		141
196	100		110	127	
197	102		130		130

[a] Dose in mg/kg given i.p. on days 1–5.



R	
151	H
153	Me
198	Et
199	Pri
200	$(CH_2)_5CH_3$
201	$(CH_2)_8CH_3$
202	$(CH_2)_{10}CH_3$
203	Ac
204	$COCH{=}CHCH_3$
205	$CO(CH_2)_4CH_3$
206	$CO(CH_2)_6CH_3$
207	$CO(CH_2)_8CH_3$
208	COPh

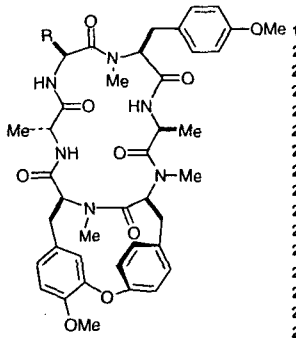
Table XIX Antitumor activity of Ether and Ester Derivatives of RA-V against P-388 Leukemia in mice

Compound	Dose[a]	T/C (%) 0.05	0.5	2.0	4.0	10.0
151 (RA-V)		131.1	152.5	164.2	165.3	187.4
153 (RA-VII)		138.6	156.7	164.2	173.6	
198		137.3	165.4	162.2	toxic	
199		142.2	175.1	105.4	toxic	
200		110.3	137.3	153.5	173.0	
201		112.5	141.5	150.1	155.4	
202		115.4	108.7	121.2	123.1	
203		133.3	155.6	168.9		194.7
204		124.1	148.5	162.3		189.6
205		133.6	143.2	151.6		197.2
206		126.7	146.7	166.7		168.9
207		122.0	146.3	151.6		150.6
208		127.8	185.6	175.6		183.3

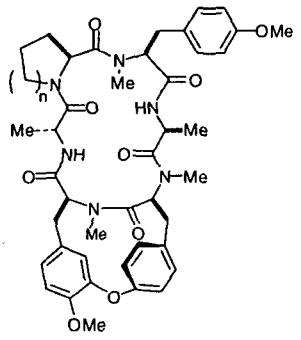
[a] Dose in mg/kg given i.p. on days 1–5.

Table XX Cytotoxicity of RA Analogs against P388 and KB cells

Compound	IC_{50} (μg/mL) P388	KB
153	0.0013	0.0023
149	0.011	0.024
209	0.034	0.030
210	0.14	0.084
211	0.031	0.059
212	0.011	0.019
213	0.027	0.031
214	0.18	0.14
215	0.99	1.9
216	0.048	0.052
217	0.020	0.042
218	0.017	0.040
219	0.030	0.014
220	>10	>10
221	0.0083	0.0060
222	0.32	0.37
223	0.29	0.31
224	0.079	0.074
225	0.079	0.21

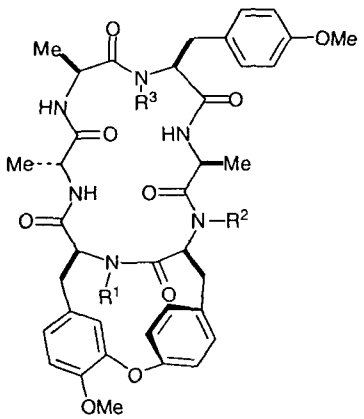


149 CH_2OH
209 $CH_2CH_2CO_2Me$
210 $(CH_2)_2OH$
211 $(CH_2)_3OH$
212 $(CH_2)_4OH$
213 $CH{=}CH_2$
214 $CH_2CH{=}CH_2$
215 $CH_2CH_2CH_3$
216 CH_2CHO
217 CH_2CO_2H
218 $CH_2CH_2SCH_3$
219 $(CH_2)_3N_3$
220 $(CH_2)_3NH_2$
221 CH_2SAc
222 $CH_2S)_2$–
223 CH_2SH



224 n = 1
225 n = 2

As mentioned above, peptide **153** exists in two or three stable conformational states in solution. However, the proline analog **224** showed only one conformation in various solvents, and extensive NMR experiments revealed that this conformation is very similar to the major conformer of peptide **153**. Since analog **224** showed potent cytotoxicity, it is concluded that the major conformation of peptide **153** is at least in part responsible for the activity. The pipecolic acid analog **225** showed similar conformational and biological tendencies.

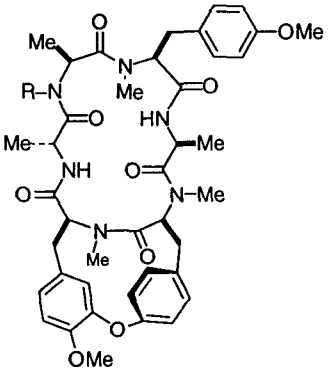


153 $R^1 = R^2 = R^3 = Me$
226 $R^1 = H, R^2 = R^3 = Me$
227 $R^1 = R^2 = Me, R^3 = H$
228 $R^1 = R^3 = Me, R^2 = H$
229 $R^1 = Me, R^2 = R^3 = H$
230 $R^1 = R^3 = H, R^2 = Me$
231 $R^1 = R^2 = H, R^3 = Me$
232 $R^1 = R^2 = R^3 = H$

N-Desmethyl derivatives **226** – **232** of RA-VII (**153**) were synthesized and evaluated using L1210 cells.[119] Analogs **228**, **229**, **231**, and **232** were found to be biologically inactive (IC_{50}, >10 μg/mL), while **226** and **227** were essentially equipotent with **153** (0.0007–0.002 μg/mL). 1D and 2D ^{1}H-NMR studies of these analogs revealed a role for *N*-methylaltion in the key conformational aspects of the natural agents. The *N*-methyl group of Tyr-5 residue is essential for maintenance of the conformational and biological properties of peptide **153**; the *N*-

methyl group of Tyr-3 residue is not essential, and its removal leads to an exclusive population of a single biologically active conformation; and removal of the *N*-methyl group of Tyr-6 residue does not alter the conformational or biological properties of peptide **153**.

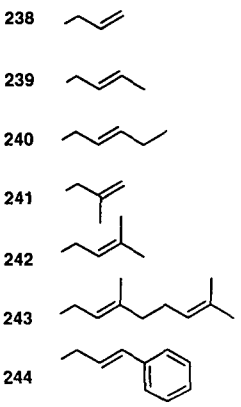
Although a lack of understanding as to how to chemically manipulate the molecule hampered derivatization of peptide **153**, the Ala-2 amide nitrogen proved to be effectively alkylated with reactive alkylating agents under the selected conditions. Analogs **233** – **269** were prepared by direct alkylation or further derivatization.[120-122] Simple alkyl analogs **233** – **244** retained potent cytotoxicity, and some of them (*e.g.*, **242** and **243**) showed more promising antitumor activity than peptide **153** in terms of maximum *T/C* values.



R

153 H
233 Me
234 Et
235 $(CH_2)_2CH_3$
236 $(CH_2)_3CH_3$
237 $(CH_2)_4CH_3$

R



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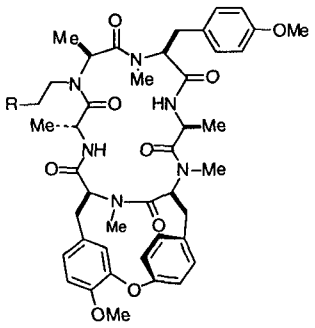
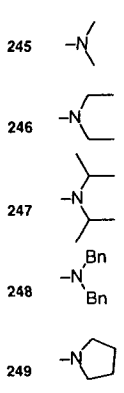


R



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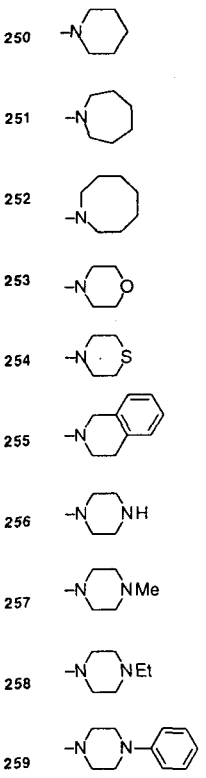

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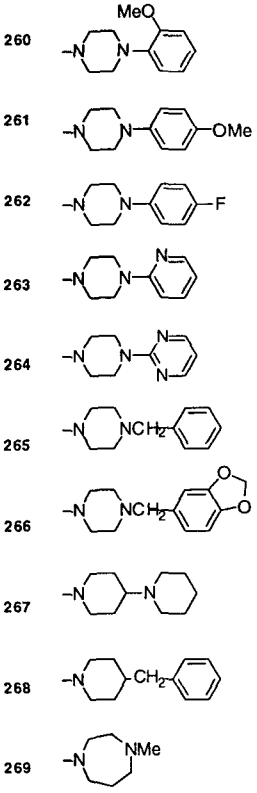
R



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The cytotoxicity of the amine analogs **245 – 269**, which are believed to improve the water solubility of the peptide, varied though some showed *in vivo* antitumor activity; the activity, however, was weaker than the parent peptide **153**.

Table XXI Cytotoxicity of RA Analogs against P388 and KB Cells

Compound	IC_{50} (μg/mL) P388	KB	Compound	IC_{50} (μg/mL) P388	KB
153 (RA-VII)	0.0013	0.0023	**251**	0.015	0.034
233	0.0012	0.0077	**252**	0.12	0.49
234	0.035	0.035	**253**	0.071	0.081
235	0.0032	0.0097	**254**	0.031	0.060
236	0.010	0.024	**255**	0.032	0.19
237	0.018	0.063	**256**	0.11	0.19
238	0.015	0.013	**257**	0.065	0.072
239	0.0076	0.018	**258**	0.74	0.56
240	0.010	0.022	**259**	0.039	0.16
241	0.0090	0.027	**260**	0.11	0.48
242	0.058	0.064	**261**	0.036	0.12
243	0.044	0.062	**262**	0.029	0.073
244	0.0094	0.030	**263**	0.033	0.099
245	0.12	0.19	**264**	0.025	0.057
246	0.21	0.46	**265**	0.021	0.051
247	0.25	0.40	**266**	0.084	0.23
248	0.19	0.48	**267**	0.30	0.4
249	0.13	0.70	**268**	0.088	0.21
250	0.19	1.1	**269**	1.4	1.7

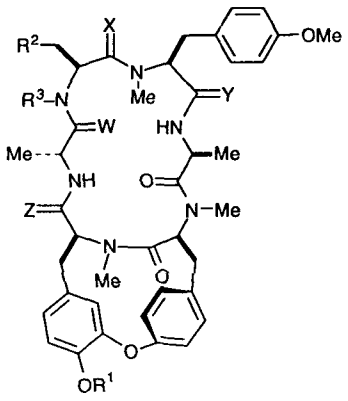
When these peptides were treated with thionating reagents, thioamides **284–292** were obtained.[123,124] They retained potent cytotoxicity, and monothioamides at Tyr-3 residue showed 2 – 4 fold more potent activity than the parent analogs (Table XXII). The NMR study revealed that these thionated analogs showed very similar conformational properties to the parent peptides in solution. Nickel borohydride reduction of thioamide **270** gave desoxopeptide **279** and its borane complex **280**. Analog **279** showed very different conformational properties around 18-membered ring moiety to peptide **153** in both crystal and solution form, which may explain its loss in activity. Although complex **280** gradually decomposed to compound **279** under the assay conditions, the borane complex **280**, although possessing a similar conformation to peptide **153**, showed only weak activity. These results emphasize the importance of the 18-membered ring structure for full expression of biological activity.

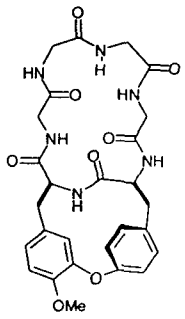
The importance of the 18-membered ring moiety for the activity is also verified by synthesizing the simplified analogs **281** and **282**, which showed very weak (L1210, IC_{50} = 2 μg/mL for **281**) or no (>10 μg/mL for **282**) activity.[125] Although backbone modified analogs which differ markedly in conformation from the major conformer of peptide **153** generally loose activity, the analog **283** possessing an 19-membered ring structure showed good activity both in vitro (P388, IC_{50} = 0.019 μg/mL) and *in vivo* (Table XXIII).[126]

Table XXII RA Thioamide Analogs and Their Cytotoxicity against P388 Cells

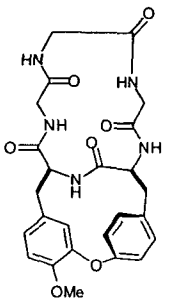
no.	R^1	R^2	R^3	W	X	Y	Z	IC_{50} (μg/mL)
153	Me	H	H	O	O	O	O	0.0013
270	Me	H	H	O	O	S	O	0.00058
271	Me	H	H	O	O	S	S	0.0017
272	Me	H	H	O	O	O	S	0.0026
273	Me	H	H	S	O	S	O	0.0044
274	Me	H	H	O	S	S	O	0.0013
151	H	H	H	O	O	O	O	0.0027
275	H	H	H	O	O	S	O	0.00088
209	Me	CH_2CO_2Me	H	O	O	O	O	0.034
276	Me	CH_2CO_2Me	H	O	O	S	O	0.0041
238	Me	H	allyl	O	O	O	O	0.015
277	Me	H	allyl	O	O	S	O	0.0038
239	Me	H	crotyl	O	O	O	O	0.0076
278	Me	H	crotyl	O	O	S	O	0.0032
279	Me	H	H	O	O	H_2	O	>10
280[a]	Me	H	H	O	O	H_2	O	1.0

[a] BH_3 complex at Ala-4 NH.





281



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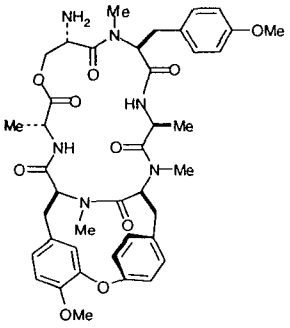


283

Table XXIII Antitumor Activity of RA-VII Analogs against P388 Leukemia in Mice

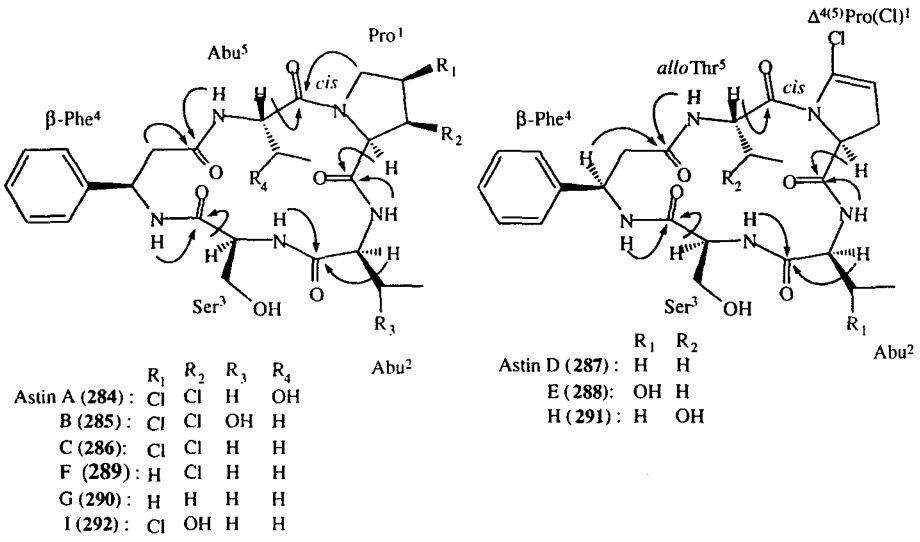
	T/C (%)						
# / Dose[a]	0.4	0.8	1.6	3.13	6.25	12.5	25.0
153 (RA-VII)	144	144	152	163	toxic		
239	112		127		155		
240	113		125		155		
242	123		132		160	174	
243	119		131		148		164
251	111		117		128	141	
254	105		105		129	143	
262			111		118	136	
264	105		119		137	133	
268			111		127	130	
283		133		158		176	170

[a] Dose in mg/kg given i.p. on days 1–5.

B. Astins from *Aster tataricus*

1. Structures of Astins

The first member of the astin family of cyclic pentapeptides containing a dichlorinated proline residue was isolated in 1993 from the biologically active extracts of the roots of *Aster tataricus* (Compositae).[127,128]. *Aster tataricus* is known as a Chinese medicine containing several terpenoids and saponins, and is also popular as a garden flower.[129] The *n*-butanol extract of this plant showed potent antitumor activity. Chromatographic purification guided by antitumor activity led to the isolation of three new cyclic pentapeptides; astins A - C (**284 - 286**).[128] Their structures, containing a novel dichlorinated proline residue, were determined by 2D NMR and FAB MS spectroscopy, as well as by degradation, followed by HPLC analysis using Marfey's method (Fig. 34).[130] Astin A is constructed of L-*allo*-threonine, L-Serine (Ser), β-phenyl alanine (β-Phe), L-α-aminobutyric acid (Abu), and L-β,γ-dichlorinated proline (Pro(Cl_2)) residues. *Allo*-threonines, though rare in nature, are found as constituents of biologically active peptide.[131,132] Astin A was sequenced based on HMBC correlations and The MS fragmentation pattern.[128, 133]



	R_1	R_2	R_3	R_4
Astin A (**284**):	Cl	Cl	H	OH
B (**285**):	Cl	Cl	OH	H
C (**286**):	Cl	Cl	H	H
F (**289**):	H	Cl	H	H
G (**290**):	H	H	H	H
I (**292**):	Cl	OH	H	H

	R_1	R_2
Astin D (**287**):	H	H
E (**288**):	OH	H
H (**291**):	H	OH

Fig. 34 Structures of astins A - I (**284-292**), and some important HMBC correlations; Pro was provisionally numbered as a first amino acid. Arrows show HMBC correlations.

Later, cyclic astins D - I (**287 - 292**) were isolated as minor constituents and subsequently characterized using degradation and NMR experiments.[134-136] Cyclic astins possess several different types of unique chlorinated proline residues. The β, γ-dichlorinated proline is contained in astins A, B, and C, a $\Delta^{4(5)}$, δ-chlorinated proline in astins D, E, and H, a β-chlorinated proline in astin F, and a β-hydroxy-γ-chlorinated proline in astin I. All of the chlorine and hydroxy group configurations in the above proline residues were elucidated to be β-orientated by NOEs, ^1H coupling and HMBC correlations, as shown in Fig. 35.

Furthermore, an acyclic astin, astin J (**293**) has been isolated.[137] D. Cheng *et al.* reported this kind of acyclic peptide, asterinins A - C, from the same plant.[138] Base-catalyzed cleavage of astins A, B, and C with a β, γ-dichlorinated proline resulted in acyclic peptides with pyrrole rings, which were considered to be produced by dechlorination and aromatization from Pro(Cl_2) to pyrrole under basic conditions, following the cleavage of the amide bond in Pro.

Acyclic astin J was also produced from cyclic astin C by hepatic microsomal biotransformation in rats (Fig. 36).[139]

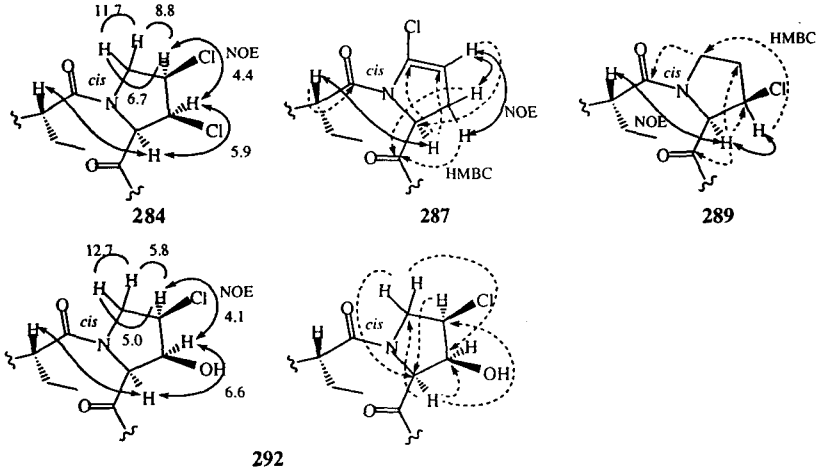


Fig. 35 NOE, HMBC and coupling correlations around Pro residues of **284**, **287**, **289** and **292**; Arrows show NOEs, dashed arrows show HMBC, and numbers show coupling constants.

Currently, synthetic studies of all astins are in progress,[140,141] however, total synthesis of astins A - C with the *cis* β, γ-dichlorinated proline has yet to be achieved.

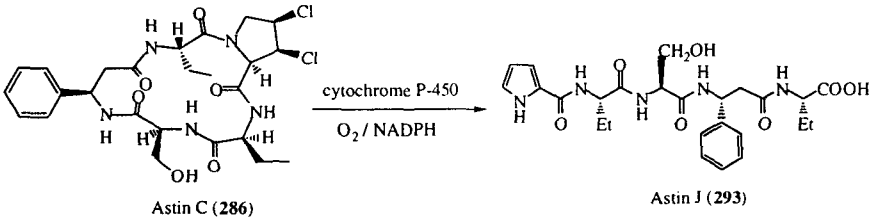


Fig. 36 Hepatic microsomal biotransformation from astin C (**286**) to astin J (**293**)

2. Conformational Analysis

A crystal structure of astin B (**285**) has been reported (Fig. 37).[133] The solid state conformation exhibits one *cis* peptide bond between Abu^5 and Pro^1. This structural feature is the main difference between the solid state structures of the astins, cyclochlorotine[142] and islanditoxin.[143] The latter two compounds are toxic metabolites of yellow rice mold, *Penicillium islandicum* Sopp., whose occurrence on a variety of foodstuffs constitutes a human health hazard. In cyclochlorotine, only the astin Abu^5 residue is replaced with serine, yet the molecule adopts a stable type I β-turn conformation with a *trans* proline amide bond and a transannular hydrogen bond.[142]

Solution conformational analysis of astin B in DMSO-d_6 based on 2D-NMR techniques, temperature effects on NH protons, rate of hydrogen-deuterium exchange, vicinal NH-CαH coupling constants, and NOE experiments has been reported (Fig. 38).[144] Calculations of molecular mechanics and restrained molecular dynamics were applied to determine the energetic preferences of various conformations of astin B. Distances involving the three intramolecular hydrogen bonds and NOE correlations were used for the refinements using the AMBER program. These results indicated that the conformation in solution was, on the whole, homologous to that observed in the solid state. Astin B, with a *cis* configuration in a proline amide bond, differed from cyclochlorotine isolated from *Penicillium islandicum*, which showed an all *trans* amide configuration. Furthermore, NMR and molecular dynamics studies suggested that astin B took a different backbone conformation from those of astins A and C.[145]

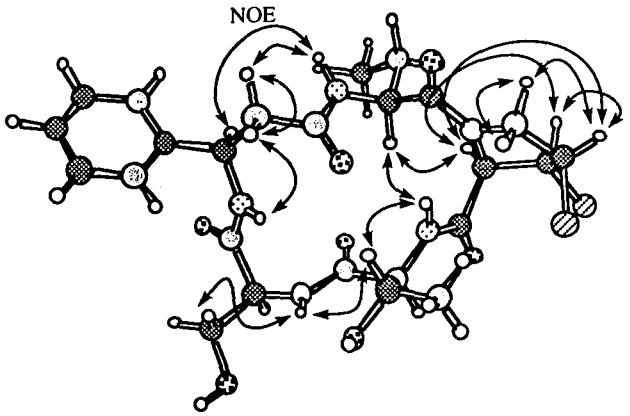


Fig. 38 NOE enhancements of astin B (**285**); The arrows show the NOE relationships confirmed by a phase sensitive NOESY experiment in DMSO-d_6 at 303K.

3. Biological Activities

Astins A - C, containing chlorines and an *allo* Thr and characterized by one *cis* peptide bond, showed antitumor activity, as determined by the total packed cell volume method using Sarcoma 180 ascites in mice.[146] The effectiveness of this activity was evaluated in terms of the tumor growth ratio (GR (%) = (test group packed cell volume / control group packed cell volume) x 100). The GR values of astins A, B and C were 40% (++) at a dose of 0.5 mg/kg/day, 26% (++) at 0.5 mg/kg/day and 45% (+) at 5 mg/kg/day, respectively, given for 5 consecutive days.[128, 133] The effective doses of astins A and B were ten-fold stronger than that of astin C. Astins D - J did not inhibit tumor growth at 10.0 mg/kg/day.[139] Various congeners without dichlorinated proline residues, prepared from astins A - C by chemical conversion and a hepatic microsomal biotransformation in rats, did not show antitumor activities either, suggesting that the 1,2-*cis*-dichlorinated proline residues play an important role in the antitumor activity of astins. Itokawa *et al.* suggested that astins A and C, with weaker activity than astin B, have different backbone conformations to astin B, and that these backbone conformations affect antitumor activity.[145] However, the presence of *cis* dichlorinated proline residues was

concluded to be a more important structural motif for astins to show antitumor activity on S-180A.

The backbone conformational difference between astin B and astin C[145] was maintained by backbone modification using Lawesson's reagents. The produced thionated derivatives, [Ser-3-ψ(CS-NH)-β-Phe-4]astin A (thioastin A) , [Ser-3-ψ(CS-NH)-β-Phe-4]astin B (thioastin B) and [Ser-3-ψ(CS-NH)-β-Phe-4]astin C (thioastin C), showed more promising antitumor activity than the corresponding astins.[147,148]

Though there are few differences between the peptide sequences of the astins and those of cyclochlorotine and islanditoxin, astins exhibited antitumor activity and only hepatotoxicity was shown by cyclochlorotine and islanditoxin.[116] The fact that minor structural changes cause such a noticeable change in biological activity in both the astins and the toxins is of interest.

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