Structure – Activity Relationships of the Compounds from Plant Resources

March, 2007

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Part1 Introduction

1−1 Background

Cancer is a major cause of human's death in advanced country with heart disease and cerebrovascular disease, and its extermination is a worldwide problem. Cancer is one of lifestyle diseases that appear to increase in frequency as countries become more industrialized and people live longer.

The treatment method of cancer includes surgical treatment, radiotherapy, and chemotherapy. In them, the chemotherapy of cancer has advanced remarkably, after the antimetabolite drug, methotrexate (MTX) was developed in 1950's.[1] MTX competitively inhibits the metabolism of folic acid and it inhibits the synthesis of DNA as a result.[2]

Many of anti-cancer agents are derivatives of natural products isolated from culture medium of microorganisms or plant resources.

Plants are the primary sources of medicines since ancient times and continue to provide mankind with new remedies. Some of the known anti-cancer agents from plants are shown in Figure 1-1.

The first plant alkaloid used as anti-cancer agent was vinca alkaloid including vincristine and vinblastine which were isolate from *Catharanthus roseus.*[3] The vinca alkaloids binds to tubulin dimmers causing disassembly of microtubule structures. Disruption of the microtubules arrests mitosis in metaphase.

Camptothecin[4] is a plant secondary metabolite from *Camptotheca acuminate.* Camptothecin affects the activity of the enzyme topoisomerase I, whose normal action is to cleave, unwind, and relegate DNA. When camptothecin binds to topoisomerase I, it will be able to cleave but not to religate DNA. Thereby, camptothecin causes single strand breaks in DNA. Irinotecan is camptothecin derivative.

Etoposide is an inhibitor of the enzyme topoisomerase II.[5] It derives from podophyllotoxin, a toxin found in the American Mayapple.

Paclitaxel (Taxol®) was isolated from the bark of Pacific yew tree, *Taxus brevifolia.*[6] Paclitaxel binds to the β subunit of tubulin. The resulting microtubule/paclitaxel complex does not have the ability to disassemble. This hyper-stabilization of the microtubule arrests mitosis in the different manner of vinca alkaloid. Paclitaxel has become an effective for lung, ovarian, breast cancer, and

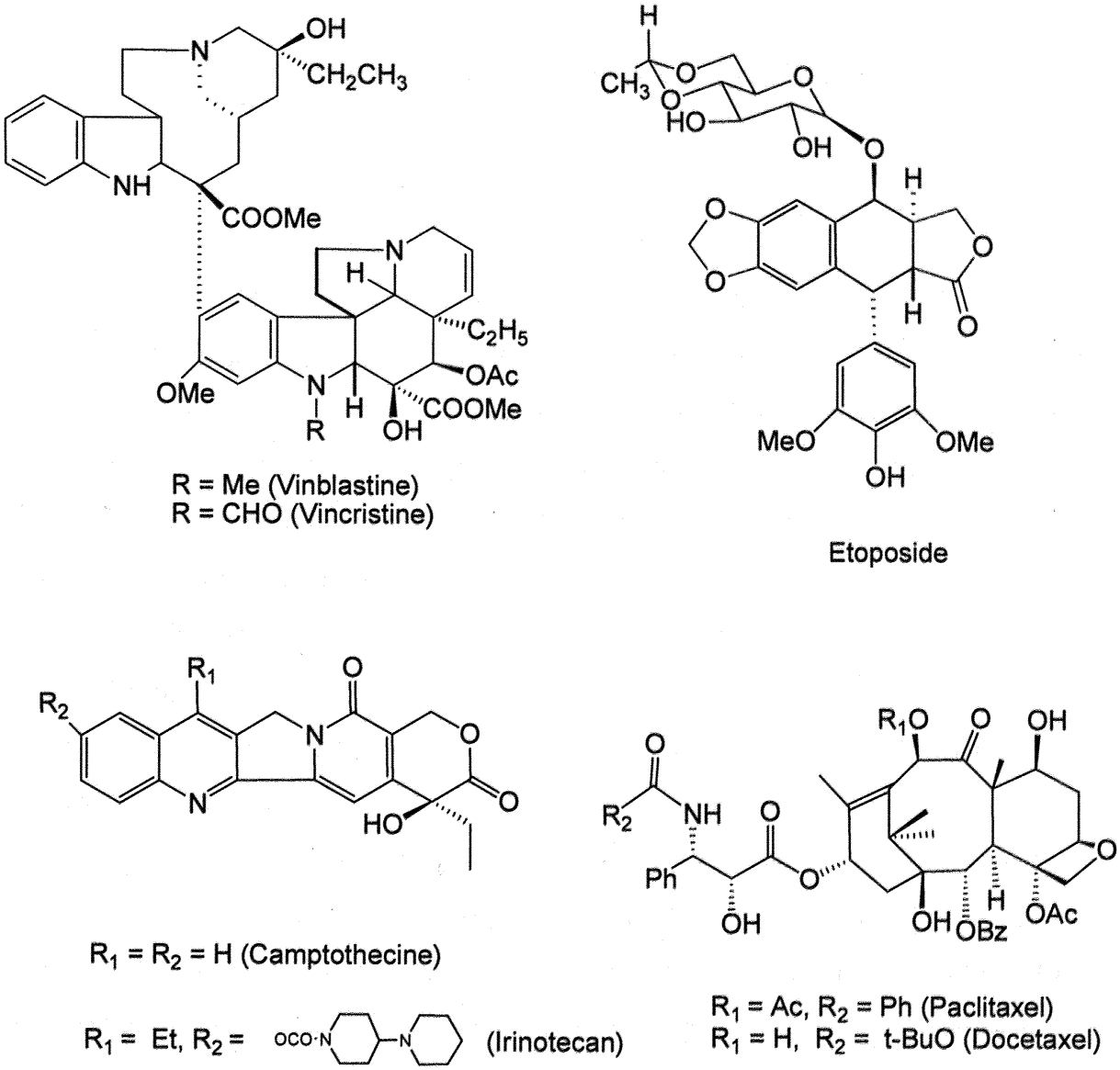


Figure 1-1. Anti-cancer agents from plant resourses

advanced forms of Kaposi's sarcoma.

Though many kinds of anti-cancer drug have been developed, cancer is still a major cause of death.

One of the problems in cancer chemotherapy is intrinsic or primary drug resistance. There are only a few drugs that are effective in the treatment, although nearly 50 antineoplastic drugs are in use. Especially, broad-spectrum resistance to the structurally diverse drugs named multi-drug resistance (MDR) is a serious obstacle in cancer chemotherapy.

Recently, some of the mechanisms of drug resistance in cancer cells have been elucidated. Those mechanisms involve inactivation of drug, alteration in drug target, increased DNA repair and inhibition of drug-induced apoptosis and decreased intracellular drug accumulation. Among the different mechanisms involved, MDR phenotype is associated with decreasing intracellular drug accumulation caused by over expression of MDR proteins such as p-glycoprotein (P-gp) and multidrug resistance proteins (MRP).[7)]

The P-gp mediated MDR is the first discovered MDR[8)] and it is most widely observed mechanism in clinical MDR.

As shown in Figure 1–2(a), P-glycoprotein is a transmembrane protein which is 1280 amino acids long and consists of two homologous halves of 610 amino acid joined by flexible linker region of 60 amino acids.[9)] Each half has an N-terminal hydrophobic domain containing six transmembrene domains followed by a hydrophilic domain containing a nucleotide binding site.

The site(s) in MDR transporters interact with drug-substrates are probably encoded in the transmemblene domains. Detailed mutagenesis studies of P-gp and photochemical labeling with reactive drug- derivatives revealed that transmembrane helices 5 and 6, helices 11 and 12, as well as the short cytoplasmic loops connecting these helices, are involved in the formation of an extended drug-binding sites.[10)]

When viewed from above the plasma membrane, P-gp is donut shaped 6-fold symmetry, a diameter of about 10 nm and a large central pore of about 5 nm in diameter. It has a thickness in the plane of the plasma membrane of about 8 nm (Figure 1–2 (b)).[11)]

P-gp extrudes the drugs utilizing the energy by hydrolysis of ATP.[12)] It was initially hypothesized that P-gp forms a hydrophilic pathway and drugs that are transported from the cytoplasm to the extracellular medium through the central pore.

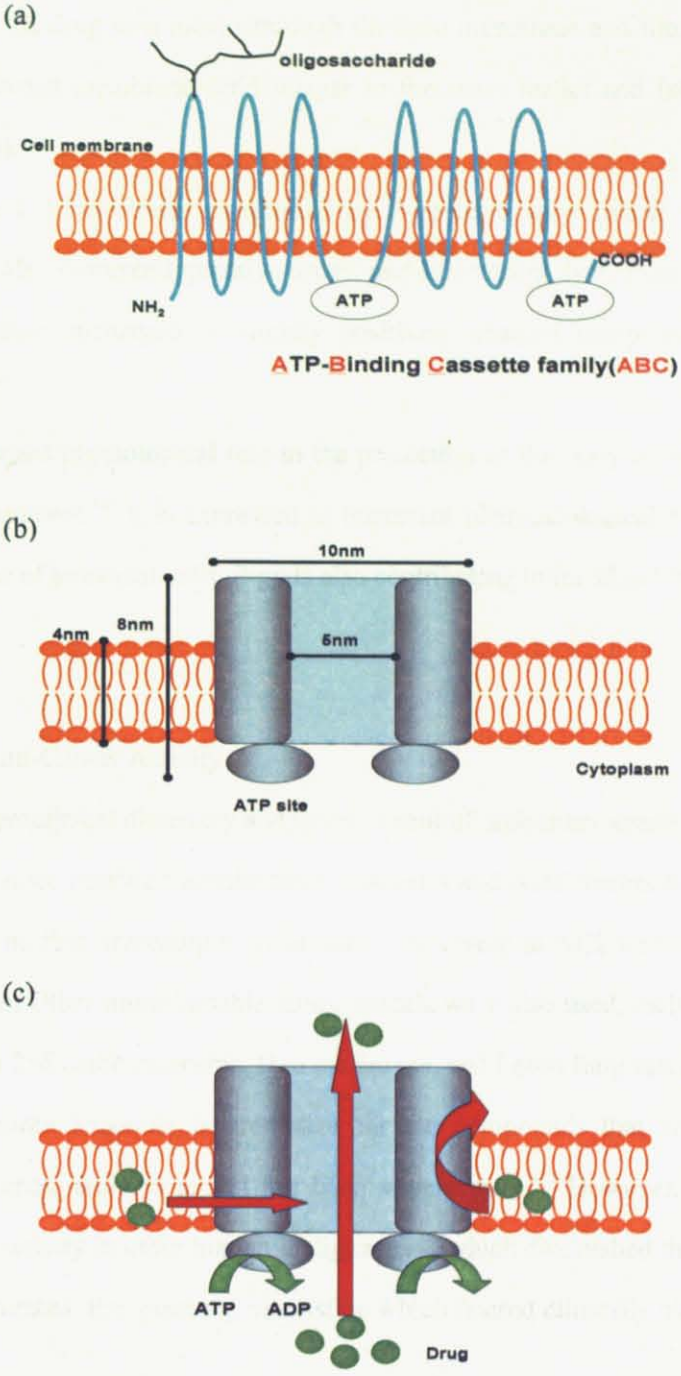


Figure 1-2 Schematic illustration of P-glycoprotein

(a) Polypeptide chain connected with oligosaccharide

(b) Diameter, pore size and thickness

(c) Efflux model of the drugs

It has been further proposed that P-gp acts like a hydrophobic vacuum cleaner or flippase. In this model P-gp intercepts the drug as it moves through the lipid membrane and flips the drug from the inner leaflet of the plasma membrane lipid bilayer to the outer leaflet and into the extracellular medium (Figure 1-2(c)).[13)]

As shown in Table 1–1, substrates transported by P-gp have very diverse structure including anticancer drugs, steroids, immunosuppressive drugs, and fluorescent dyes. P-gp is a transporter for large hydrophobic, either uncharged or slightly positively charged compounds which may be 200–1800 Da in size.[14)]

P-gp plays an important physiological role in the protection of the body against toxic chemicals occurring in the environment.[15)] It is expressed in important pharmacological barriers, such as the brush border membrane of intestinal cells. P-gp is also contributing to the blood-brain barrier.[16)]

1–2 Biological Assay

A) Evaluation of the Anti-Cancer Activity

Early strategies for preclinical discovery and development of anticancer agents were largely based upon testing agents in mice bearing transplantable leukemias and solid tumors derived from murine and human sources. The first screening models used extensively at NCI were *in vivo* L1210 and P388 murine leukemias. Other transplantable tumor models were also used, including sarcoma 180, carcinoma 755, Walker 256 carcinosarcoma, B16 melanoma, and Lewis lung carcinoma.

In them, L1210 muirne leukemia is predictive for the compounds that are effective against leukemias and lymphomas, and the model has been widely used.[17)] However, this model is less effective at predicting activity in other human malignancies, which diminished the rate of success in the treatment of solid tumors. For example, vincristine which is used clinically did not be selected in this model.

In 1975, NCI established the screening model using the P388 murine model as a primary screen to select candidates for screening in the tumor panel. However, the researchers were unable to correlate compound activity against tumors in animal models to clinical activity based on tumor histology. In 1985, NCI began the development of an *in vitro* cell-line-based screen representing the major classes of solid tumors that would permit the testing of compounds against a broad panel of human tumors.

Table 1-1 Characterization of substrates of P-glycoprotein

Cancer drugs	Doxorubicin Daunorubicin Vinblastine Vincristine Actinomysin D Paclitaxel Teniposide Etoposide
Steroids	Aldosterone Hydrocortisone Cortisol Corticosterone Dexamethasone
Immunosupressive drugs	Cyclosporin A FK506
Fluorescent dye	Rhodamine-123 Calcein AM

- Large hydrophobic, either uncharged or slightly positively charged compounds
- 200—1800 Da in size

The original hypothesis was that this screen would be able to identify compounds with activity against cancers of a particular tissue and that human tumor cell lines would be better predictors than mouse tumors for compound activity against solid tumors in humans.

Since 1990, NCI has used the human tumor cell line *in vitro* screen as its primary assay. The screen is currently composed of 60 human tumor cell lines, representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney.

The pattern of response of the cell lines as a group can be used to rank a compound according to the likelihood of sharing common mechanisms. The COMPARE algorithm qualifies this pattern and searches an inventory of screened agents to compile a list of the compounds that have the most similar patterns of cellular sensitivity and resistance.[18)]

In Japan, Japanese Foundation for Cancer Research (JFCR) established a panel of new 39 cell lines of various human cancers and developed a database of their chemosensitivities.[19)] Drugs were profiled in terms of their "fingerprints", patterns of differential activity against the cell lines. There was a significant correlation between a drug's fingerprint and its mode of action, as observed in the NCI panel of 60 cell lines. Therefore their cell-line panel is a powerful tool to predict the modes of action of new compounds.

Though the above-mentioned screening systems are useful tool to search the mode of action of the compound, it takes large time and labor. This work aimed to elucidate the structure-activity relationships (SAR) of the numerous compounds.

The author established the new convenient screening system to be suitable for this purpose. For evaluation of the strength of cell growth inhibition activity, three kinds of the cell-lines were used. Cytotoxicities of the compounds were investigated by using normal human fibroblasts cell line (WI-38), malignant tumor cell line (VA-13) induced from WI-38 by infection with SV-40 virus, and human liver tumor cell line (HepG2). When a compound showed weaker cytotoxicity against WI-38 than that against VA-13, it is expected to be a desirable lead compound for anti-cancer agent which is free of side effect. Cell line HepG2 was induced from human liver tumor, and normally liver is the organs playing roll of detoxication against the toxic substances by expressing some kinds of the efflux transporter protein such as MRP2. It is also reported that the sensitivity of HepG2 cells to the anti-cancer agent, paclitaxel was increased by co-administration of known MDR modulator verapamil.[20)] That is HepG2 cells have the feature of drug resistance to paclitaxel.

From this point of view, when the cytotoxicity of the compound against HepG2 is efficiency comparing with paclitaxel, it might be expected that the compound has MDR reversal activity.

B) Evaluation of the MDR reversal activity

The reliable detection and quantitative determination of the clinically relevant levels of the MDR proteins would be extremely important in the clinical treatment of various cancerous diseases. Combination chemotherapy treatment protocols could be adjusted and drug-resistance reversing agent could be applied accordingly.

The calcein assay was adopted as convenient primary screening of the MDR modulator.[21-23] This assay uses the fluorogenin dye, calcein acetoxymethyl ester (calcein AM) as a substrate for efflux activity of p-gp. Calcein AM is a nonfluorescent, highly lipid soluble dye that can rapidly penetrate the plasm membrane of the cell. Ester bond are cleaved by endogenous esterase, formed calcein AM into intensely fluorescent calcein. MDR cells expressing high levels of p-gp extrude calcein from the plasma membrane as illustrated in Figure 1-3. Inhibition rate of p-gp activity can quantify by measurement of the increase in intracellular calcein fluorescence.

1–3 Purpose of this work

Overcoming MDR in clinical is sure to become an improvement of treatment for a lot of cancer patients. Pharmacological modulation seems to be the first choice at present.

MDR modifying agents, which inhibit the function of the MDR proteins are good candidates for such a pharmacological modulation. These compounds are expected to increase the cytotoxic action of MDR-related drugs by preventing the extrusion of anti-cancer drugs from target cells. Co-application of a non-toxic MDR-modulating compound with combination chemotherapy may significantly improve the cancer cure rate.

As summarized in Table 1-2, the first generation consisted of drugs that were already in clinical use. Calcium channel blockers, quinine derivatives, calmodulin inhibitors and the immunosupressive agent were all shown to interact with the MDR transporters in vitro and *in vivo*.

The second generation modulators consisted of derivatives of the first generation compounds, which have less pronounced effect on their original target, but retained their modulatory effects. The third generation modulators are molecules sepecifically devised to interact with specific MDR transporters such as reversin 121 and 205 (Peptide chemosensitizers), MS-209 (qunoline) or

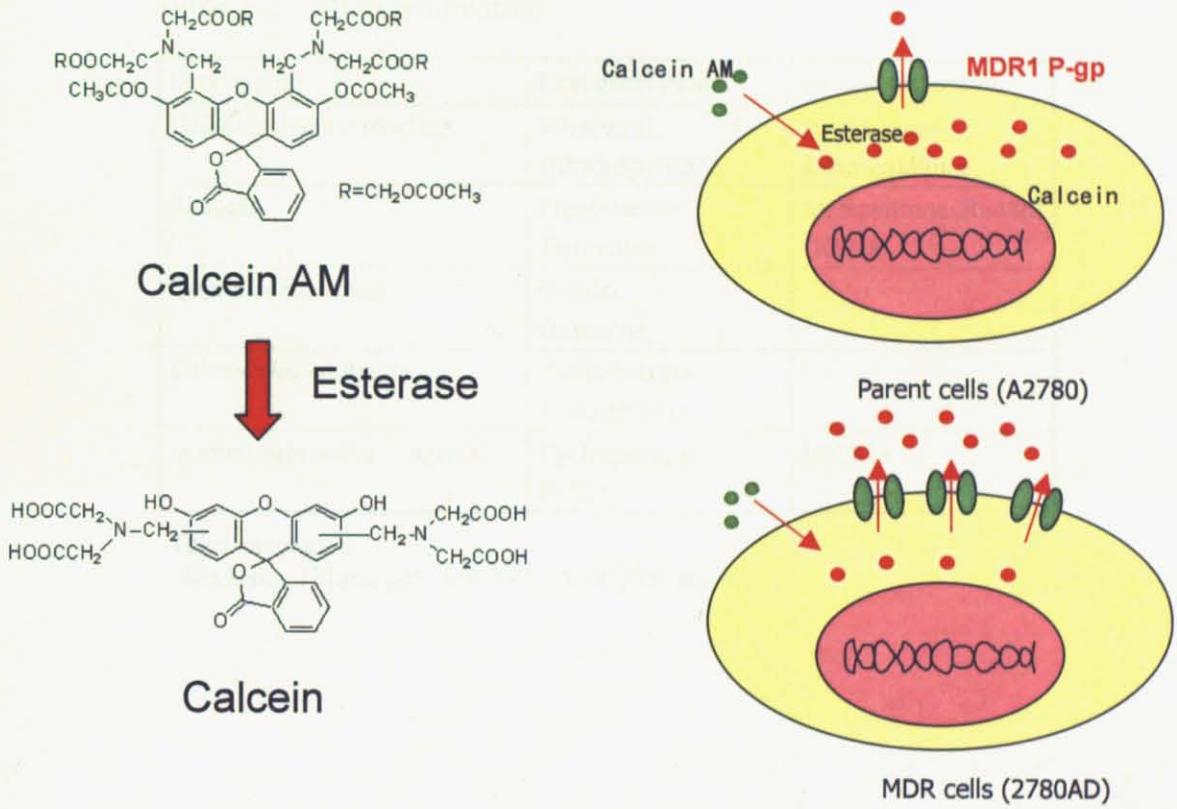


Figure 1–3 Principle of the calcein assay

Table 1-2 MDR modulators

Drug types	First generation	Second generation
Calcium channel blockers	Verapamil Dihydropyridine	R-verapamil Dexniguldipine
Steroids	Progesterone Tamoxifen	Mefepristone(Ru486) GG918
Quinine derivatives	Quinine Quinidine	
Calmodulin inhibitors	Phenothiazine Thioxanthene	
immunosupressive agents	Cyclosporin A FK506	PSC833

Third generation
Reversins 121and 205, MS-209, LY335979, etc.

LY335979 (cyclopropyldibenzosuberane). From the above-mentioned viewpoint, this study aimed to develop the potential and unique MDR reversal agents with or without anti-cancer activity. The former agents with anti-cancer activity expected to be lead compounds for new type of anti-cancer agents and the latter agents without anti-cancer activity expected to be lead compounds for MDR modulators used with anti-cancer agents.

In this report, the author investigated anti-cancer activity and MDR reversal activity of variety of the compound isolated from the plant resources and their derivatives. Further more the structure-activity relation ships of those compounds were described, and the important factors for the expression of those activities were elucidated.

In part2 and part 3, the author investigated the structure-activity relationships of some taxoid as MDR modulator and anti-cancer agent. Paclitaxel (Taxol®) is the most famous taxoid as potent anti-cancer agent. Since the discovery of paclitaxel as an important anticancer agent, a number of bioactive paclitaxel derivatives have been isolated from the plants and the cell cultures of *Taxus* species and prepared by chemical syntheses. In part 2, the author focused some baccatin III analogues derived from taxinine, a major compornent of *T. cuspidate* (0.1% from fresh needles and stems).[24)]

Interestingly, some non-taxol-type taxoids have been reported to possess significant MDR reversal activity. Taxuyunnanine C is one of the major C-14 oxygenated non-taxol-type toxoid produced by cell culture of *Taxus* spp. in high yields (ca. 5–10% of the dry weight). The structure-activity relationships of taxyunnanine C and its analogues was investigated in part 3.[25)]

In part 4, the author reported the structure-activity relationships of some lignans as MDR modulator.

Lignans are a class of secondary plant metabolites produced by oxidative dimerization of two phenylpropanoid units. Althogh their molecular backbone consists only of two phenylpropan (C6-C3) units, lignans show an enormous structural diversity. Many lignans show physiological activity such as anticancer, antioxidant, antimicrobial, anti-inflammatory and immnosupressive activities[26-28)].

In this part, some structurally distinguishable groups of lignans are examined about their anti-cancer and MDR reversal activities. Those are tetrahydrofuran lignans, secolignans, dibenzylbutyrolactone, and dibenzylbutanediol lignans.[29-31)]

Part2 Structure-Activity Relationships of 4-Deacetylbaccatin III Analogues as Multidrug Resistance Modulator and Anti-Cancer Agent

2–1 Introduction

In cancer chemotherapy, occurrence of multidrug resistance (MDR) in cancer cells caused by repeated administration of anticancer agents is a serious problem. One mechanism of MDR is overexpression of the P-glycoprotein (P-gp), which is the efflux pump of anticancer drugs.[32,33] P-gp is a transporter for a wide range of reagents utilizing energy by hydrolysis of ATP. P-gp is expressed on the small intestine, capillary of brain, kidney, and liver. Its physiological role is considered as a defense mechanism against the toxic materials in the cell. When P-gp is expressed on the cell membrane of a cancer cell, it transports various kinds of anti-cancer agents from inside of the cell to the outside. Many taxane derivatives, which showed MDR reversal activity, have been reported.[34—42] In our laboratory, the isolation of MDR reversal agents from *T. caspidata,* [43—45] their production by callus culture, [46—50] and the synthesis of taxinine NN-1, the most efficient MDR reversal agents in natural taxane.[43), 51] have been reported.

Structure-activity relationship (SAR) studies of the functional groups of taxanes revealed that substitutions of the OH group at the C-7 position to hydrophobic side groups are effective for MDR reversal activity.[52]

Many taxane derivatives for SAR studies were synthesized from 10-deacetyl-baccatin III or 14β-hydroxybaccatin III with an acetoxy group at the C-4 position.[38, 39, 41, 42] It has been reported that deacetylation at the C-4 position result in the loss of cytotoxicity in pacritaxel.[53] From the viewpoint of MDR reversal agent, it is desirable that the compound has no cytotoxicity and has high MDR reversal activity at the time when it is used with an anticancer reagent toward MDR cancer cells.

2–2 Experimental Method

2–2–1 Chemistry

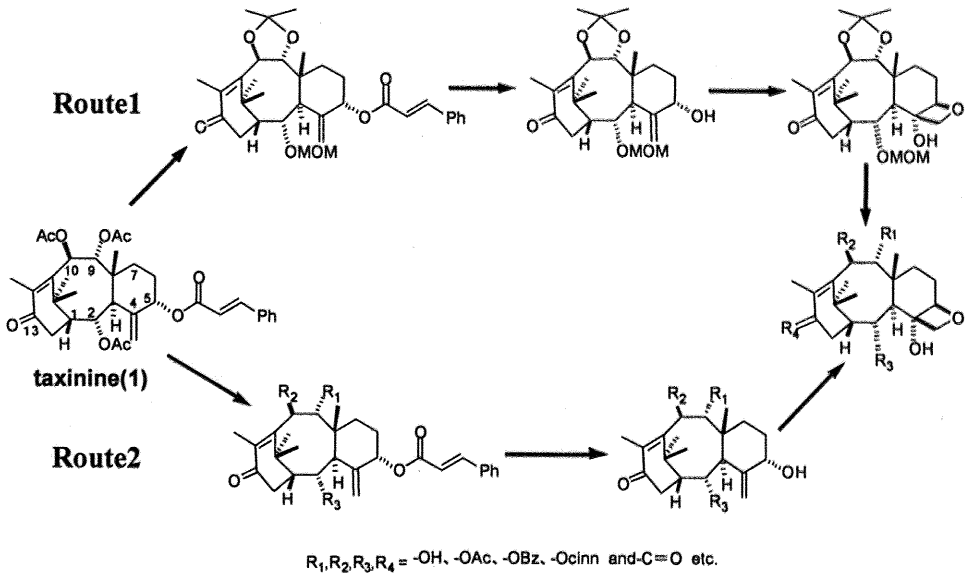
We synthesized some taxane derivatives with a 5β,20-epoxy ring (4,5-oxetane ring) and with hydroxyl group at the C-4 position from taxinine (**1**) along the route 1 and route 2 shown in Scheme 2-1.

Scheme2-2 and 2-3 show the details of the route1. After the hydrolysis of **1**, the 9, 10-dihydroxyl groups of the resulting **1a** were protected as acetonide and successive hydrolysis of the acetoxyl group at C-2 in the resulting **1b** gave the 2-hydroxy derivative **1c.** Further protection of **1c** with MOMCl in a mixture of DIEA and dichloroethane gave compound **1d.** Hydrolysis of **1d** in the mixture of 1,4-dioxane and MeOH gave the 5-hydroxyl derivative **1e**. Oxidation of double bond at C-4 with osmium tetraoxide gave the compound **1f**. After protection of generated primary hydroxyl group and introduction of leaving group into the secondary hydroxyl group, the formation of oxetane ring was achieved by cyclization reaction.

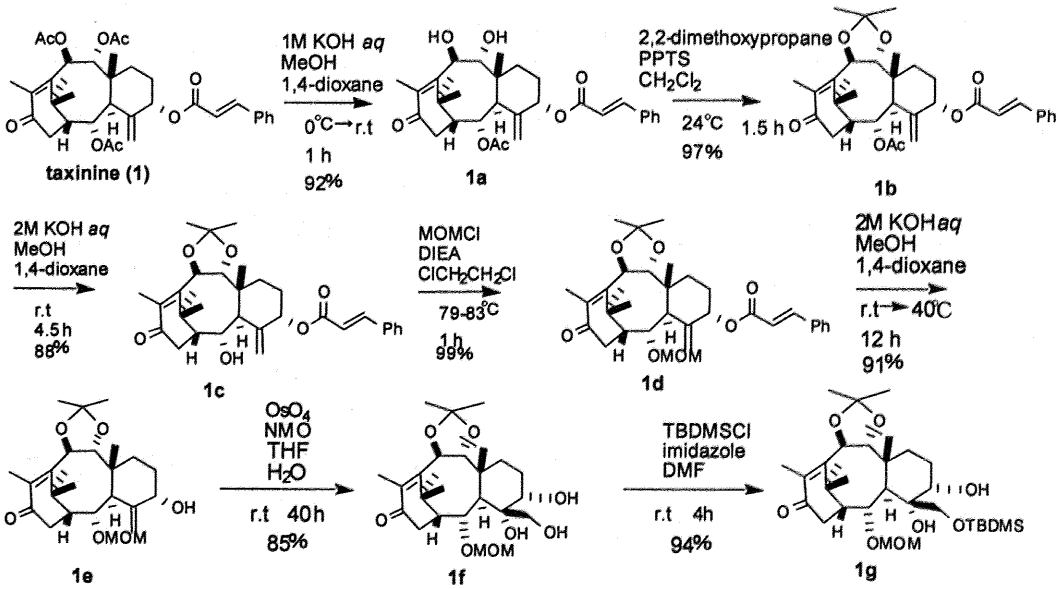
Scheme 2-4 to 2-8 show the details of the route2. After the hydrolysis of **1**, the 9, 10-dihydroxyl groups of the resulting **2a** and **2b** were protected as acetonide. Hydrolysis of resulting **2d** gave 2-hydroxy derivative **2c** and 2, 5-dihydroxy derivative **2e** (Scheme 2-4). Substitution of the hydroxyl group to benzoyloxy group at C-2 of **2c** gave the compound **2f** (Scheme 2-5). After ortho-esterification of acetoxy groups at C-9 and C-10 of **2f,** regioselective hydrolysis at C-9 or C-10 was achieved by changing the transposition conditions (Scheme 2-6, Table2-1). Regioselective hydrolysis at C-9 or C-10 of 2-acetoxy derivative **2k** was also achieved by changing the transposition conditions (Scheme 2-7, Table2-2). The hydroxyl group at C-9 in **2i** was oxidated by Jones reagent. Hydrolysis of resulting **2n** gave 5-hydroxy derivative **2o**. Oxidation of the double bond at C-4 with osmium tetraoxide gave the compound **2p**. After protection of the generated primary hydroxyl group and introduction of the leaving group into the secondary hydroxyl group, the formation of oxetane ring was achieved by cyclization reaction (Scheme 2-8).

The structures of the compounds employed to evaluate their biological activities are shown in Figure 2-1. They are thirteen kinds of the synthesized taxoids (**5**—**17**) with hydroxyl group at C-4 position and three kinds of known taxan derivatives bearing acetoxy group at C-4 position, Taxol (**2**), 10-deacetylbaccatin III (**3**), and baccatin III (**4**).

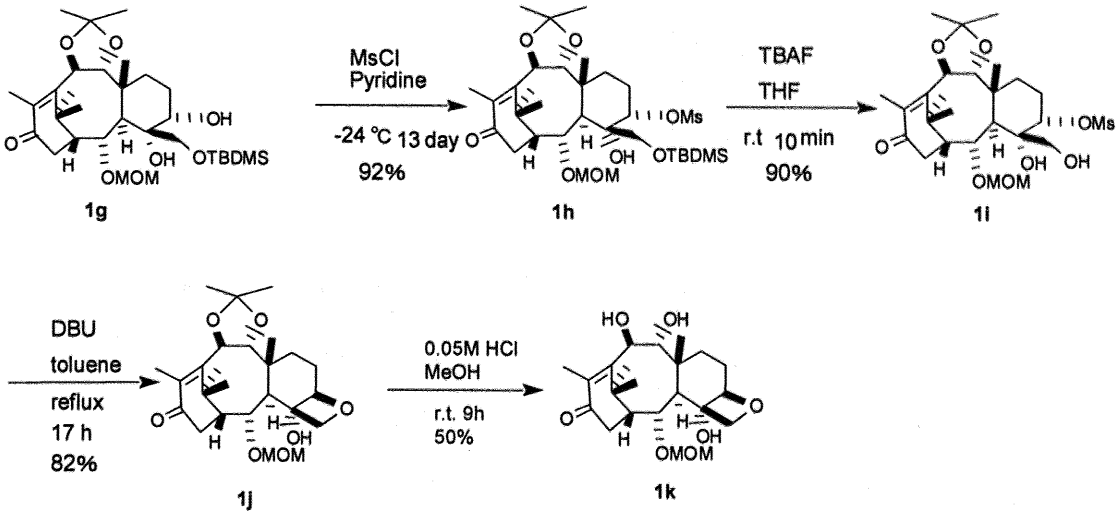
Scheme 2-1.



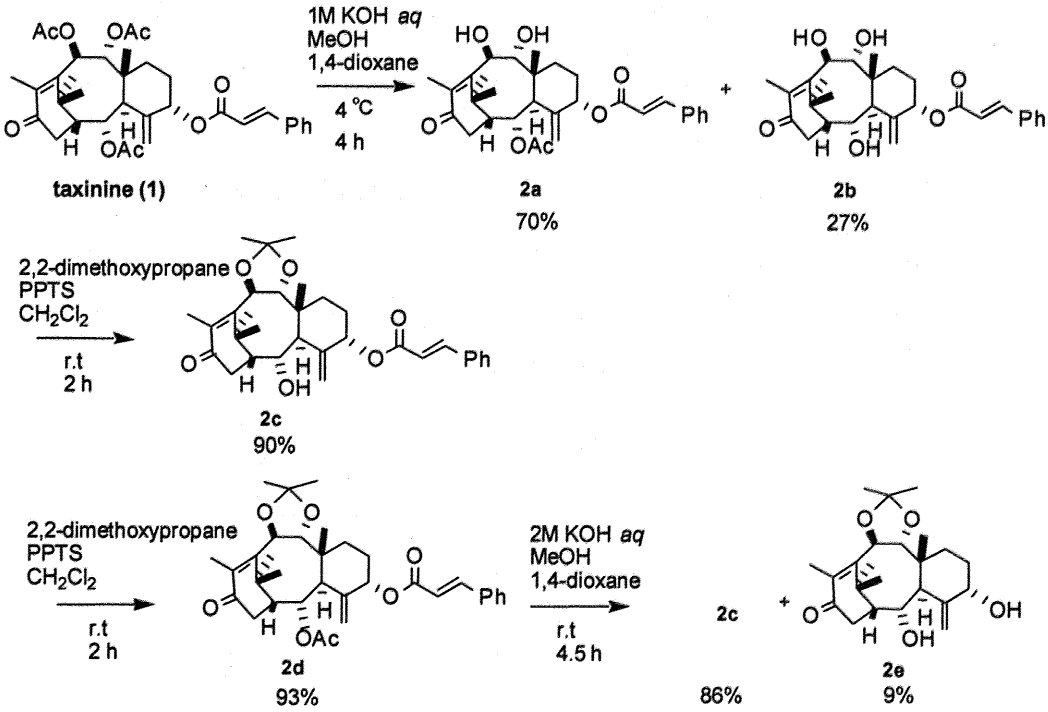
Scheme 2-2.



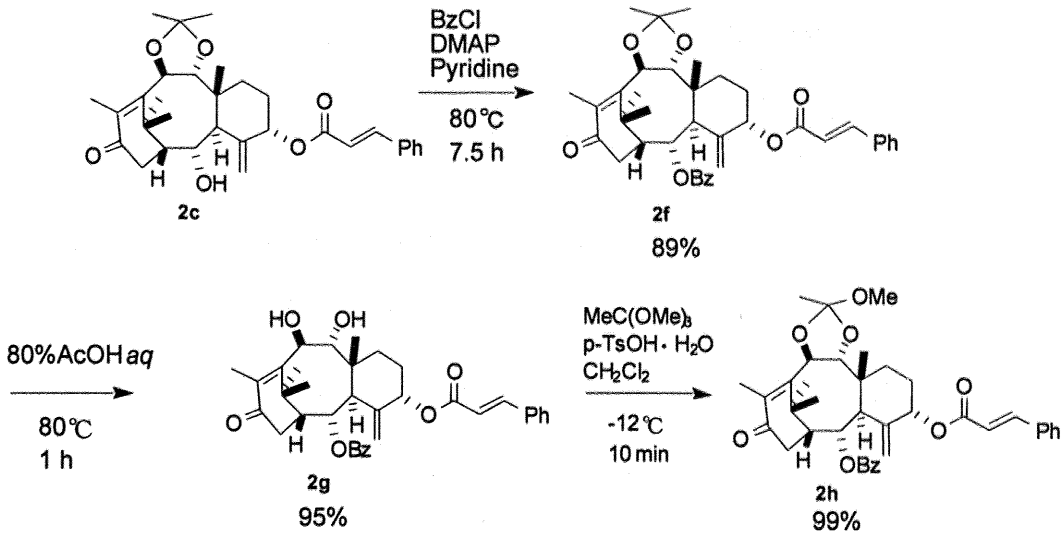
Scheme 2-3.



Scheme 2-4.



Scheme 2-5.



Scheme 2-6.

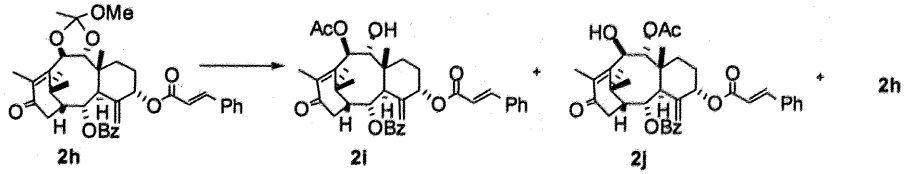
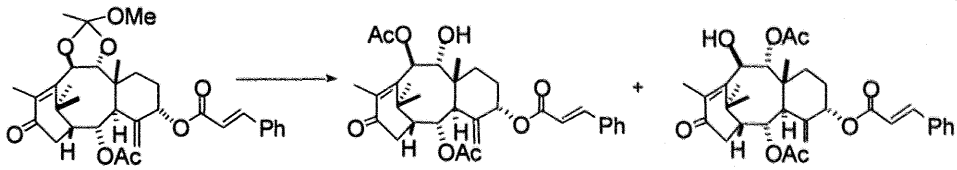


Table 2-1. Transposition conditions for the regioselective hydrolysis at C-9 or C-10

entry	reagents	conditions	yields (%)		
			2i	2j	2h
1	CH_2Cl_2 10% AcOH *aq* (AcOH 40eq)	-2 ℃ ~ rt, 5.5 h	-	-	98
2	CH_2Cl_2 p-TsOH.H_2O (0.2eq) H_2O (2eq)	-12 ℃, 65 min	21	74	-
3	CH_2Cl_2 CF_3COOH (0.2eq) H_2O (2eq)	-3 ℃, 40 min	28	70	-
4	Toluene 50% AcOH *aq* (AcOH 40 eq)	50 ℃ ~ 80 ℃, 4 h	40	53	-
5	Benezene 50% AcOH *aq* (AcOH 20 eq)	80 ℃ (reflux), 23 h	37	15	45
6	50% AcOH *aq* (0.05M)	100 ℃ (reflux), 50 min	60	37	-
7	Toluene 80% AcOH *aq* (AcOH 40 eq)	95 ℃, 9.5 h	71	19	-

Scheme 2-7.

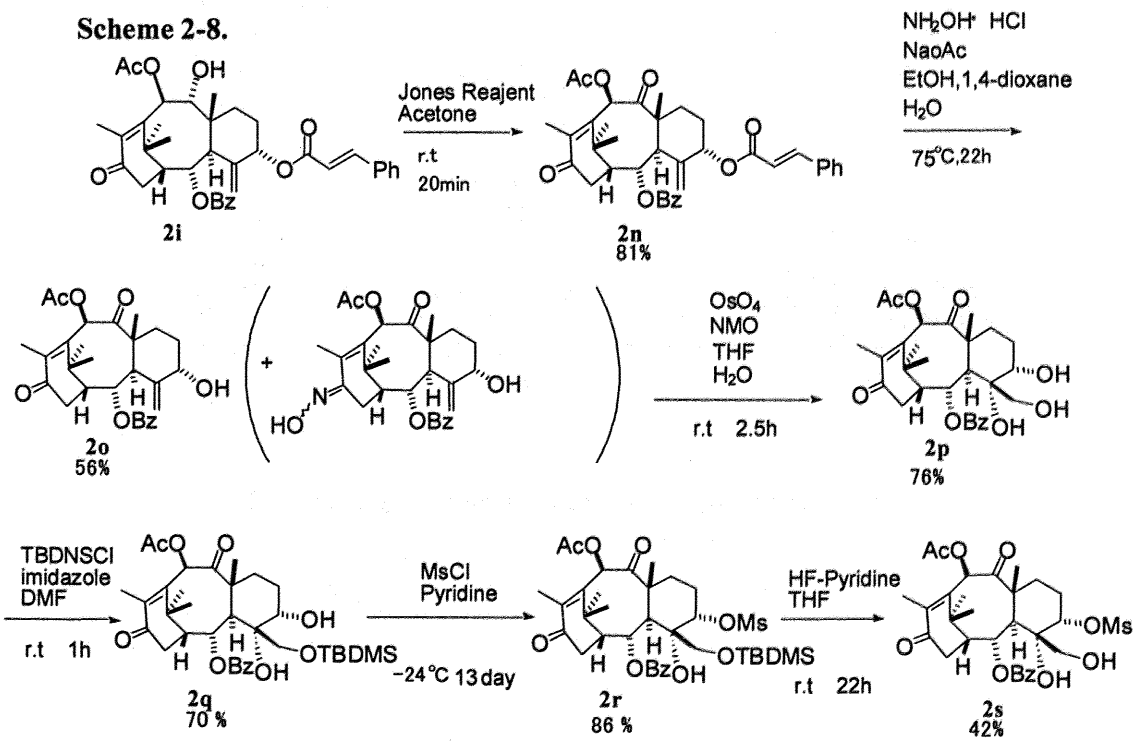


2k **2l** taxinine NN-3 **2m** taxinine NN-14

Table 2-2. Transposition conditions for the regioselective hydrolysis at C-9 or C-10

entry	reagents	conditions	yields (%)		
			2l	**2m**	**2k**
1	CH_2Cl_2 p-TsOH.H_2O (0.2eq) H_2O (2eq)	-12℃, 65 min	21	74	-
2	Toluene 80% AcOH *aq*(AcOH 40 eq)	95℃, 9.5 h	71	19	-

Scheme 2-8.



2i **2n** 81% **2o** 56% **2p** 76% **2q** 70 % **2r** 86 % **2s** 42%

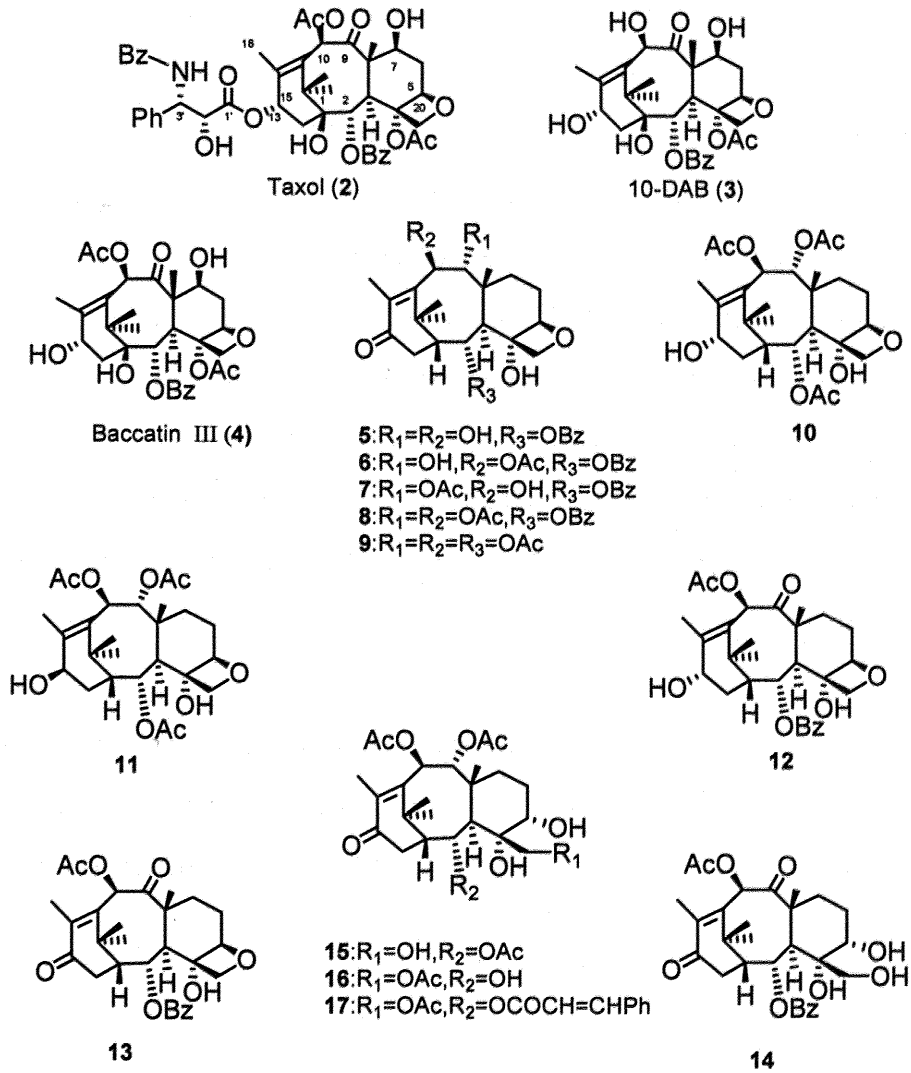


Figure 2-1. Structures for the comounds

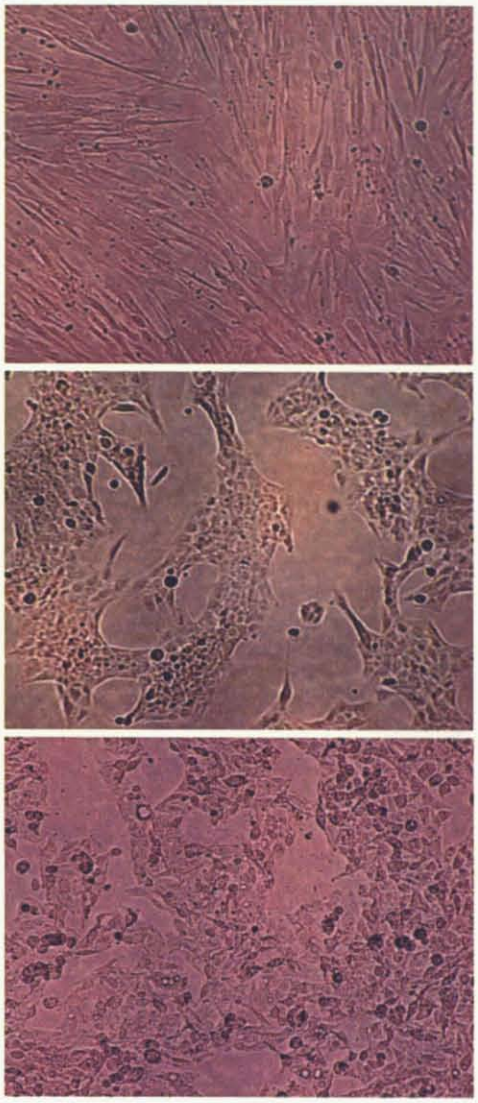
2–2–2 Cell Growth Inhibition

Normal human lung fibroblast cells WI-38、SV40-transformed WI-38 cells VA-13 or human liver tumor cells HepG2 are available from the Institute of Physical and Chemical Research(RIKEN), Tsukuba, Ibaraki, Japan. WI-38 cells were maintained in Eagle's MEM medium (Nissui Pharmaceutical Co., Japan). VA-13 cells were maintained in RITC 80-7 medium (Asahi Technoglass Co., Japan) . HepG2 cells were maintained in MEM medium (Invitrogen). All of media were supplemented with 10 %(v/v) fetal bovine serum (FBS) (FILTRON PTY LTD., Australia) together with kanamycin. and incubated at 37C° in a humidified atmosphere of 5% CO2. Figure 2-2 shows the photomicrographs of the three kinds of the cultivated cells for 72h.

100 μl of medium containing about 5,000 cells in 96 well was incubated in a humidified atmosphere of 5% CO2 for 24h. Then the test compounds dissolved in dimethyl sulfoxide (DMSO) were added to the well and continued further 48h at the same conditions. Coloration substance, WST-8[2-(2-methyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt] was added to the medium. The resulting formazan concentration was determined by the absorption at 450nm. Figure 2-3 shows the principle of the coloration reaction.

Figure 2-4 shows coloration of the culture medium in the 96-well microplate after the addition of coloration substance.

Cell viability (%) was calculated as [(experimental absorbance-background absorbance)/(control absorbance-background absorbance)]x100.The viability at different concentration of compounds was plotted and the concentration result in a 50% inhibition of growth was calculated as IC_{50}.



a) WI-38

Normal human fibroblast cells

b) VA-13

Malignant tumor cells induced malignant tumor cell induced from WI-38 by SV-40 virus

c) HepG2

Human liver tumor cells

Figure 2-2. Human cell lines for the evaluation of the cell growth inhibitory activities of the compounds.

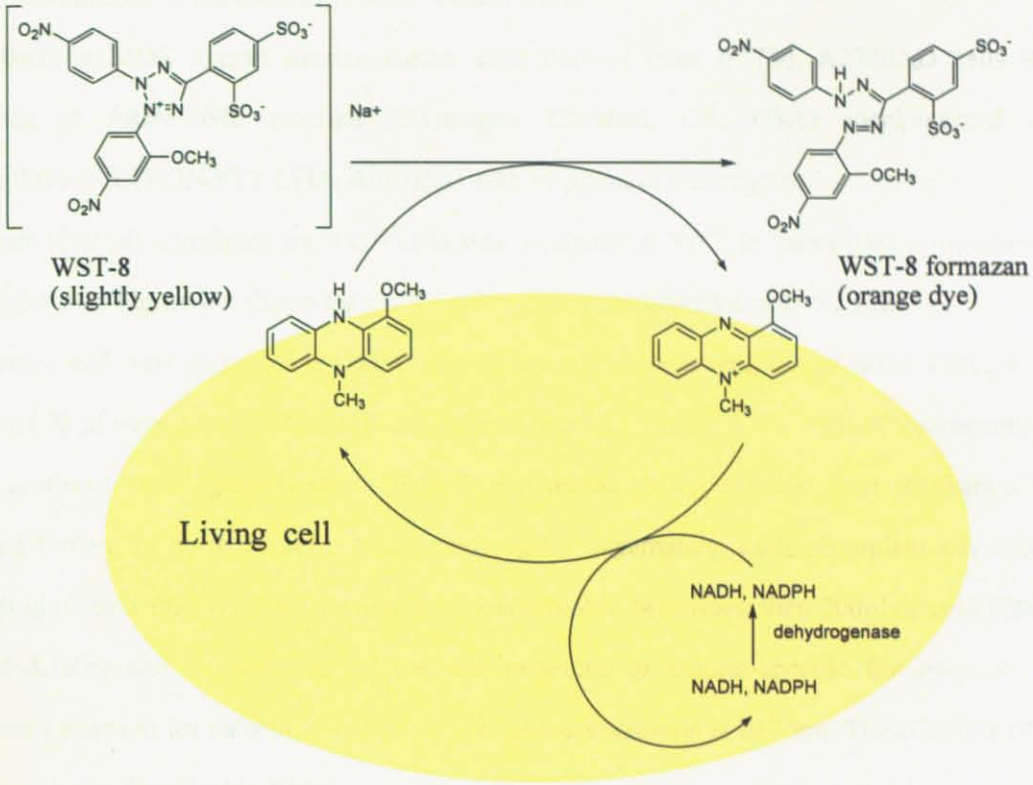


Figure 2-3. Principle of WST-8 assay

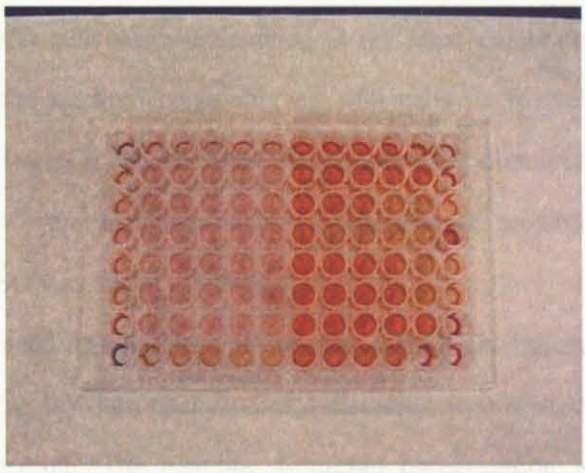


Figure 2-4. Coloration of the culture medium in the 96-well plate by WST-8 assay method

2–2–3 Accumulation of the Calcein in MDR Cancer Cells

A2780AD is MDR human ovarian cancer cells derived from A2780. A2780AD cells were maintained in PRIM-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10%(v/v)FBS (FILTRON PTY LTD., Australia) with 80 μg/ml of kanamycin.

Medium (100 μl) containing ca. $1x10^5$cells was incubated at 37C° in humidified atmosphere of 5% CO2 for 24h. Figure 2-5 shows the photo -micrographs of cultivated cells for 72h.

Test compounds were dissolved in DMSO and diluted with phosphate buffered saline PBS (-). Test samples of 50 μl were added to the medium and incubated for 15min. Then, 50μl of fluorogenic dye calcein acetoxymethyl ester [1μM in PBS(-)] was added to the medium, and incubation was continued further for 60 to 180min. After removing the supernatant, each microplate was washed with 200 μl of cold PBS (-). The washing step was repeated two times, then 200μl of cold PBS (-) was added. Retention of resulting calcein was measured as calcein-specific fluorescence. The absorption maximum for calcein is 494nm, and emission maximum is 517 nm. The principle of the calcein assay was described in Part1.

Verapamil and taxinine NN-1, the known MDR modulator were used as positive control.

2–2–4 MDR Reversal Activity of the Compound toward MDR Cancer Cells

Both A2780 and A2780AD cells were maintained at the same conditions as mentioned in the section 2-2-3. 100 μl of the medium containing ca. 400 cells in 96 well were incubated in a humidified atmosphere of 5% CO2 for 24h. Test compounds were dissolved in DMSO and diluted with PBS (-). Then, 50μl of the test compounds was added to the medium. Taxol, adriamycin or vincristin dissolved in DMSO was added to the medium.

The incubation was continued further 7days at the same conditions. Coloration substance, WST-8 [2-(2-methyl-4-nitrophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to the medium. The resulting formazan concentration was determined by the absorption at 450nm.

Cell viability (%) was calculated as [(experimental absorbance-background absorbance)/(control absorbance-background absorbance)]x100.The viability at different concentration of compounds was plotted and the concentration result in a 50% inhibition of growth was calculated as IC_{50}.

2–3 Result

2–3–1 Accumulation of the Calcein in MDR Cancer Cells

The effects of fifteen kinds of taxane derivatives, **3—17,** on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined. The experimental data are shown in table 2-3 to 2-9 and figure 2-5 to 2-11. The effects of the structure change of the compounds on the increasing amount of calcein in MDR 2780AD cells were summarized in table 2-10(a) and (b).

BaccatinIII (**4**) and 10-deacetylbaccatin III (**3**) showed no activity toward calcein accumulation in MDR 2780AD cells. Among the compounds possessing an oxetane ring, compounds **6—8** with a benzoyloxy group at C-2 and an acetoxy group at C-9 and/or C-10 showed significant effect on the calcein accumulation and the strength of their activities is almost the same. However the corresponding compound **5** with hydroxyl groups at both C-9 and C-10 showed weaker activity than those of **6—8**. These results suggested that compound **5** possessing hydroxyl groups at both C-9 and C-10 positions decreased the activity of the cellular accumulation of calcein in MDR 2780AD cells.

Compound **9** with a 13-carbonyl, 5β,20-epoxy ring (4,5-oxetane ring), and three acetoxyl groups at C-2, C-9, and C-10 showed significant activity toward calcein accumulation in MDR 2780AD cells. On the other hand, the corresponding 13α- and 13β-hydroxyl derivatives, **10** and **11**, the reduction products of the carbonyl group at C-13 of **9**, lost the activity.

The structure change of **6** to **13** is the hydroxyl group at C-9 of **6** to a carbonyl group of **13**. Since the activity of **6** and **13** is almost the same, the effect of carbonyl and hydroxyl groups at C-9 showed the same efficiency. Compound **12** with structure change of the 13-carbonyl group of **13** to a 13α-hydroxyl group showed further stronger activity. These results showed that the 13α-hydroxyl group is more efficient than the 13-carbonyl group in this case. The structure-activity relationship of compounds **9—13** probably indicated that the existence of one carbonyl group at C-13 or C-9 is desirable for the expression of the activity.

Compound **12** showed the strongest activity among the compounds tested but **4** had no activity for calcein accumulation in MDR 2780AD cells. The structure changes from **4** to **12** occur at the C-1, C-4, and C-7 positions. Two hydroxyl groups at C-1 and C-7 of **4** were lost in **12** and the acetoxyl group at C-4 of **4** was displaced by a hydroxyl group in **12**. These changes of functional groups of **4** to **12** enhanced remarkably the accumulation of calcein in MDR 2780AD cells. Compound **14** possessing 5α-,20-dihydroxyl groups instead of a 5β,20-epoxy ring (4,5-oxetane ring) in **13** showed

weaker activity than that of **13**. Analogously, compound **15** possessing 5α-,20-dihydroxyl groups in stead of 5β,20-epoxy ring in **9** showed no activity of accumulation of calcein in 2780 AD cells, although **9** had significant activity. These results indicated that the 5β,20-epoxy ring (4,5-oxetane ring) is an important functional group and 5α-,20-dihydroxyl group has no effect for the expression of activity of the taxane derivatives above-mentioned. On the contrary, compound **16** possessing 5α-hydroxy-20-acetoxy groups instead of the 5α,20-dihydroxyl groups of **15** showed significant activity. Compound **17**, 2-cinnamoyloxy derivative of **16,** showed more efficient activity than that of **16**. This enhancement of the activity was induced by the change of the functional group at C-2 from hydroxyl group to a cinnamoyloxyl group.

The above-mentioned compounds, **5—17**, are all oxygenated at C-2, C-4, C-5, C-9, C-10, C-13, and C-20 positions and synthesized for this research from taxinine, which is the major component of the Japanese yew tree, *Taxus caspidata.*

In fifteen taxane derivatives tested in this research, ten compounds showed moderate to strong activity on calcein accumulation in MDR 2780AD cells. These results seemed to indicate that the taxane skeleton has special meaning toward the MDR reversal activity although the strength of activity depends on the combination of the functional groups on the taxane skeleton.

Exp. 2-1

Table2-3. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3172 ± 83
Verapamil	0.25	3284 ± 179
	2.5	3425 ± 448
	25	4016 ± 974
Taxinine NN-1	0.25	3361 ± 491
	2.5	3915 ± 817
	25	5213 ± 1103
10-Deacetyl -baccatinIII (3)	0.25	3013 ± 145
	2.5	2855 ± 344
	25	2950 ± 488
BaccatinIII (4)	0.25	2957 ± 224
	2.5	2657 ± 557
	25	2563 ± 474

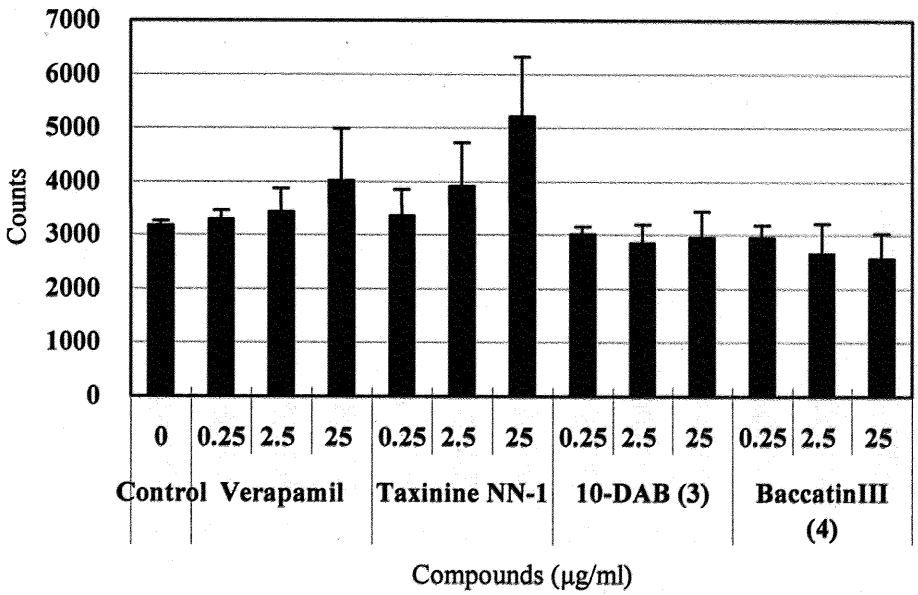


Figure 2-5.

Exp.2-2

Table2-4 Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3698 ± 74
Verapamil	0.25	3688 ± 68
	2.5	3775 ± 648
	25	4731 ± 1033
Taxinine NN-1	0.25	3942 ± 387
	2.5	4528 ± 544
	25	5365 ± 739
5	0.25	3901 ± 145
	2.5	4066 ± 344
	25	3906 ± 488
6	0.25	3662 ± 652
	2.5	3786 ± 480
	25	5163 ± 177

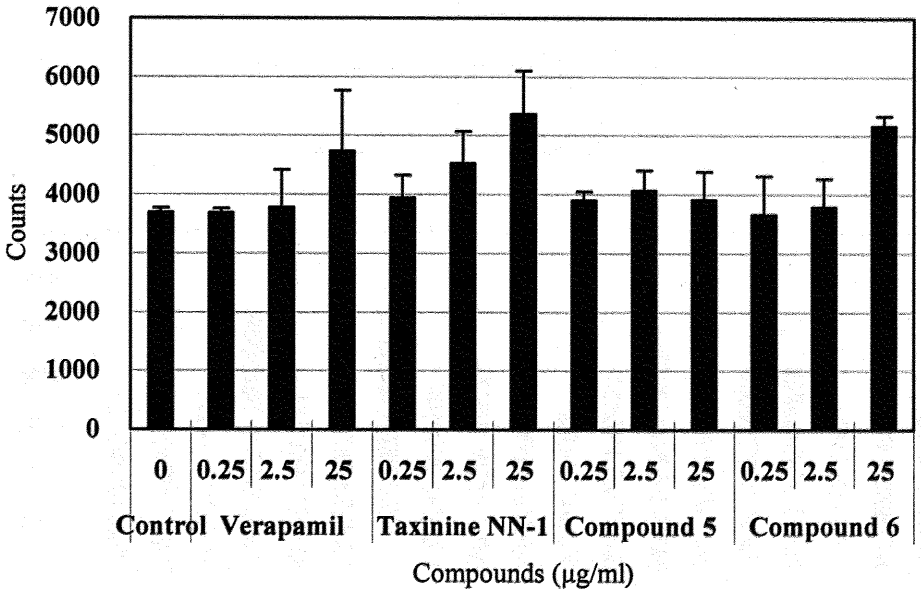


Figure 2-6.

Exp.2-3

Table2-5. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3402 ± 247
Verapamil	0.25	3472 ± 295
	2.5	3674 ± 159
	25	5181 ± 173
Taxinine NN-1	0.25	3582 ± 584
	2.5	4328 ± 684
	25	5836 ± 357
7	0.25	3900 ± 145
	2.5	4003 ± 344
	25	4559 ± 488
8	0.25	3288 ± 139
	2.5	4060 ± 230
	25	4599 ± 321
9	0.25	3916 ± 271
	2.5	4084 ± 441
	25	4173 ± 371
10	0.25	3197 ± 191
	2.5	3230 ± 425
	25	3229 ± 234
11	0.25	2781 ± 394
	2.5	3238 ± 234
	25	3260 ± 159

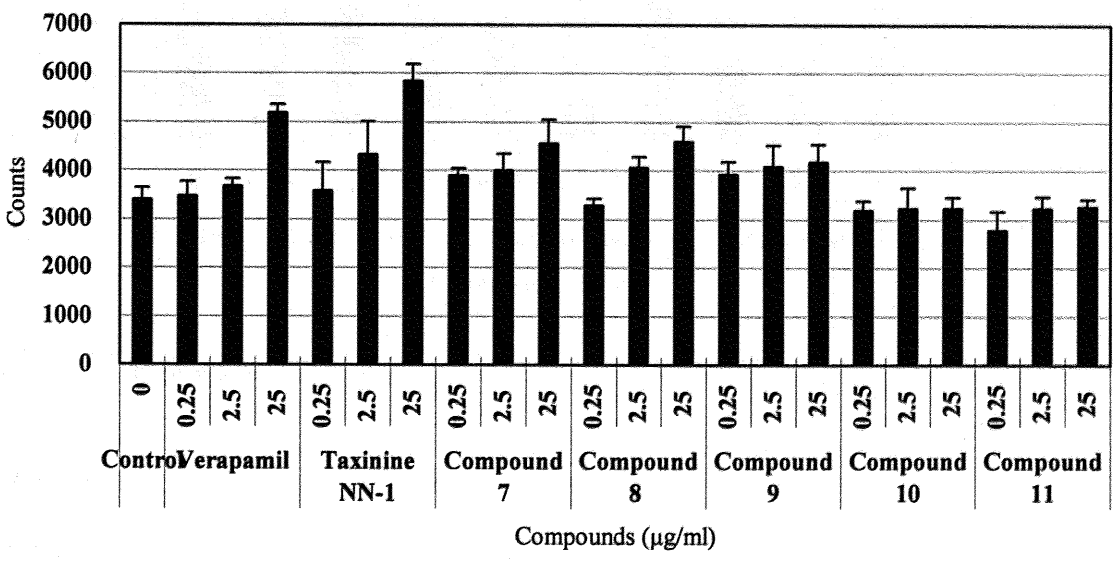


Figure 2-7.

Exp.2-4

Table2-6. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	2380 ± 175
Verapamil	0.25	2517 ± 319
	2.5	2542 ± 164
	25	3548 ± 88
Taxinine NN-1	0.25	2112 ± 416
	2.5	3091 ± 424
	25	4289 ± 383
12	0.25	2291 ± 145
	2.5	3071 ± 344
	25	3868 ± 488

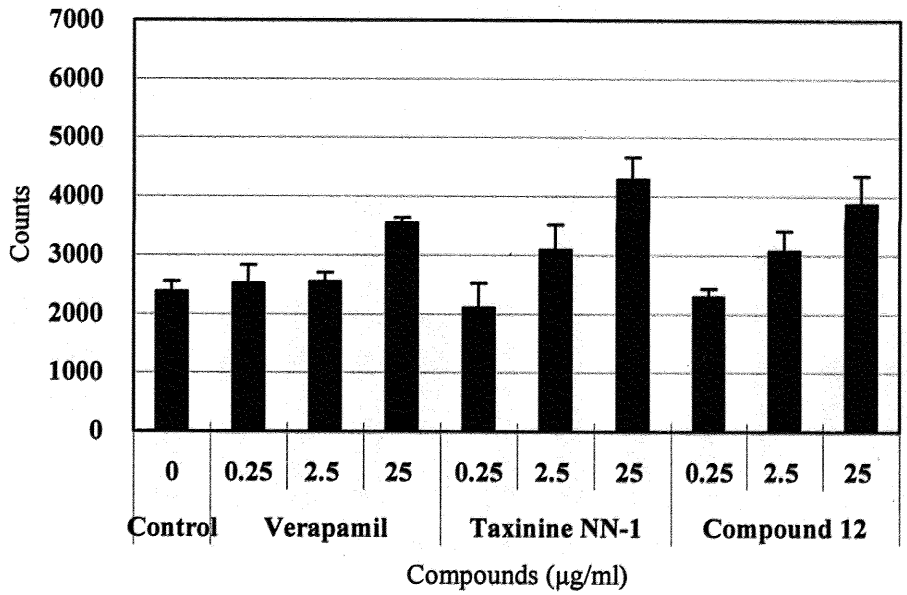


Figure 2-8.

Exp.2-5

Table2-7. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3751 ± 62
Verapamil	0.25	3743 ± 75
	2.5	4080 ± 296
	25	5937 ± 180
Taxinine NN-1	0.25	3706 ± 19
	2.5	4716 ± 73
	25	7233 ± 291
13	0.25	3774 ± 145
	2.5	4499 ± 344
	25	5150 ± 488
15	0.25	2982 ± 172
	2.5	3528 ± 897
	25	3308 ± 216

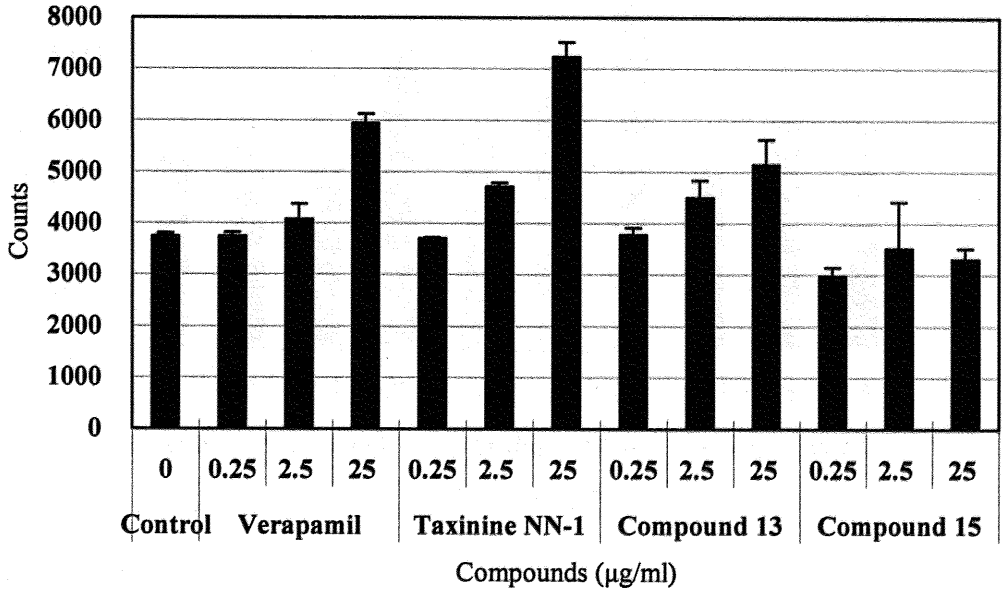


Figure 2-9.

Exp.2-6

Table2-8. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well±SD
Control	0	3402 ± 247
Verapamil	0.25	3472 ± 295
	2.5	3289 ± 675
	25	5181 ± 173
Taxinine NN-1	0.25	3582 ± 584
	2.5	4328 ± 684
	25	5836 ± 357
14	0.25	3003 ± 145
	2.5	3883 ± 344
	25	3795 ± 488

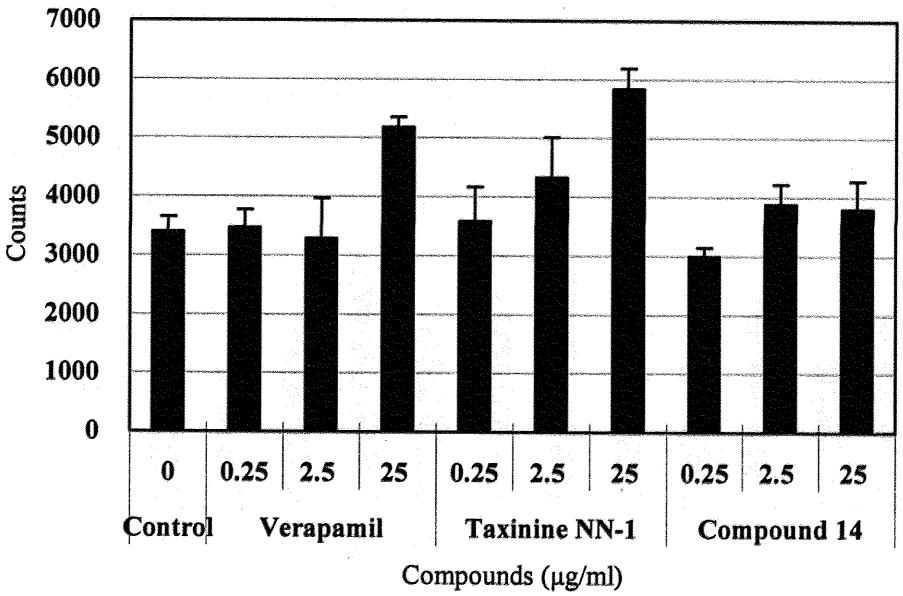


Figure 2-10.

Exp.2-7

Table2-9. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ±SD
Control	0	2853 ± 355
Verapamil	0.25	2396 ± 389
	2.5	2778 ± 530
	25	4280 ± 405
Taxinine NN-1	0.25	3086 ± 417
	2.5	3544 ± 803
	25	4555 ± 958
16	0.25	2506 ± 145
	2.5	2769 ± 344
	25	3585 ± 488
17	0.25	2523 ± 550
	2.5	3299 ± 73
	25	4141 ± 233

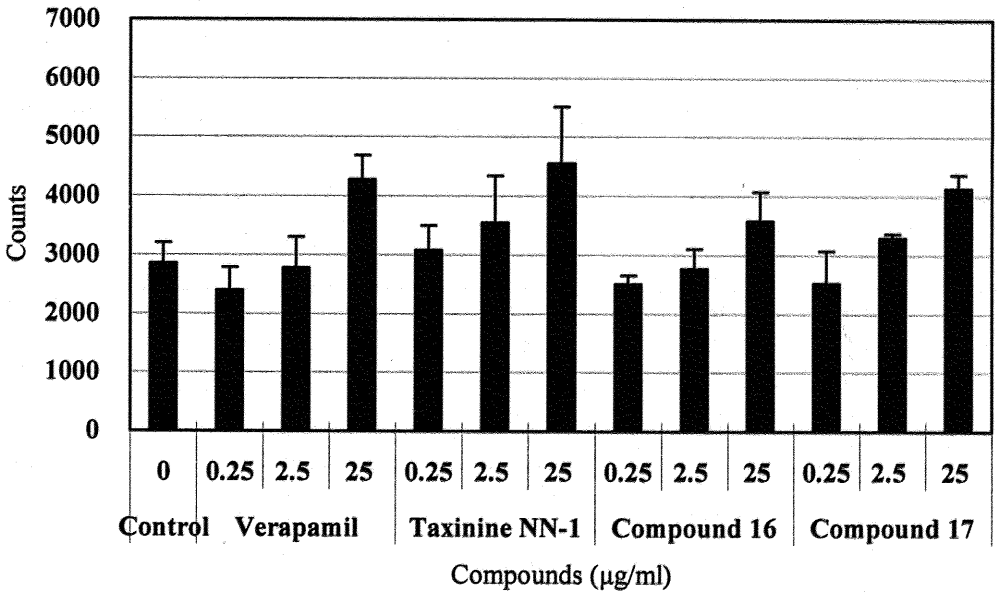
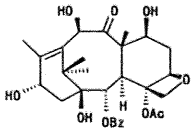
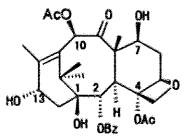
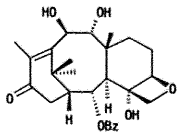
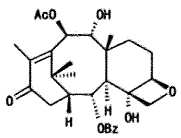
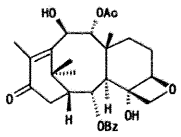
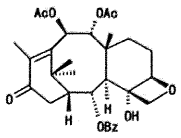
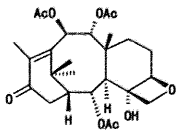
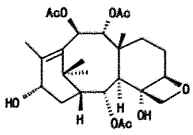


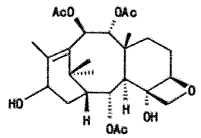
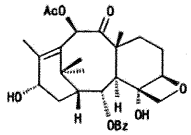
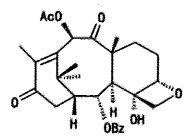
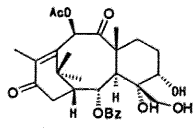
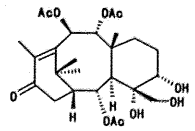
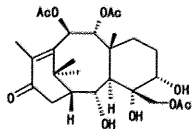
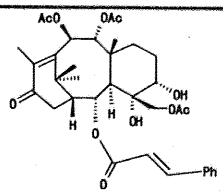
Figure 2-11.

Table 2-10 (a). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound	Structure	μg/ml	Calcein accumulation[a] % control[b]	%verapamil	%Taxinine NN-1
10-Deacetyl -baccatinⅢ (3)		0	100	100	100
		0.25	95	92	90
		2.5	90	83	73
		25	93	73	57
BaccatinⅢ (4)		0	100	100	100
		0.25	93	90	90
		2.5	84	78	78
		25	81	64	64
5		0	100	100	100
		0.25	105	106	99
		2.5	110	108	90
		25	106	83	73
6		0	100	100	100
		0.25	99	99	93
		2.5	102	100	84
		25	140	109	96
7		0	100	100	100
		0.25	115	112	109
		2.5	118	109	92
		25	134	88	78
8		0	100	100	100
		0.25	97	95	92
		2.5	119	111	94
		25	135	89	79
9		0	100	100	100
		0.25	115	113	109
		2.5	120	111	94
		25	123	81	72
10		0	100	100	100
		0.25	94	92	89
		2.5	95	88	75
		25	95	62	55

[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b]The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

Table 2-10 (b). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound	Structure	μg/ml	Calcein accumulation[a] % control[b]	%verapamil	%Taxinine NN-1
11		0	100	100	100
		0.25	82	80	78
		2.5	95	88	75
		25	96	63	56
12		0	100	100	100
		0.25	96	91	108
		2.5	129	121	99
		25	163	109	90
13		0	100	100	100
		0.25	101	101	102
		2.5	120	110	95
		25	137	87	71
14		0	100	100	100
		0.25	88	86	84
		2.5	114	118	90
		25	112	73	65
15		0	100	100	100
		0.25	79	80	80
		2.5	94	86	75
		25	88	56	46
16		0	100	100	100
		0.25	88	105	81
		2.5	97	100	78
		25	126	84	79
17		0	100	100	100
		0.25	88	105	82
		2.5	116	119	93
		25	145	97	91

[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b]The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

2–3–2 MDR Reversal Activity of the Compound toward MDR Cancer Cells

The effect of the compound **12** on the cytotoxicity of taxol, adriamycin (ADM) and vincristine (VCR) toward MDR 2780AD cells and its parental cells (A2780) was tested comparing with verapamil, which is a well-known MDR reversal agent (Table 2-11, Figure 2-12). The IC_{50} values of taxol for A2780 and MDR 2780AD cells were 0.7 and 535 nM, respectively. When compound **12** was added at a final concentration of 0.2, 2.0 and 10 μM, the IC_{50} values of taxol for MDR 2780AD cells were shifted to 375, 10 and 1 nM respectively. The enhancing effect of **12** for taxol was comparable to that of verapamil. Compound **12** is also effective for ADM and VCR. The IC_{50} values of ADM for A2780 and MDR 2780AD cells were 13 nM and 909 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 556, 107 and 17 nM in the presence of **12** at a final concentration of 0.2, 2.0 and 10 μM, respectively. The IC_{50} values of VCR for A2780 and 2780AD cells were 0.9 nM and 895 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 336, 30, and 7 nM in the presence of **12** at a final concentration of 0.2, 2.0, and 10 μM, respectively. The enhancing effects of **12** for taxol, ADM, and VCR were the same levels as that of verapamil toward MDR 2780AD cells. On the other hand, compound **12** showed no enhancing effect for taxol, ADM, and VCR toward the parental 2780 cells. Thus, compound **12** can modulate the multidrug resistance of cancer cells as well as verapamil *in vitro*.

Table 2-11. Effect of the compound 12 on the cytotoxicity of anticancer agents toward A2780 and MDR 2780AD cells[a]

Cell lines	MDR modulator (μM)		IC_{50}(nM) of anticancer agents		
			Taxol	ADM	VCR
2780	no modulator		0.7	13	0.9
	Verapamil	0.2	0.7	11	1
		2	0.8	9	0.8
		10	0.9	14	1.1
	12	0.2	0.7	8	1
		2	0.9	10	0.8
		10	0.8	7	0.8
2780AD	no modulator		535	909	895
	Verapamil	0.2	197	615	563
		2	11	109	29
		10	0.9	59	7
	12	0.2	375	556	336
		2	10	107	30
		10	1	17	7

[a] Enhancing effects of verapamil and compound **12** on the cytotoxicity of Taxol, adriamycin (ADM) and vincristin (VCR) toward A2780 cells and MDR A2780 (2780AD)cells were determined in the presence of 0.2, 2.0 and 10 μM of each compounds.

[b] The values represent means of triplicate determination.

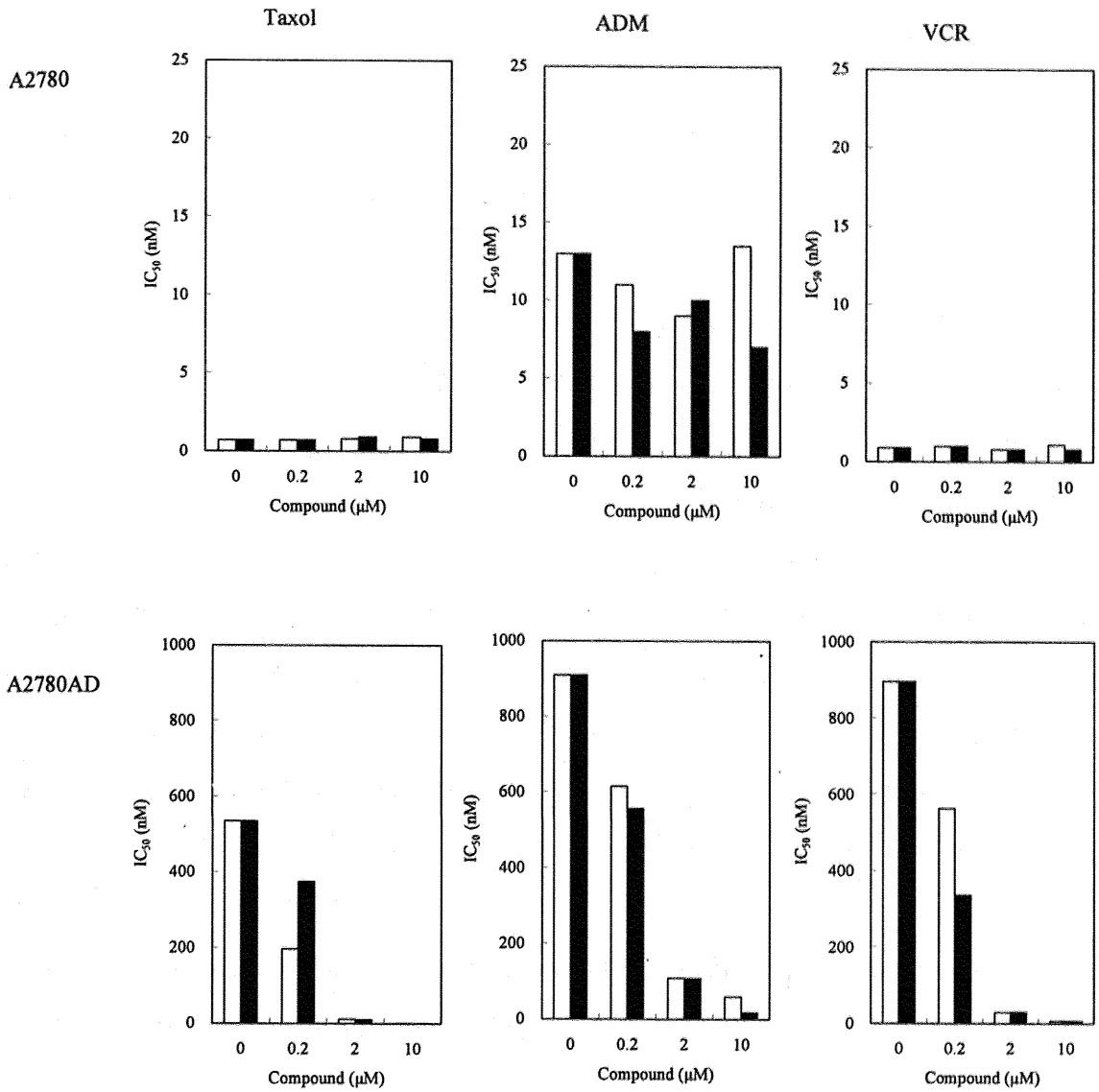


Figure 2-12. Effect of the compound 12 on the cytotoxicity of anticancer agents toward A2780 and MDR 2780AD cells

Enhancing effects of verapamil (■) and compound **12** (□) on the cytotoxicity of Taxol, adriamycin (ADM) and vincristin (VCR) toward A2780 cells and MDR A2780 (2780AD)cells were determined in the presence of 0.2, 2.0 and 10 μM of each compounds.

2–3–3 Cell Growth Inhibition

Typical dose-response curves for the cytotoxicities of Taxol and adriamycin used as positive control toward WI-38, VA-13, and HepG2 cells were shown in Figure 2-13.

Cell growth inhibitory activity (IC_{50}) of compounds **4—17** to three different cell lines was examined and summarized in Table 2-12 (a) and (b).

The three cell lines employed in this experiment are human lung fibroblast cells (WI-38), malignant lung tumor cells (VA-13) induced from WI-38, and human liver cancer Hepatoma G2 cells (HepG2). Compound **8** showed the smallest IC_{50} values toward VA-13 and HepG2 in compounds **5—8**. The results suggested that displacement of the acetoxyl group at C-9 and /or C-10 of **8** with a hydroxyl group induced the decrease of activity in **5**, **6**, and **7**. Cytotoxic activity of **9** against WI-38, VA-13 and HepG2 decreased remarkably on displacement of the 2α-benzoyloyl group of **8** with an acetoxyl group. Compound **13** showed moderate and weak activities to VA-13 and HepG2, respectively. Compound **14** with 5α-,20-dihydroxyl groups instead of 5α,20-oxetane ring in **13** showed weaker activity to VA-13 than that of **13**. Compounds **15** and **16** with a 5α-,20-dihydroxyl and 5α-hydroxy-20-acetoxy groups respectively showed significant activity toward VA-13. On the contrary, compound **17,** 2-cinnamoyloxy derivative of **16,** showed significant activity not to VA-13 but HepG2.

These results suggested that some modifications of the functional groups on the taxane skeleton induced a new cytotoxicity to a different cell line.

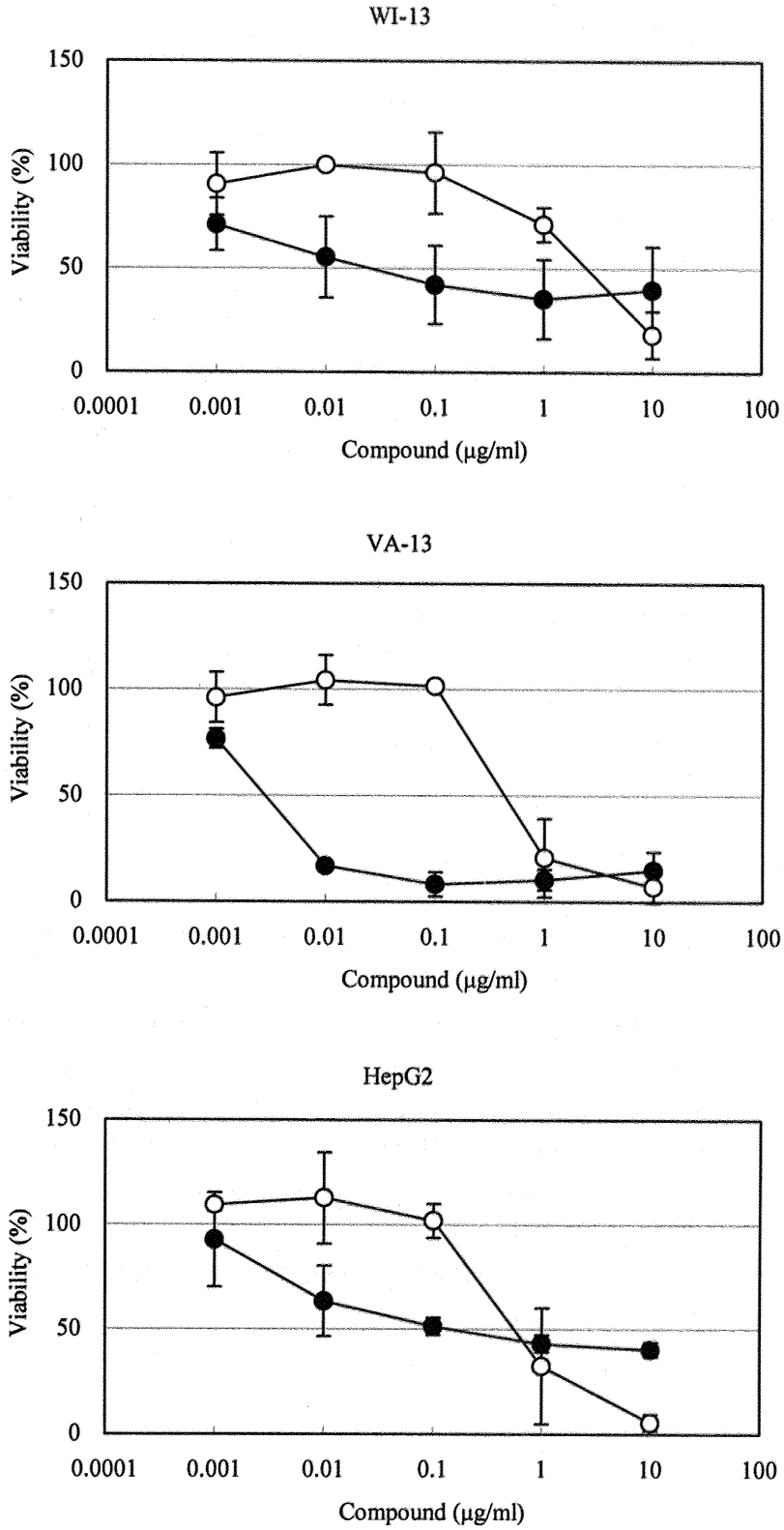
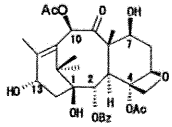
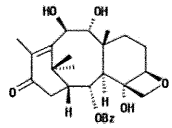
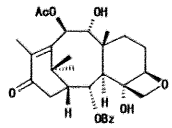
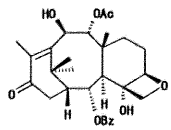
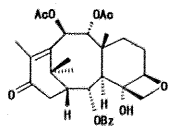
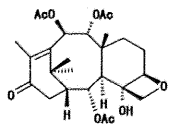
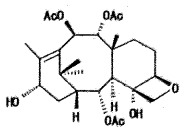


Figure 2-13. Cell growth inhibition activities of the anti-cancer agents

●: Taxol

○: Adriamycin

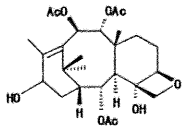
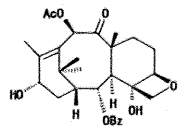
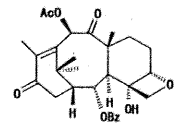
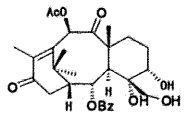
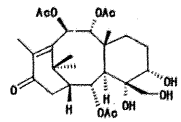
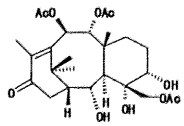
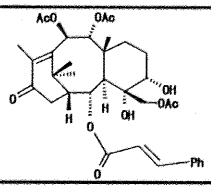
Table 2-12 (a). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compound			Cell line	Cytotoxicity (IC_{50})[a]		
	Structure	M.W.		μg/ml	μM	Selectivity[b]
Baccatin Ⅲ (4)		586.60	WI-38 VA-13 HepG2	16 0.84 9.9	27 1.4 17	1.00 19.2 1.62
5		470.55	WI-38 VA-13 HepG2	>100 >100 68	>213 >213 144	1.00 – >1.48
6		512.59	WI-38 VA-13 HepG2	42 >100 78	82 >195 152	1.00 <0.42 0.54
7		512.59	WI-38 VA-13 HepG2	0.67 67 43	1.3 131 84	1.00 0.01 0.02
8		554.63	WI-38 VA-13 HepG2	0.82 52 7.3	1.5 94 13	1.00 0.02 0.11
9		492.56	WI-38 VA-13 HepG2	77 >100 >100	156 >203 >203	1.00 <0.76 <0.76
10		494.57	WI-38 VA-13 HepG2	8.4 82 >100	17 165 >202	1.00 0.10 <0.08

[a] IC_{50} represent means of duplicate determination.

[b] The values are the ratio of IC_{50} (μM) (VA-13/WI-38, or HepG2/WI-38)

Table 2-12 (b). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compound			Cytotoxicity (IC_{50})[a]			
	Structure	M.W.	Cell line	μg/ml	μM	Selectivity[b]
11		494.57	WI-38	76	154	1.00
			VA-13	>100	>202	<0.76
			HepG2	>100	>202	<0.76
12		512.50	WI-38	54	106	1.00
			VA-13	77	150	0.70
			HepG2	70	136	0.78
13		510.50	WI-38	7.4	14	1.00
			VA-13	7.9	16	0.93
			HepG2	30	59	0.25
14		510.57	WI-38	57	112	1.00
			VA-13	60	117	0.96
			HepG2	33	65	1.72
15		528.50	WI-38	85	161	1.00
			VA-13	10	18	8.77
			HepG2	>100	>189	<0.85
16		510.57	WI-38	>100	>196	1
			VA-13	8.5	17	>11.5
			HepG2	>100	>196	–
17		640.72	WI-38	68	106	1.00
			VA-13	82	128	0.83
			HepG2	2.1	3.3	31.9

[a] IC_{50} represent means of duplicate determination.

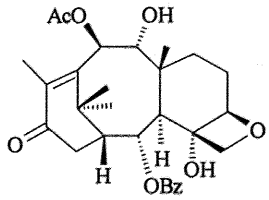
[b] The values are the ratio of IC_{50} (μM) (VA-13/WI-38, or HepG2/WI-38)

2–4 Conclusion

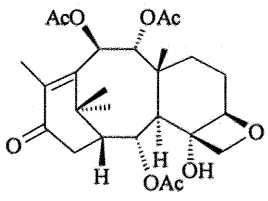
As shown in Figure 2-14, compounds **6**, **9**, and **12** showed MDR reversal activities but have no cytotoxicity. These compounds are expected to be lead compounds of MDR cancer reversal agents.

Compounds **13**, **15**, and **16** showed significant cytotoxic activity toward VA-13. Among them, **13** and **16** showed significant MDR reversal activity and are expected to be a new type of anticancer reagents (Figure 2-15 (a)). On the other hand, cytotoxic activity of compounds **8** and **17** against HepG2 are the same level or more than that of taxol (**2**) or baccatinIII (**4**). Since compounds **8** and **17** also showed significant MDR reversal activity, they are expected as lead compounds of new type anticancer agents (Figure 2-16 (b)).

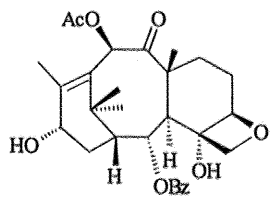
Compound **13** with a carbonyl group at C-13 showed both MDR reversing and cytotoxic activities in vitro. On the other hand, the corresponding 13α-hydroxy derivative **12** showed efficient MDR reversing activity but its cytotoxic activity decreased drastically. Introduction of an isoserine moiety to the 13α-hydroxyl group of taxane derivatives increases cytotoxic activity remarkably as shown in the change from baccatin III (**4**) to taxol (**2**) in Figure 2-13. Compound **12** is also expected to be a lead compounds of anti-MDR cancer reagents or anticancer reagents after introduction of the isoserine moiety on the13α-hydroxyl group of **12**.



6



9

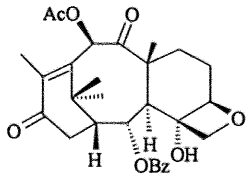


12

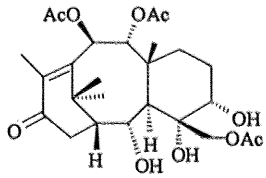
Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
6	82	>195	152	140
9	156	>203	>203	123
12	106	150	136	163

*Concentration of compounds : 25 μ g/ml

Figure 2-14. Candidates as MDR modulator



13

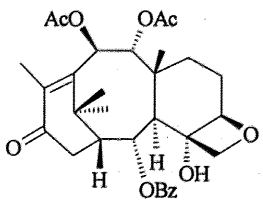


16

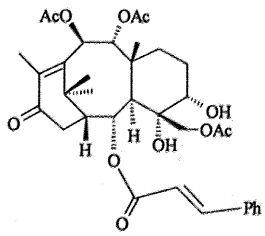
Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
13	14	16	59	137
16	>196	17	>196	126

*Concentration of compounds : 25 μ g/ml

Figure 2-15 (a). Candidates as anti-cancer agents with MDR reversal activity



8



17

Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
8	1.5	94	13	135
17	106	128	3.3	145

*Concentration of compounds : 25 μ g/ml

Figure 2-15 (b). Candidates as anti-cancer agents with MDR reversal activity

2–5 Summary

1,7-Deoxy-4-deacetylbaccatin III (**12**) and its six analogues **6**—**9**, **13**, and **14**, which were synthesized from taxinine, showed significant activity as MDR reversal agent by the assay of the calcein accumulation toward MDR human ovarian cancer 2780AD cells. The most effective compound **12** in this assay is actually efficient for the recovery of cytotoxic activity of paclitaxel (taxol®), adriamycin (ADM), and vincristine (VCR) toward MDR 2780AD cells at the same level toward parental 2780 cells. This activity of **12** is very interesting because baccatin III (**4**) has no such MDR reversal activity but has cytotoxic activity. The essential functional groups inducing such a difference in biological activity between **4** and **12** are 4α-acetoxyl for **4** and 4α-hydroxyl for **12**. In seven compounds possessing MDR reversal activity, compound **12** is the most desirable compound for anti-MDR cancer reversal agent, because it has the highest accumulation ability of anticancer agent in MDR cancer cells and weak cytotoxic activity. Compounds **8** and **13** showed significant cytotoxic activity toward HepG2 and VA-13, respectively, as well as MDR reversal activity. They are expected to become lead compounds for new types of anticancer reagent or anti-MDR cancer reagent.

Part3 Structure–Activity Relationships of Taxuyunnanine C Derivatives as Multidrug Resistance Modulator and Anti-Cancer Agent

3-1 Introduction

In cancer chemotherapy, occurrence of multidrug resistance (MDR) of cancer cells caused by repeated administration of anticancer agents is a serious problem. One of the mechanisms of MDR is overexpression of P-glycoprotein (P-gp).[32, 33] P-gp is originally a transporter for a wide range of reagent and its physiological role is a defense mechanism against the toxic materials in cells. P-gp utilizes energy induced by hydrolysis of ATP for their transport from inside to the outside of cells.

Many taxane derivatives with MDR reversal activity have been reported.[34–42] In our laboratory, the isolation and structure determination of taxoids, which showed the increase of cellular accumulation of vincristine in MDR cancer cells have been previously reported.[43—50] In them, taxinine NN1 and taxinine NN-11 bearing a cinnamoyloxy group and a hydroxyl group on the skeleton exhibited especially strong MDR reversal activity.[45, 51]

Taxuyunnanine C (**18**) and its 14-acyloxy analogs (**19—22**) are the major metabolites from callus cultures of *Taxus* species in high yields. Their activity toward the accumulation of vincristine in MDR 2780AD cells have been reported and it was deduced that a hydrophobic less-hindered alkyl side chain of the C-14 acyloxy group of those compounds played some role to increase the activity.[48]

As a part of our study of structure-activity relationships (SAR) of taxiods as MDR reversal agents, I reported here the synthesis and SAR of a series of new 14-oxygenated taxoids derived from taxuyunnanine C (**19**) and its analogs which are produced by callus cultures of *T. cuspidata*.[48, 54, 55]

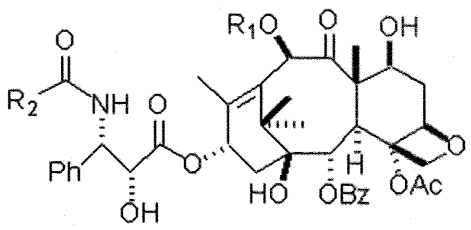
3-2 Experimental Methods

3-2-1 Chemistry

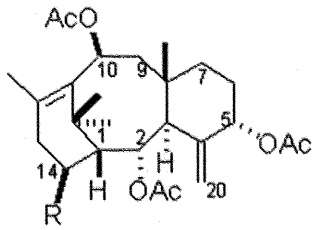
As shown in Scheme 3-1, 14-deacetyltaxuyunnanine C (**23**), 10-deacetyltaxuyunnanine C (**24**), 5-deacetyltaxuyunnanine C (**25**) with a hydroxyl group at the C-14, 10, or 5 position and 10,14-dideacetyl -taxuyunnanine C (**26**) with two hydroxyl groups at the C-14 and 10 positions by hydrolysis of **1**. 14-Butanoyloxy- (**23a**), 14-pentanoyloxy- (**23b**), 14-benzoyloxy- (**23c**), and 14-cinnamoyloxy- (**23d**) derivatives of **23** were obtained by the acylation of **23** by the corresponding acylchloride. Cinnamoylation of 10-deacetyltaxuyunnanine C (**24**) and 5-deacetyltaxuyunnanine C (**25**) gave **24a** and **25a** bearing a cinnamoyloxy group at C-10 or C-5, respectively. Cinnamoylation of 10, 14-dideacetyltaxuunanine C (**26**) gave 10- and 14-monocinnamoyloxy analogs, **26b** and **26a**, and a 10,14-dicinnamoyloxy analog **26c.**

14-Deacetoxytaxuyunnanine C (**23f**) was obtained by Chugaev reaction of **23** (Scheme 3-2). 2-Benzoyloxy analog, **27a** was prepared from the corresponding 2-deacetyltaxuyunnanine C (**27**), which was isolated from the callus culture of *T.cuspidata* (Scheme 3-3) as a minor product.[52]

For the preparation of 7- or 9-oxygenated derivatives of **18** and its analogs **21** and **22**, we used a biotransformation. Thus, taxoids, **28—30**, bearing a hydroxyl group at the C-7 or C-9 position was obtained by treatment of taxoids **18, 21,** and **22** with *Absidia. coerulea* IFO 4011. Compounds **28—30** were further converted to the corresponding cinnamoyl esters, **28a**, **29a**, and **30a** (Scheme 3-4).

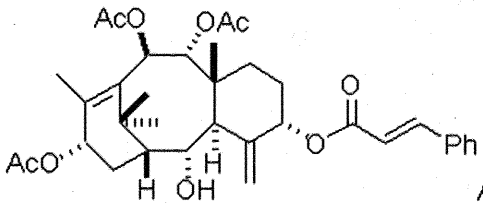


paclitaxel R_1 = OAc, R_2 = Ph
docetaxel R_1 = H, R_2 = *t*-BuO

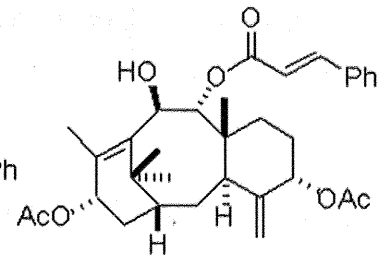


18 R = OAc (taxuyunnanine C)
19 R = r
20 R = s
21 R = t
22 R = u

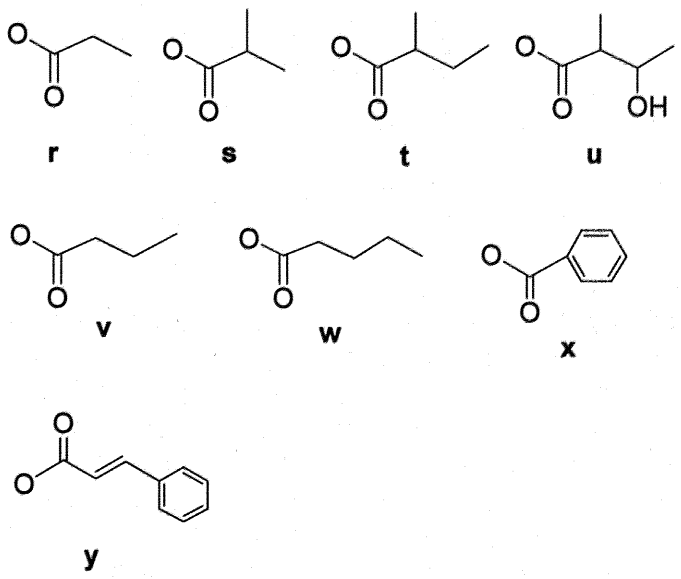
taxuyunnanine C and its analogs



taxinine NN-1



taxinine NN-11



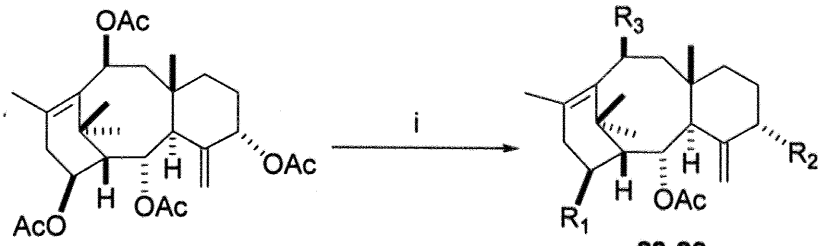
r **s** **t** **u**

v **w** **x**

y

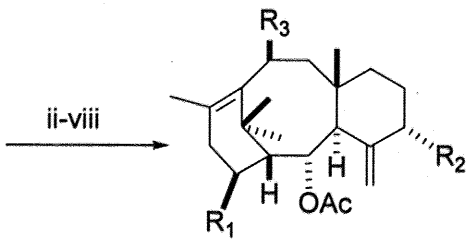
Figure 3-1. Acyloxy groups r-y in the structures of Taxuyunnanine C derivatives

Scheme 3-1



18 **23-26**

23 R_1 = OH, R_2= R_3 = OAc
24 R_1 = R_2 = OAc, R_3 = OH
25 R_1 = R_3= OAc, R_2= OH
26 R_1=R_3= OH, R_2 = OAc

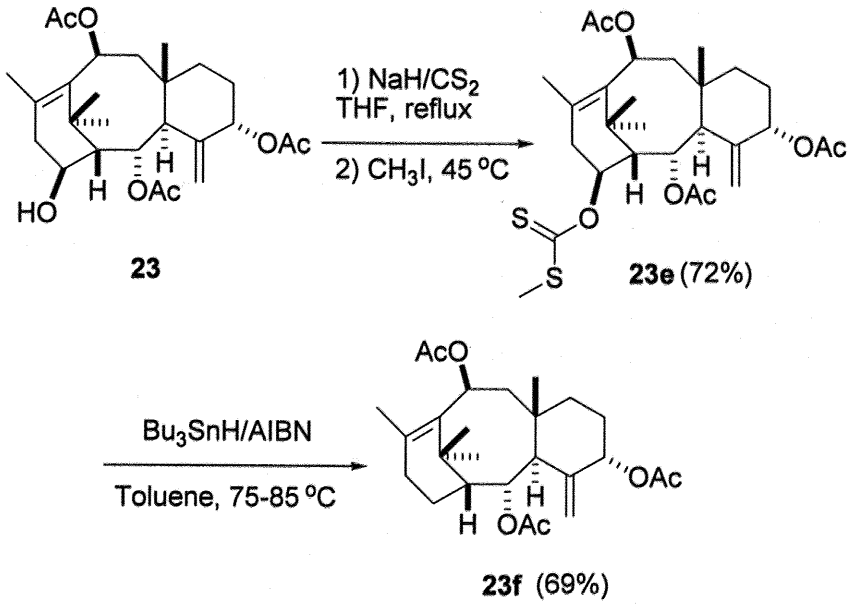


23a-26c

23a R_1= v, R_2 = R_3 = OAc
23b R_1 = w, R_2= R_3 = OAc
23c R_1 = x, R_2 =R_3 = OAc
23d R_1 = y, R_2 = R_3 = OAc
24a R_1 = R_2 = OAc, R_3 = y
25a R_1 = R_3 = OAc, R_2 = y
26a R_1 = y, R_2 = OAc, R_3 = OH
26b R_1 = OH, R_2 = OAc,R_3 = y
26c R_1 = R_3 = y, R_2 = OAc

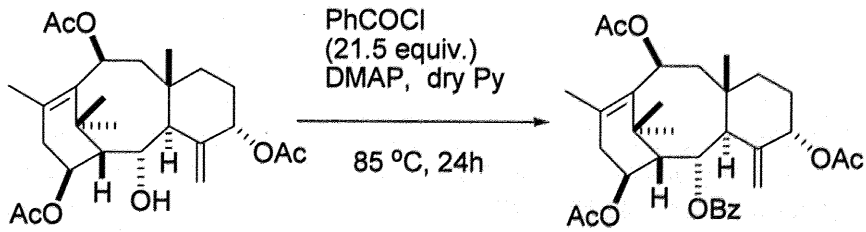
Regents and conditions: (i) **23**, 1M K_2CO_3 (5 equiv.), 1:3 THF-MeOH, 45 °C, 7h (23.9% for **23,** 4.3% for **24**, 0.5% for **25**, 20.7% for **26**); (ii) **23**, $CH_3(CH_2)_2COCl$ (13 equiv.), DMAP, dry Py, 80 °C, 12.5h (87.6 % for **23a**); (iii) **23**, $CH_3(CH_2)_3COCl$ (13 equiv.), DMAP, dry Py, 80 °C, 13.5h (88.0 % for **23b**); (iv) **23**, PhCOCl (10 equiv.), DMAP, dry Py, 80 °C, 15h (92.7 % for **23c**); (v) **23**, PhCH=CHCOCl (8 equiv.), DMAP, dry Py, 85 °C, 16h (92.8 % for **23d**); (vi) **24**, PhCH=CHCOCl (8 equiv.), DMAP, dry Py, 85 °C, 13h (91.5 % for **24a**); (vii) **25**, PhCH=CHCOCl (20 equiv.), DMAP, dry Py, 85 °C, 19h (80% for **25a**); (viii) **26**, PhCH=CHCOCl (2.2 equiv.), DMAP, dry Py, 85-90 °C, 23h (8.4% for **26a**, 29.1% for **26b**, 5.9% for **26c**).

Scheme 3-2



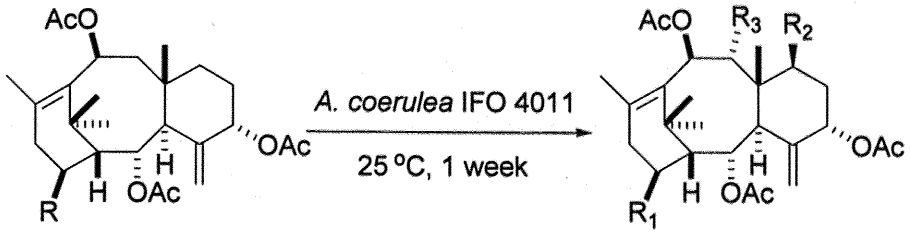
23 **23e** (72%) **23f** (69%)

Scheme 3-3



27 **27a** (90.9%)

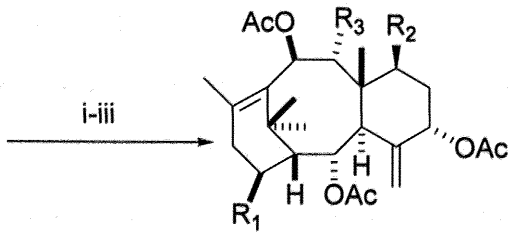
Scheme 3-4



18, **21**, and **22**

28 — **30**

28 R_1 = OAc, R_2 = H, R_3 = OH
29 R_1 = t, R_3 = H, R_2 = OH
30 R_1 = u, R_3 = H, R_2 = OH



28a — **30a**

28a R_1 = OAc, R_2 = H, R_3 = y
29a R_1 = t, R_3 = H, R_2 = y
30a R_1 = u, R_3 = H, R_2 = y

Regents and conditions: (i) **28**, PhCH=CHCOCl (10 equiv.), DMAP, dry Py, 85–90 oC, 9 h (84.6% for **28a**); (ii) **29**, PhCH=CHCOCl (10 equiv.), DMAP, dry Py, 85-90 oC, 10 h (88.8% for **29a**); (iii)**30**, PhCH=CHCOCl (6 equiv.), DMAP, dry Py, 85-90 oC, 5.5 h (13.9% for **30a**).

3–2–2 Cell Growth Inhibition

Normal human lung fibroblast cells WI-38、SV40-transformed WI-38 cells VA-13 or human liver tumor cells HepG2 are available from the Institute of Physical and Chemical Research(RIKEN), Tsukuba, Ibaraki, Japan. WI-38 cells were maintained in Eagle's MEM medium(Nissui Pharmaceutical Co., Japan). VA-13 cells were maintained in RITC 80-7 medium(Asahi Technoglass Co., Japan) . HepG2 cells were maintained in MEM medium (Invitrogen). All of media were supplemented with 10 %(v/v) fetal bovine serum (FBS) (FILTRON PTY LTD., Australia) together with kanamycin. and incubated at 37C° in a humidified atmosphere of 5% CO2.

100 μl of medium containing about 5,000 cells in 96 well was incubated in a humidified atmosphere of 5% CO2 for 24h. Then the test compounds dissolved in dimethyl sulfoxide (DMSO) were added to the well and continued further 48h at the same conditions. Coloration substance, WST-8 [2-(2-methyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono -sodium salt] was added to the medium. The resulting formazan concentration was determined by the absorption at 450nm. Figure 2-3 shows the principle of the coloration reaction.

Cell viability (%) was calculated as [(experimental absorbance-background absorbance)/(control absorbance-background absorbance)]x100.The viability at different concentration of compounds was plotted and the concentration result in a 50% inhibition of growth was calculated as IC_{50}.

3–2–3 Accumulation of the Calcein in MDR Cancer Cells

2780AD cells were maintained in PRIM-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10%(v/v)FBS (FILTRON PTY LTD., Australia) with 80 μg/ml of kanamycin. Medium (100 μl) containing ca. $1x10^5$cells was incubated at 37 °C in humidified atmosphere of 5% CO2 for 24h.

Test compounds were dissolved in DMSO and diluted with phosphate buffered saline PBS (-). Test samples of 50 μl were added to the medium and incubated for 15min. Then, 50μl of fluorogenic dye calcein acetoxymethyl ester [1μM in PBS(-)] was added to the medium, and incubation was continued further for 60min. After removing the supernatant, each microplate was washed with 200 μl of cold PBS (-). The washing step was repeated two times, then 200μl of cold PBS(-) was added. Retention of resulting calcein was measured as calcein-specific fluorescence. The absorption maximum for calcein is 494nm, and emission maximum is 517 nm. The principle of the calcein assay was described in Part 1.

Verapamil and taxinine NN-1, the known MDR modulator were used as positive control.

3-2-4. Accumulation of the vincristine in MDR 2780AD cells[53)]

MDR 2780AD cells (1 x 10^6 cells/well) were seeded in a 24-well plate and cultured for 18h before the assay. The cells were treated with 1 x 10^5 dpm of [^{3}H]-VCR (222 Gbp/mmol; Amersham Pharmacia Biotech, Tokyo, Japan) in the presence or absence of verapamil or taxoids. Immediately after incubation for 2h at 37 °C, the cells were washed five times with ice-cold phosphate-buffered saline containing 0.1mg/ml of nonradioactive VCR and lysed with 500 μL of 0.2 M NaOH. After incubation for 45 min at 56 °C,lysates were neutralized with 2 M asetic acid, and the radioactivity was counted in ACS II (Amersham Pharmacia Biotech).

3–2–5 MDR Reversal Activity of the Compound toward MDR Cancer Cells

Both A2780 and 2780AD cells were maintained at the same conditions as mentioned in cellular accumulation of calcein at the section of material and method. 200 μl of the medium containing ca. 400 cells in 96 well were incubated in a humidified atmosphere of 5% CO2 for 24h.

Then, 1μl of the test compounds dissolved in DMSO was added to the medium. Taxol, adriamycin or vincristin dissolved in DMSO was added to the medium.

The incubation was continued further 5days at the same conditions. Coloration substance, WST-8[2-(2-methyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4- disulfophenyl) -2H-tetrazolium, mono -sodium salt] was added to the medium. The resulting formazan concentration was determined by the absorption at 450nm.

Cell viability (%) was calculated as [(experimental absorbance-background absorbance)/(control absorbance-background absorbance)]x100.The viability at different concentration of compounds was plotted and the concentration result in a 50% inhibition of growth was calculated as IC_{50}.

3-2-6 Evaluation for the Mode of Action of the Compound using Panel of Human Cancer Cell Lines[54,55,56)]

The cells were plated at proper density in 96-well plates in RPMI-1640 medium with 5% fetal bovine serum and allowed to attach overnight. The cells were exposed to drugs for 48h. Then, the cell growth was determined according to the sulforhodamine B assay, described by Skehan et al.[57)]

Three dose response parameters were calculated for each experimental agent, growth inhibition of 50% (GI_{50}) which was the drug concentration resulting in a 50% reduction in the net protein increasing (as measured by SRB staining) in control cells during the drug incubation, the drug concentration resulting in total growth inhibition (TGI), and the drug concentration resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning (LC_{50}), which indicated a net loss of cells following treatment. The mean graph, which shows the differential growth inhibition of the drug in the cell line panel, was drawn based on a calculation using a set of GI_{50}. To analyze the correlation between the mean graphs of drugs A and B, the COMPARE computer algorithm was developed according to the method described by Paull et al. Peason correlation coefficients were calculated using the following formula: $r = (\Sigma(x_i - x_m)(y_i - y_m))/(\Sigma(x_i - x_m)^2\Sigma(y_i - y_m)^2)^{1/2}$, where x_i and y_i are log GI_{50} of drug A and drug B, respectively, against each cell line, and x_m and y_m are the mean values of x_i and y_i, respectively.

3-3 Result

3-3-1 Accumulation of the Calcein in MDR Cancer Cells

The effects of taxoids prepared as described in 3-2-1 on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined. The experimental data are shown in table 3-1 to 3-9 and figure 3-2 to 3-10. These results are summarized in Table 3-10 (a), (b), and (c).

Taxuyunnanine C (**18**), and its monodeacetyl analogs, **23—25** and **27** and its 9-hydroxylated analog (**28**) showed weak to significant activity toward calcein accumulation in MDR 2780AD cells. In them, compounds **23** and **25**, in which the acetoxyl groups at C-14 or C-5 of **18** were displaced by a hydroxyl group respectively, showed stronger activity than that of the parental compound, taxuyunnanine C (**18**). On the other hand, compounds **24, 27**, and **28,** in which the acetoxy groups at C-10 or C-2, or one hydrogen at C-9 of **18** were displaced by a hydroxyl group respectively, showed weaker activity than that of the parental compound, taxuyunnanine C (**18**). Taxoid **23f** in which the acetoxy group at C-14 of **18** was displaced by hydrogen, showed no activity. Compounds **29** and **30** with a hydroxyl group at the C-7 position showed lower activity than those of their parental compounds, **21** and **22**. These results of bioassay suggested that the substitution of the acetoxyl group at C-14 or C-5 of **18** with a more polar substituent such as a hydroxyl group is desirable to increase the activity toward calcein accumulation in MDR 2780AD cells. On the other hand, the substitution of the acetoxyl group at C-2, C-10 or one hydrogen at C-9 of **18** or at C-7 of **21** and **22** with a more polar substituent such as a hydroxyl group is an undesirable change to increase the activity.

Then, we investigated the influence of functional groups at C-14 of **18**. The activity of **18**, its seven kinds of C-14 acyloxy analogs (**19**, **20**, **21**, **22**, **23a**, **23b**, **23c**, and **23d**), 14-deacetyltaxuyunnanine C (**23**), and 14-deacetoxytaxuyunnanine C (**23f**) were compared. The activity order of the compounds was **22** > **23** = **20** > **23c** ≥ **18**= **23d** ≥ **19** = **21** ≥ **23a** ≥ **23b** >> **23f**. These results suggested that the compounds with hydrophilic substituents at C-14 such as **22** and **23** and the compounds with bulky acyloxy substituents at C-14 such as **20** and **23c** are desirable to increase the activity. On the contrary, the compounds possessing acyloxy groups with less hindered hydrophobic propyl and butyl side chains such as **23a** and **23b** showed lower activity than that of the parental compound **18**. Compound **23f**, in which the acetoxy group at C-14 of **18** was displaced by hydrogen, lost the activity completely.

Since taxinine derivatives, taxinine NN1 and taxinine NN-11 bearing a cinnamoyloxy group exhibited strong MDR reversal activity, we expected the special effect of an aromatic acyloxy group such as a cinnamoyloxy group or a benzoyloxy group on taxusyunnanin C derivatives in calcein accumulation in MDR 2780AD cells. We examined the activity of ten kinds of cinnamoyloxy analogs of **18**. The order of the activity was **26a** > **30a** > **24a** = **23d** = **27a** = **28a** ≥ **25a** > **26b** > **29a** >> **26c**. In them, compound **26a** with a cinnamoyloxy group at C-14 and a hydroxyl group at C-10 showed the strongest activity. On the contrary, **26b**, regioisomer of **26a**, with a cinnamoyloxy group at C-10 and a hydroxyl group at C-14 showed very weak activity. Compound **30a** with a cinnamoyloxy group at C-7 and a polar acyloxy group, 2-metyl-3-hydroxybutanoyloxy group at C-14 showed significant activity. Interestingly, **29a** with a cinnamoyloxy group at C-7 and less polar 2-metylbutanoyloxy group at C-14 lost activity. Other analogs of **18**, **23d**, **24a**, **25a**, **28a** which possess one cinnamoyloxy group at C-14, C-10, C-5, and C-9, and compound **27a** with one benzoyloxy group at C-2 showed almost the same activity with that of **18**. Compound **26c** with two cinnamoyloxy groups at C-10 and C-14 showed no activity. In conclusion, compound **26a** with a cinnamoyloxy group at C-14 and a hydroxyl group at C-10 has the strongest activity in twenty-six test samples of taxuyunnanine C analogs toward calcein accumulation in MDR 2780AD cells.

Exp. 3-1(a)

Table3-1. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	897 ± 84
Verapamil	0.25	1206 ± 44
	2.5	1361 ± 33
	25	1757 ± 81
Taxinine NN-1	0.25	1158 ± 5
	2.5	1442 ± 42
	25	2017 ± 76
18	0.25	1163 ± 6
	2.5	1268 ± 100
	25	1141 ± 96
20	0.25	1190 ± 38
	2.5	1244 ± 38
	25	1326 ± 26
22	0.25	730 ± 91
	2.5	839 ± 137
	25	1510 ± 25
23	0.25	937 ± 26
	2.5	773 ± 202
	25	1325 ± 65

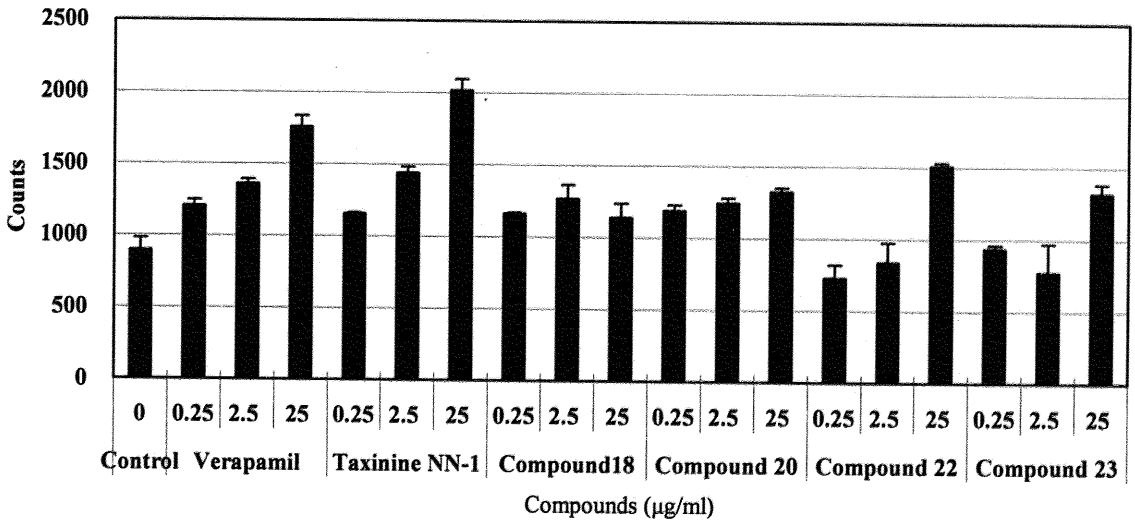


Figure 3-2

Exp. 3-1(b)

Table3-2. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	897 ± 84
Verapamil	0.25	1206 ± 44
	2.5	1361 ± 33
	25	1757 ± 81
Taxinine NN-1	0.25	1158 ± 5
	2.5	1442 ± 42
	25	2017 ± 76
23d	0.25	1100 ± 130
	2.5	1132 ± 134
	25	1123 ± 267
25	0.25	1056 ± 78
	2.5	1060 ± 21
	25	1260 ± 110
26a	0.25	963 ± 39
	2.5	1179 ± 120
	25	1702 ± 246

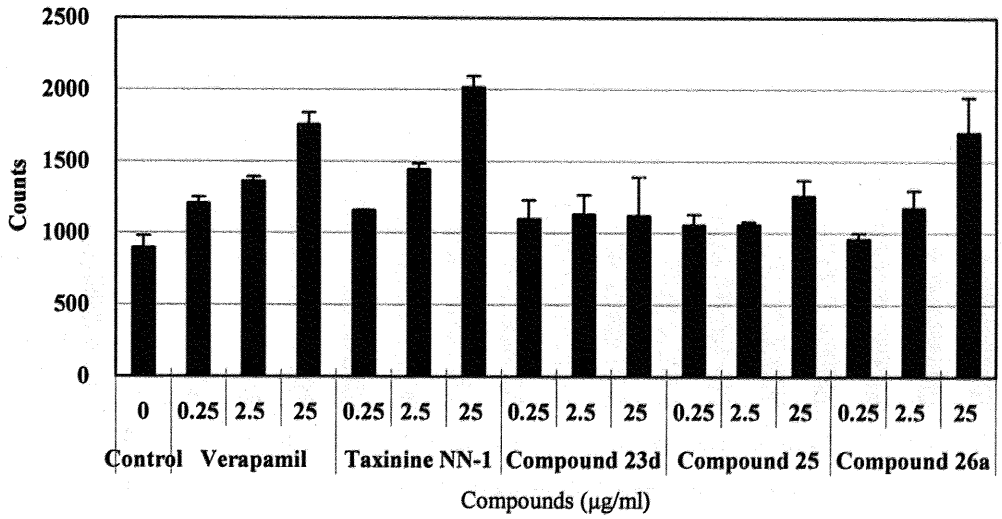


Figure 3-3

Exp. 3-2(a)

Table3-3. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3653 ± 176
Verapamil	0.25	3743 ± 159
	2.5	4161 ± 161
	25	5556 ± 81
Taxinine NN-1	0.25	3855 ± 136
	2.5	4712 ± 264
	25	5884 ± 518
19	0.25	3782 ± 243
	2.5	3593 ± 57
	25	4347 ± 462
21	0.25	3669 ± 99
	2.5	3144 ± 277
	25	4331 ± 725
23a	0.25	3669 ± 99
	2.5	3144 ± 277
	25	4331 ± 725
23b	0.25	3669 ± 99
	2.5	3144 ± 277
	25	4331 ± 725

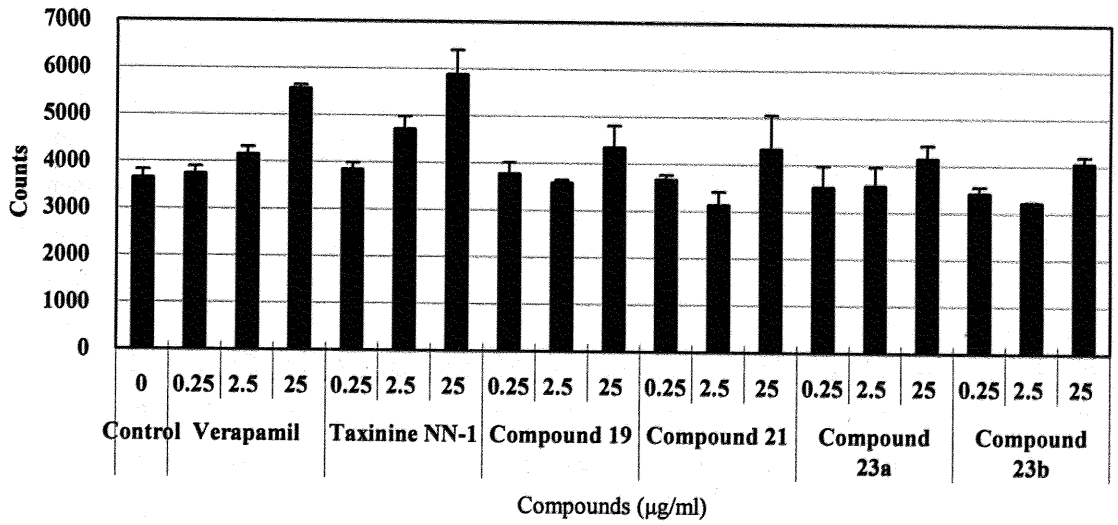


Figure 3-4

Exp. 3-2(b)

Table3-4. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3653 ± 176
Verapamil	0.25	3743 ± 159
	2.5	4161 ± 161
	25	5556 ± 81
Taxinine NN-1	0.25	3855 ± 136
	2.5	4712 ± 264
	25	5884 ± 518
23c	0.25	3295 ± 148
	2.5	3891 ± 230
	25	4863 ± 3
25a	0.25	3712 ± 266
	2.5	3469 ± 170
	25	4408 ± 412
27a	0.25	3581 ± 170
	2.5	3492 ± 234
	25	4561 ± 175
29	0.25	3430 ± 113
	2.5	3586 ± 509
	25	4027 ± 148

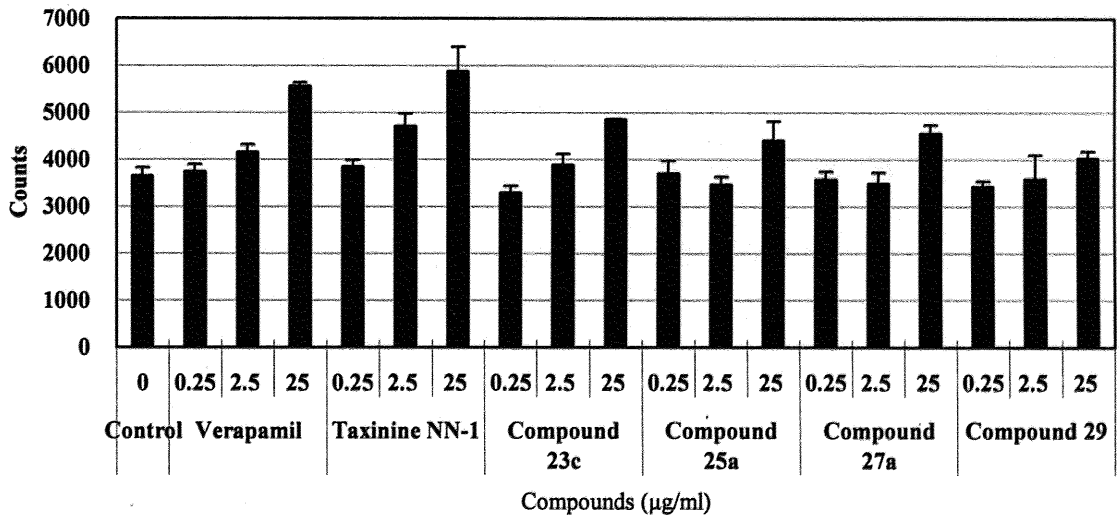


Figure 3-5

Exp. 3-3(a)

Table3-5. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	4012 ± 170
Verapamil	0.25	4365 ± 158
	2.5	4399 ± 110
	25	5254 ± 136
Taxinine NN-1	0.25	4147 ± 221
	2.5	4630 ± 45
	25	5762 ± 275
23f	0.25	3529 ± 32
	2.5	3458 ± 50
	25	4165 ± 33
24	0.25	4129 ± 191
	2.5	3918 ± 364
	25	4465 ± 489
28	0.25	4092 ± 210
	2.5	4060 ± 148
	25	4429 ± 343
28a	0.25	3449 ± 114
	2.5	3374 ± 8
	25	3894 ± 129

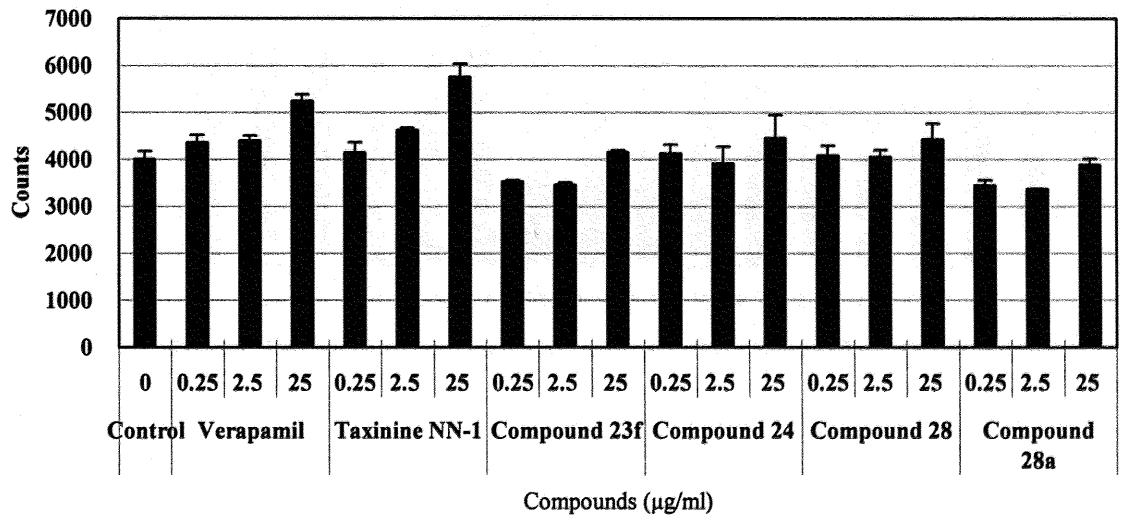


Figure 3-6

Exp. 3-3(b)

Table3-6. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	4012 ± 170
Verapamil	0.25	4365 ± 158
	2.5	4399 ± 110
	25	5254 ± 136
Taxinine NN-1	0.25	4147 ± 221
	2.5	4630 ± 45
	25	5762 ± 275
29a	0.25	3529 ± 32
	2.5	3458 ± 50
	25	4165 ± 33
30	0.25	4129 ± 191
	2.5	3918 ± 364
	25	4465 ± 489
30a	0.25	4092 ± 210
	2.5	4060 ± 148
	25	4429 ± 343

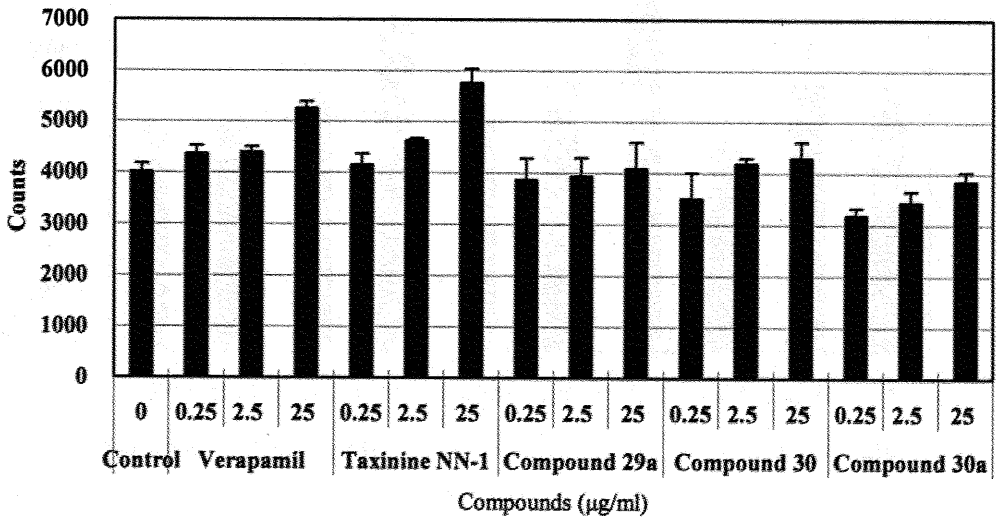


Figure 3-7

Exp. 3-4

Table3-7. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3653 ± 176
Verapamil	0.25	3743 ± 159
	2.5	4161 ± 161
	25	5556 ± 81
Taxinine NN-1	0.25	3855 ± 136
	2.5	4712 ± 264
	25	5884 ± 518
24a	0.25	3369 ± 154
	2.5	4483 ± 661
	25	4587 ± 69

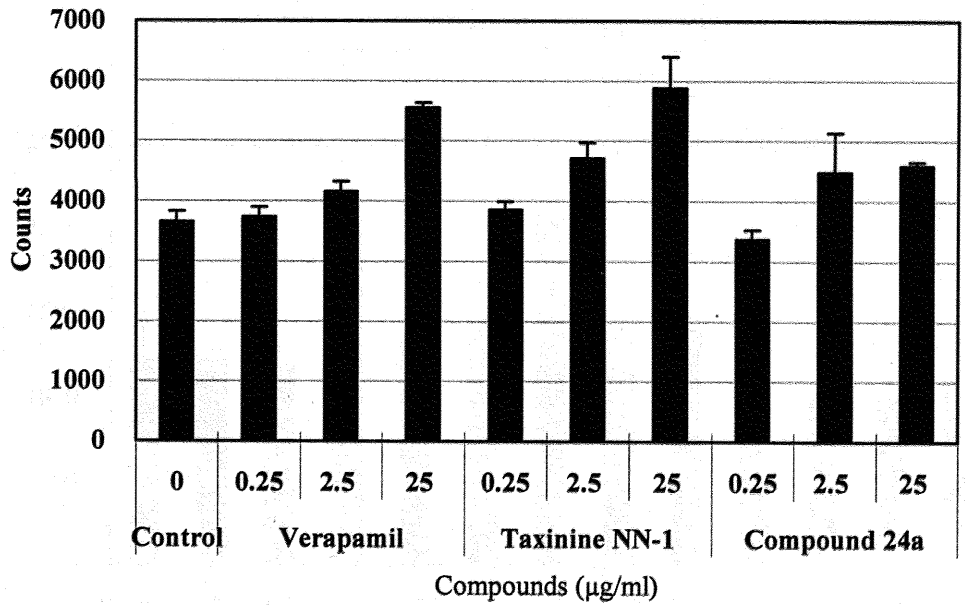


Figure 3-8

Exp. 3-5

Table3-8. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	1240 ± 203
Verapamil	0.25	1232 ± 55
	2.5	1190 ± 124
	25	2063 ± 13
Taxinine NN-1	0.25	1333 ± 34
	2.5	1586 ± 92
	25	2084 ± 151
26b	0.25	1349 ± 76
	2.5	1405 ± 86
	25	1397 ± 18
26c	0.25	1031 ± 53
	2.5	996 ± 207
	25	1115 ± 31

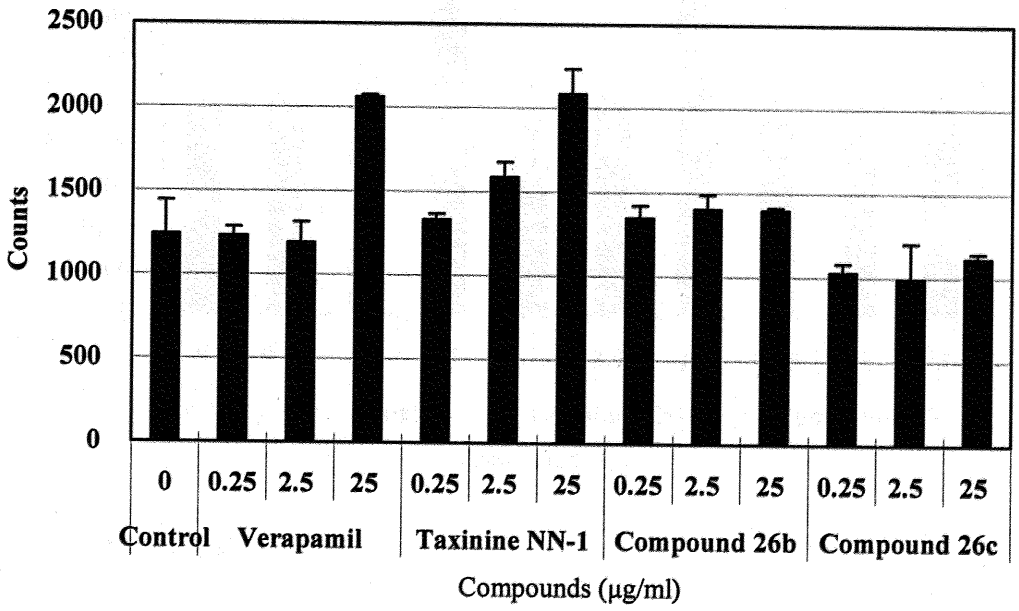


Figure 3-9

Exp. 3-6

Table3-9. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	2472 ± 123
Verapamil	0.25	2695 ± 134
	2.5	2848 ± 134
	25	4059 ± 58
Taxinine NN-1	0.25	2581 ± 169
	2.5	2692 ± 54
	25	4183 ± 338
27	0.25	2441 ± 158
	2.5	2454 ± 76
	25	2734 ± 167

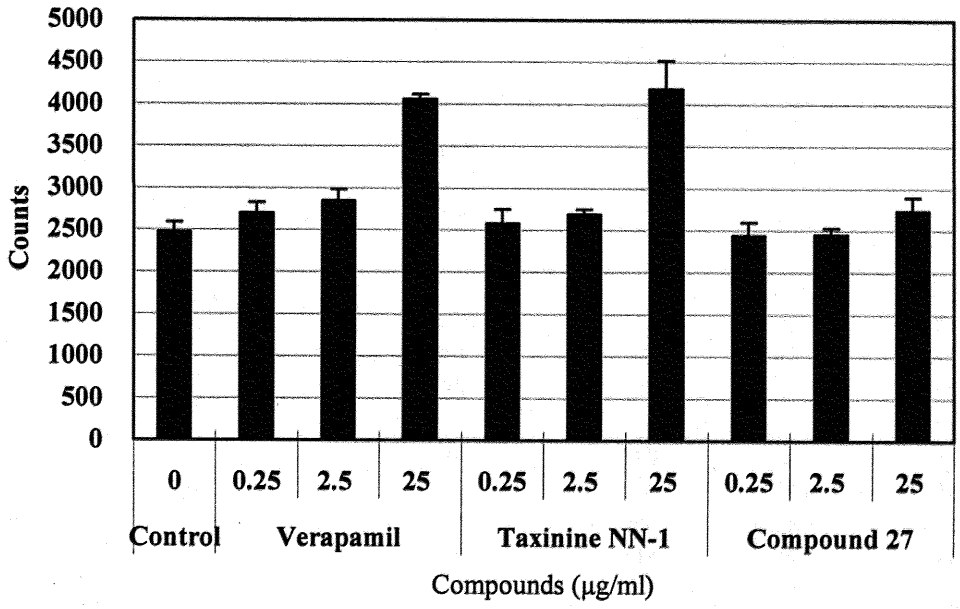
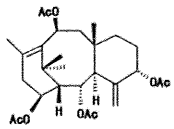
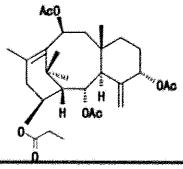
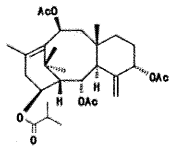
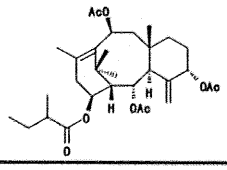
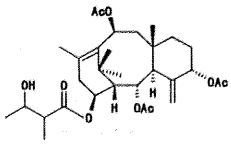
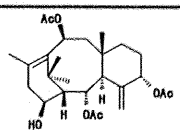
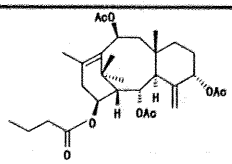
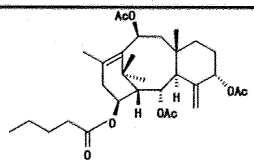
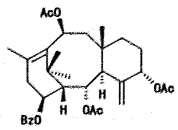


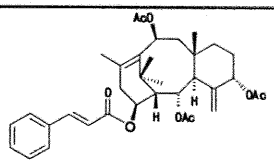
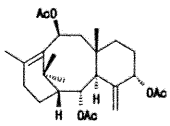
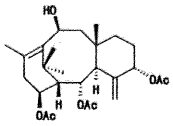
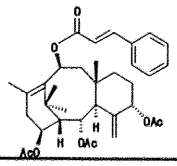
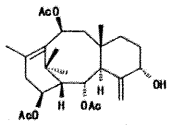
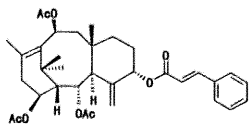
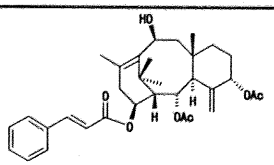
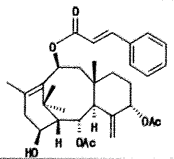
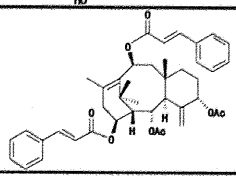
Figure 3-10

Table 3-10 (a). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound	Structure	μg/ml	Calcein accumulation[a] % control[b]	%verapamil	%Taxinine NN-1
Taxuyunnanine C (18)		0	100	100	100
		0.25	130	96	100
		2.5	141	93	88
		25	127	65	57
19		0	100	100	100
		0.25	104	101	98
		2.5	98	86	76
		25	119	78	74
20		0	100	100	100
		0.25	133	99	103
		2.5	139	91	86
		25	148	75	66
21		0	100	100	100
		0.25	100	98	95
		2.5	86	76	67
		25	119	78	74
22		0	100	100	100
		0.25	81	61	63
		2.5	94	62	58
		25	168	86	75
23		0	100	100	100
		0.25	104	78	81
		2.5	86	57	54
		25	148	75	66
23a		0	100	100	100
		0.25	97	94	92
		2.5	98	86	76
		25	114	75	71
23b		0	100	100	100
		0.25	94	91	89
		2.5	88	77	68
		25	111	73	69
23c		0	100	100	100
		0.25	90	88	85
		2.5	107	93	83
		25	133	88	83

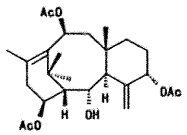
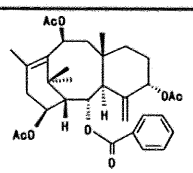
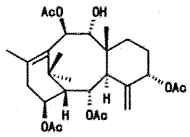
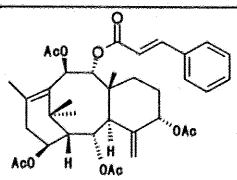
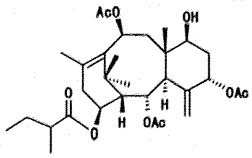
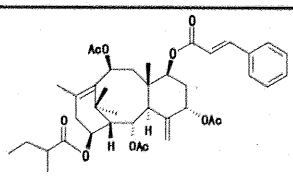
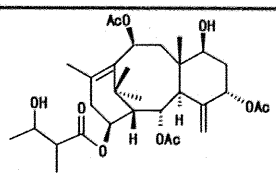
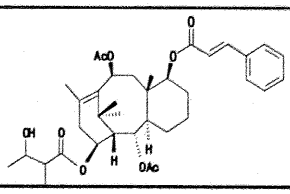
[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b]The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

Table 3-10 (b). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound	Structure	μg/ml	Calcein accumulation[a] % control[b]	%verapamil	%Taxinine NN-1
23d		0	100	100	100
		0.25	123	91	95
		2.5	126	83	79
		25	125	64	56
23f		0	100	100	100
		0.25	88	81	85
		2.5	86	79	75
		25	104	79	72
24		0	100	100	100
		0.25	103	95	100
		2.5	98	89	85
		25	111	85	77
24a		0	100	100	100
		0.25	92	90	87
		2.5	123	108	95
		25	126	83	78
25		0	100	100	100
		0.25	118	88	91
		2.5	118	78	73
		25	141	72	62
25a		0	100	100	100
		0.25	102	99	96
		2.5	95	83	74
		25	121	79	75
26a		0	100	100	100
		0.25	107	80	83
		2.5	132	87	82
		25	190	97	84
26b		0	100	100	100
		0.25	109	109	101
		2.5	113	118	89
		25	113	68	67
26c		0	100	100	100
		0.25	83	84	77
		2.5	80	84	63
		25	90	54	54

[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b] The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

Table 3-10 (c). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound	Structure	μg/ml	Calcein accumulation[a] % control[b]	%verapamil	%Taxinine NN-1
27		0	100	100	100
		0.25	99	91	95
		2.5	99	86	91
		25	111	67	65
27a		0	100	100	100
		0.25	98	96	93
		2.5	96	84	74
		25	125	82	78
28		0	100	100	100
		0.25	102	94	99
		2.5	101	92	88
		25	110	84	77
28a		0	100	100	100
		0.25	86	79	83
		2.5	84	77	73
		25	97	74	68
29		0	100	100	100
		0.25	94	92	89
		2.5	98	86	76
		25	110	72	68
29a		0	100	100	100
		0.25	96	88	93
		2.5	98	90	85
		25	102	78	71
30		0	100	100	100
		0.25	87	80	85
		2.5	104	95	90
		25	107	82	75
30a		0	100	100	100
		0.25	80	73	77
		2.5	86	78	74
		25	96	73	67

[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b]The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

3-3-2 Accumulation of the Vincristine in MDR Cancer Cells

The effects of several taxoids, **23**, **24**, **27**, **28**, **23d**, **24a**, **26a** **26b**, and **26c** on the accumulation of a widely used anticancer agent, vincristine (VCR) in MDR 2780AD cells were tested comparing with the effects of verapamil as a positive control (Table 3-11).

Compound **26a** possessing a hydroxyl group at the C-10 position and a cinnamoyloxy group at the C-14 position showed the highest activity of cellular accumulation of vincristine in MDR 2780AD cells among the taxoids subjected to the test. Compound **26b**, regioisomer of **26a**, with a cinnamoyloxy group at C-10 and a hydroxyl group at C-14 showed weaker activity than that of **26a**.Taxoid **26c** bearing cinnamoyloxy groups at both C-10 and C-14 positions showed very weak activity. The activity of taxoid **24a** with a cinnamoyloxy group at the C-10 position was higher than that of taxioid **7** with a hydroxyl group at the corresponding positions. The results were similar to the calcein assay though there were some little differences.

3-3-3 MDR Reversal Activity of the Compound toward MDR Cancer Cells

The effects of compounds **23** and **26a** on the cytotoxicity of taxol, adriamycin (ADM) and vincristine (VCR) toward MDR 2780AD cells and its parental cells (A2780) were tested comparing with verapamil, which is a well-known MDR reversal agent (Table 3-12, Figure 3-11(a)(b)(c)).

Cytotoxicity of modurators in the absence of the anticancer agents was also shown in the table 3-12 as viability (%) of the cells comparing to that in the absence of modulators.

The IC_{50} values of taxol for A2780 and MDR 2780AD cells were 2.8 and 183 nM, respectively.

When compound **23** was added at a final concentration of 0.2, 2.0 and 10 μM, the IC_{50} values of taxol for MDR 2780AD cells were shifted to 21, 26 and 5.3 nM respectively. At this time, cytotoxicity of compound **23** against 2780AD cells was equal to that of verapamil, showing more than 70% of cell viability at the concentration of 10μM, while .compoud **23** showed significant cytotoxicity against A2780 cells showing 20% of cell viability at the same concentration. From these results, compound **23** enhanced the sensitivity to taxol in MDR 2780AD cells. The IC_{50} of taxol for 2780AD cells in the presence of 10μM of compound **23** was equal to that for parental A2780 cells.

Compound **23** is also effective for ADM and VCR. The IC_{50} values of ADM for A2780 and MDR 2780AD cells were 5.7 nM and 298 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 215, 256 and 181 nM in the presence of **23** at a final concentration of 0.2, 2.0 and 10 μM, respectively. The IC_{50} values of VCR for A2780 and 2780AD cells were 3.1 nM and 311 nM,

respectively. The IC_{50} values for MDR 2780AD cells were shifted to 55, 26, and 11 nM in the presence of 23 at a final concentration of 0.2, 2.0, and 10 μM, respectively. The enhancing effects of **23** for taxol, ADM, and VCR were the same levels as that of verapamil toward MDR 2780AD cells. Thus, compound **23** can modulate the multidrug resistance of cancer cells as well as verapamil *in vitro*.

Cytotoxicity of compound **26a** against 2780AD cells in the absence of anticancer agents was more significant than that of compound **23**, showing 38% of cell viability at the concentration of 10μM, while the viability of the cell was 27% in the case of parental A2780 cells at the same concentration of compound **26a**. IC_{50} of compound **26a** for MDR 2780AD and parental A2780 was calculated as 8.0 and 6.1μM, respectively. Thus compound **26a** showed MDR reversal activity against 2780AD cells.

When compound **26a** was added at a final concentration of 0.2, 2.0 and 10 μM, the IC_{50} values of taxol for MDR 2780AD cells were shifted to 22, 4.2 and 3.9 nM respectively.

These results showed that compound **26a** enhanced the sensitivity to taxol in MDR 2780AD cells.

Compound **26a** is also effective for ADM and VCR. The IC_{50} values of ADM for A2780 and MDR 2780AD cells were 5.7 nM and 298 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 284,138 and 73 nM in the presence of **26a** at a final concentration of 0.2, 2.0 and 10 μM, respectively. The IC_{50} values of VCR for A2780 and 2780AD cells were 3.1 nM and 311 nM, respectively. The IC_{50} values for MDR 2780AD cells were shifted to 51, 39, and 23 nM in the presence of **26a** at a final concentration of 0.2, 2.0, and 10 μM, respectively. Thus, compound **26a** can modulate the multidrug resistance of cancer cells as well as verapamil *in vitro*.

Table 3-11. Effects of Compounds on the Accumulation of Vincristine (VCR) in Multidrug-Resistant Cells 2780AD

	conc. (μg/mL)	average[b] (dpm/well)	% of control[c]	activities[d]	verapamil[e] (%)
23	0.1	360	102	±	94
	1	517	147	+	79
	10	853	242	+	53
23d	0.1	336	95	±	87
	1	494	140	+	75
	10	985	280	+	62
24	0.1	324	92	±	84
	1	420	119	+	64
	10	805	229	+	51
24a	0.1	404	132	+	130
	1	641	209	+	118
	10	1385	451	++	81
26a	0.1	425	121	+	111
	1	959	272	+	146
	10	1855	527	+++	116
26b	0.1	402	114	+	105
	1	669	190	+	102
	10	1749	497	++	110
26c	0.1	320	91	±	83
	1	325	92	±	49
	10	429	122	+	27
27	0.1	306	101	±	89
	1	436	144	+	84
	10	786	259	+	74
28	0.1	303	86	-	79
	1	379	108	±	58
	10	592	168	+	37

[a]The amount of VCR accumulated in MDR2780AD cells was determined with the control in the presence of 0.1, 1, and 10 μg/ml of taxoids. [b]The values represent the mean of triplicated determination. [c]The values are the relative amount of VCR accumulated in the cell compared with the control experiment. [d]The indices are expressed on a scale of seven by the range of the relative amount of VCR accumulation as compared with the control experiment(%): +++, 501-1000%; ++, 301-500%; +, 111-300%; ±, 91-100%; –, <90%. [e]The values are expressed as the relative amount of vincristine(VCR) accumulation in the cell as compared with that of verapamil.

Table 3-12. Effect of the compounds on the cytotoxicity of anticancer agents toward A2780 and MDR 2780AD cells

Cell lines	MDR modulator	(μM)	Viability of the cell (%)[a]	IC_{50}(nM) of anticancer agents[b]		
				Taxol	ADM	VCR
A2780	no modulator	0	100	2.8	5.7	3.1
	Verapamil	0.2	93	2.7	5.8	3.4
		2	82	2.9	6.0	2.0
		10	53	3.5	7.7	0.5
	23	0.2	97	2.6	7.0	2.6
		2	95	2.3	6.0	1.6
		10	20	6.6	7.8	4.8
	26a	0.2	89	2.6	7.0	2.6
		2	74	2.3	6.0	1.6
		10	27	6.6	7.8	4.8
2780AD	no modulator	0	100	183	298	311
	Verapamil	0.2	84	36	269	41
		2	90	5.5	201	25
		10	71	4.9	164	3.9
	23	0.2	101	21	215	55
		2	96	26	256	26
		10	77	5.3	181	11
	26a	0.2	105	22	284	51
		2	86	4.2	138	39
		10	38	3.9	73	23

[a] Cyfotoxicity of the compounds were evaluated in the absence of the anticancer agents.

[b] Enhancing effects of the compounds on the cytotoxicity of Taxol, adriamycin (ADM) and vincristin (VCR) toward A2780 cells and MDR A2780 (2780AD)cells were determined in the presence of 0.2, 2.0 and 10 μM of each compounds.

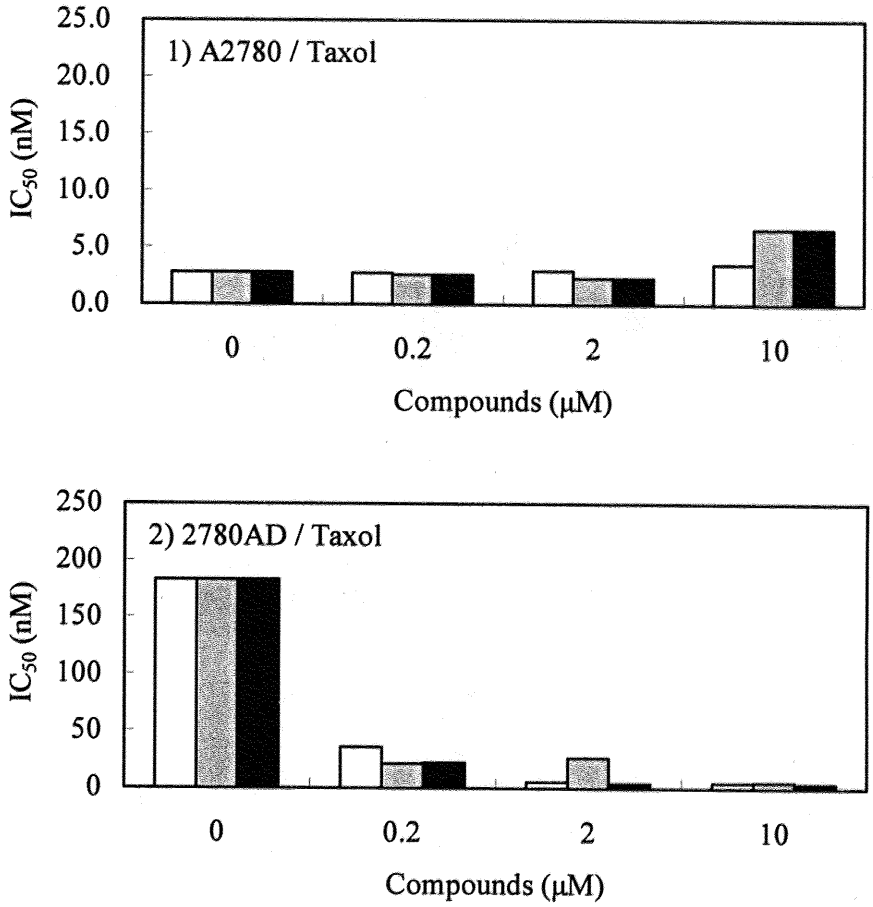


Figure 3-11(a)

Enhancing effects of verapamil (□) ,compound **23**(■) and **26a** (■)on the cytotoxicity of Taxol toward A2780 cells(1) and 2780AD cells(2) were determined in the presence of 0.2, 2.0 and 10 μM of each compounds.The values represent means of triplicate determination.

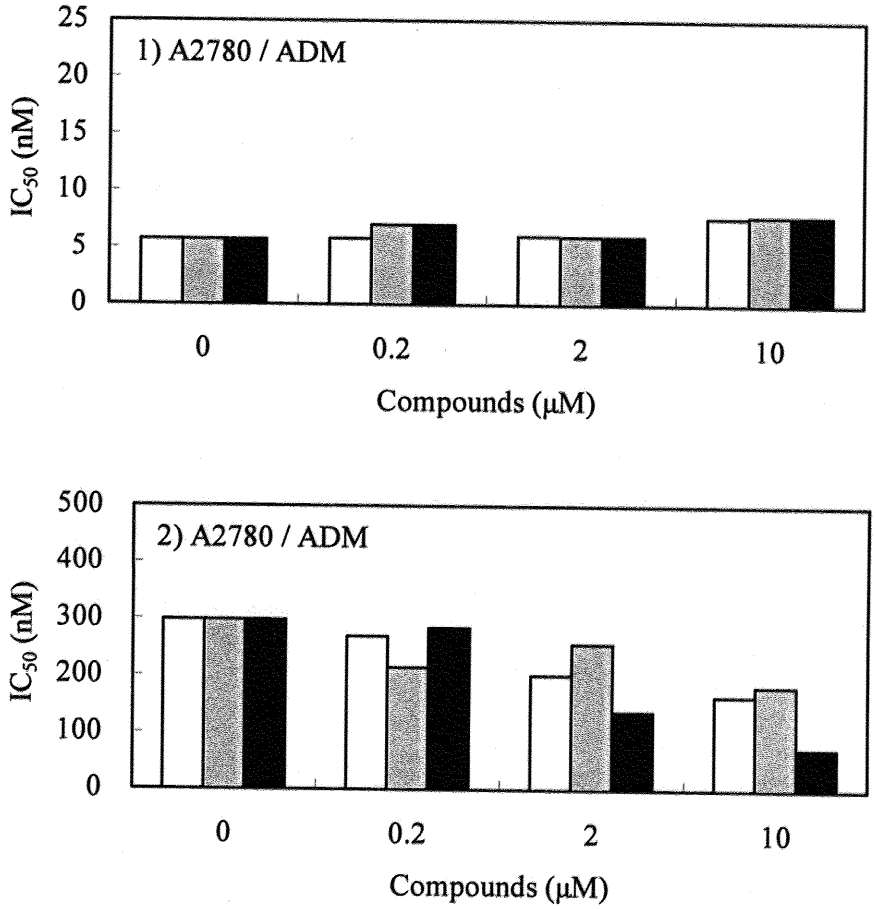


Figure 3-11(b)

Enhancing effects of verapamil (□) ,compound **23**(■) and **26a** (■)on the cytotoxicity of ADM toward A2780 cells(1) and 2780AD cells(2) were determined in the presence of 0.2, 2.0 and 10 μM of each compounds.The values represent means of triplicate determination.

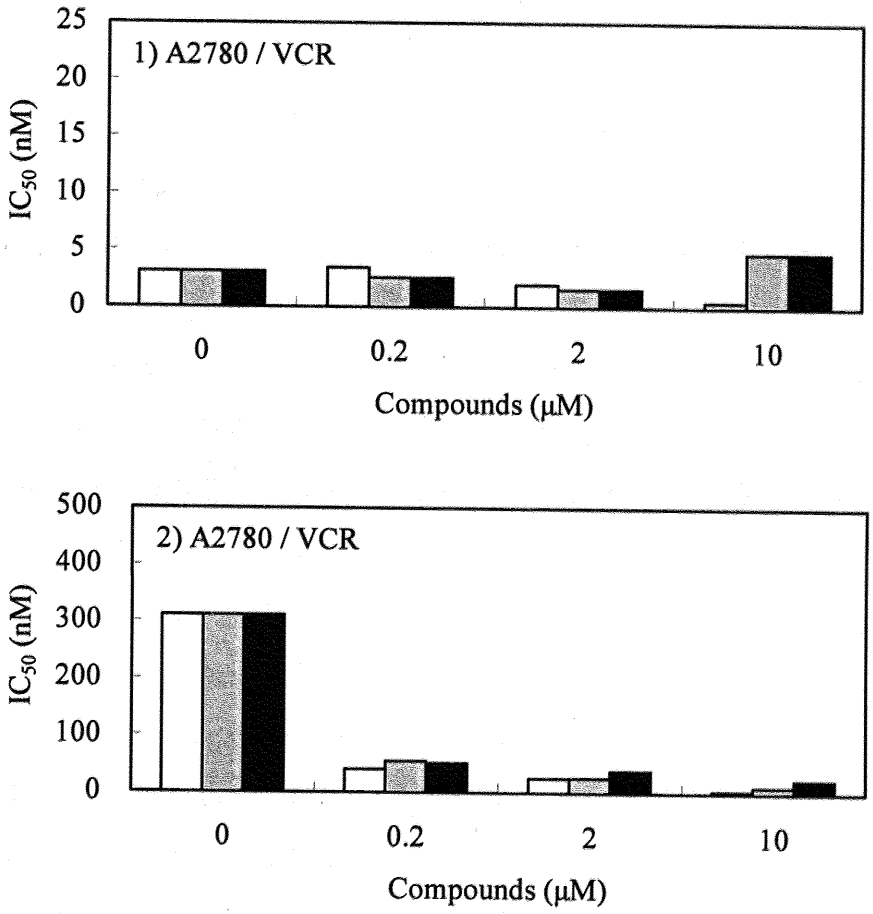


Figure 3-11(c)

Enhancing effects of verapamil (□) ,compound **23**(■) and **26a** (■)on the cytotoxicity of VCR toward A2780 cells(1) and 2780AD cells(2) were determined in the presence of 0.2, 2.0 and 10 μM of each compounds.The values represent means of triplicate determination.

3-3-4 Cell growth Inhibition

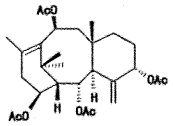
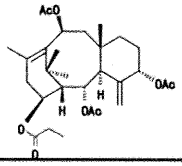
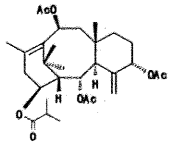
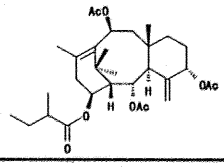
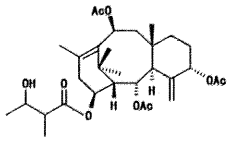
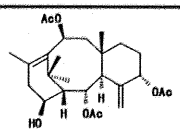
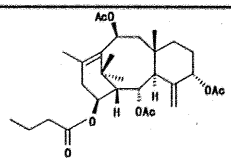
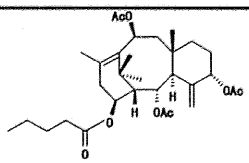
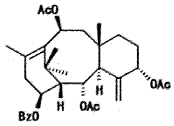
Cell growth inhibitory activity (IC_{50}) of taxoids to three different cell lines was examined and the results were summarized in Table 3-14(a), (b), and (c)).

Taxuyunnanine C (**18**) and its analogs **23—25, 27,** and **28** showed weak activity in many cases. Only taxuyunnanine C (**18**) and its C-10 deacetyl taxuyunnanine C (**24**) showed significant activity against HepG2 and VA-13, respectively. On the contrary, 14-deacetoxytaxuyunnanine C (**23f**) showed significant activities to all three cell lines. This result contrasted with the result of calcein assay and suggested that the modification of the C-14 position of the taxan skeleton significantly affected the biological activities.

The cytotoxic activity on HepG2 of taxuyunnanine C analogs, **19—21, 23a**, and **23b** bearing different kinds of acyloxy groups at C-14 increased according to the increase of hydrophobicity of their acyloxy group at C-14. In them, **23a** also showed significant cytotoxic activity on VA-13 in addition to the activity on HepG2. However, these compounds showed very weak or no cytotoxic activity against normal cell lines, WI-38. This is a desirable result in the screening of anticancer agents.

The cytotoxicity of **24a** was examined, in the results of *in vitro* primary screening of **24a** based on the 39 human cancer cell lines[17,18] shown in Table 3-14. Although the effective concentration of **7a** is rather high, differential growth inhibition is recognized. Since the result of COMPARE of **24a** is marginal ($0.5 < r < 0.75$), it possibly belongs to a unique mechanistic class and is new member of anticancer agents. On the other hand, cell growth inhibition effect (MG-MID of $GI_{50} = -4.41$) and differential growth inhibition of compound **24a** are weak. The result suggests that compound **24a** is not effective an anticancer agent.

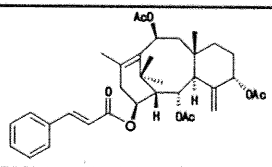
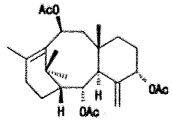
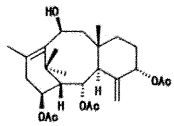
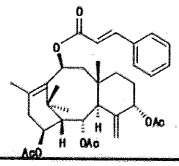
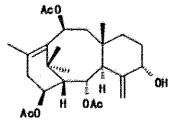
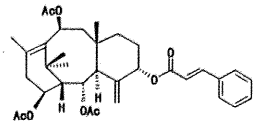
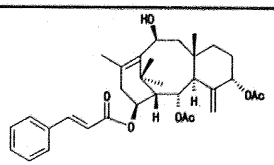
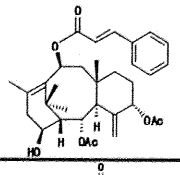
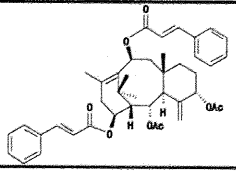
Table 3-14(a). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compounds			Cell line	Cytotoxicity (IC_{50})[a]		
	Structure	M.W.		μg/ml	μM	Selectivity[b]
Taxuyunnanine C (18)		504.6	WI-38 VA-13 HepG2	66 27 9	130 53 18	1.00 2.45 7.05
19		518.6	WI-38 VA-13 HepG2	>100 49 45	>193 94 86	1.00 >2.06 >2.23
20		532.7	WI-38 VA-13 HepG2	>100 48 15	>188 91 28	1.00 >2.07 >6.71
21		546.7	WI-38 VA-13 HepG2	>100 33 23	>183 61 42	1.00 >2.99 >4.39
22		562.7	WI-38 VA-13 HepG2	45 5 10	79 9 18	1.00 8.55 4.30
23		462.3	WI-38 VA-13 HepG2	62 37 >100	133 80 >216	1.00 1.66 <0.62
23a		532.7	WI-38 VA-13 HepG2	39 9 10	73 17 18	1.00 4.27 3.97
23b		546.7	WI-38 VA-13 HepG2	>100 31 9	>183 57 17	1.00 >3.23 >11.04
23c		566.7	WI-38 VA-13 HepG2	>100 >100 81	>176 >176 144	1.00 - >1.23

[a] IC_{50} represent means of duplicate determination.

[b] The values are the ratio of IC_{50} (μM) (WI-38/VA-13, or WI-38/HepG2)

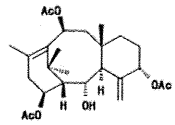
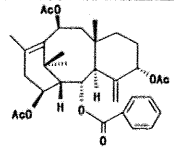
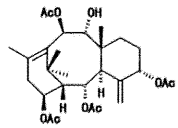
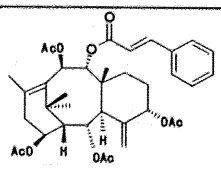
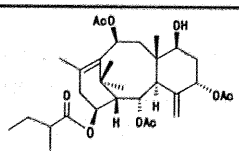
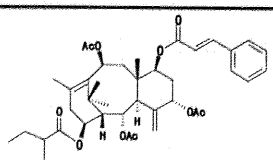
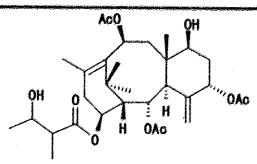
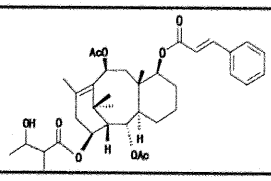
Table 3-14(b). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compounds			Cell line	Cytotoxicity (IC_{50})[a]		
	Structure	M.W.		μg/ml	μM	Selectivity[b]
23d		592.7	WI-38 VA-13 HepG2	91 >100 >100	154 >169 >169	1.00 <0.91 <0.91
23f		446.6	WI-38 VA-13 HepG2	4 5 5	9 12 10	1.00 0.73 0.87
24		520.7	WI-38 VA-13 HepG2	48 6 50	93 11 96	1.00 8.30 0.97
24a		592.7	WI-38 VA-13 HepG2	>100 >100 63	>169 >169 106	1.00 - >1.59
25		462.3	WI-38 VA-13 HepG2	69 49 85	149 105 183	1.00 1.41 0.81
25a		592.7	WI-38 VA-13 HepG2	59 74 6	99 125 10	1.00 0.79 9.95
26a		550.7	WI-38 VA-13 HepG2	7 21 9	13 37 16	1.00 0.36 0.85
26b		550.7	WI-38 VA-13 HepG2	40 6 8	72 11 15	1.00 6.25 4.70
26c		680.8	WI-38 VA-13 HepG2	>100 >100 >100	>147 >147 >147	1.00 - -

[a] IC_{50} represent means of duplicate determination.

[b] The values are the ratio of IC_{50} (μM) (WI-38/VA-13, or WI-38/HepG2)

Table 3-14(c). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compounds			Cell line	Cytotoxicity (IC_{50})[a]		
	Structure	M.W.		μg/ml	μM	Selectivity[b]
27		462.6	WI-38 VA-13 HepG2	60 48 57	130 104 122	1.00 1.26 1.07
27a		566.7	WI-38 VA-13 HepG2	71 99 29	125 175 52	1.00 0.72 2.42
28		520.6	WI-38 VA-13 HepG2	67 >100 67	129 >192 128	1.00 <0.67 1.00
28a		650.3	WI-38 VA-13 HepG2	98 >100 >100	151 >154 >154	1.00 <0.98 <0.98
29		562.7	WI-38 VA-13 HepG2	46 45 42	82 79 75	1.00 1.03 1.09
29a		692.4	WI-38 VA-13 HepG2	>100 >100 >100	>144 >144 >144	1.00 - -
30		578.7	WI-38 VA-13 HepG2	81 >100 88	140 >173 153	1.00 <0.81 0.92
30a		708.4	WI-38 VA-13 HepG2	44 35 57	62 50 80	1.00 1.25 0.78

[a] IC_{50} represent means of duplicate determination.

[b] The values are the ratio of IC_{50} (μM) (WI-38/VA-13, or WI-38/HepG2)

Table 3-14. Summary of evaluation of compound 24a based on the 39 human cancer cell lines.

Parameters of effective concentrations

	GI_{50}	TGI	LC_{50}
MG-MID	-4.41	-4.04	-4.00

Results of the COMPARE analysis

Rank	Compounds	r[a]	Molecular Targets / Drug Type
1	ICRF-154	0.581	topoisomerase
2	ICRF-193	0.580	topoisomerase
3	TNP-470	0.552	

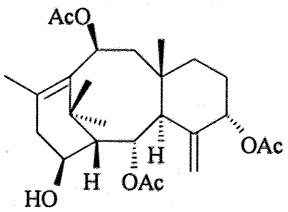
The mean graph of compound **24a** was compared with those of 200 standard compounds using the COMPARE analysis. Drugs were ordered according to the correlation coefficient. Drugs with correlation coefficients higher than 0.5 ($P < 0.001$) were included.

[a] Peason correlation coefficient.

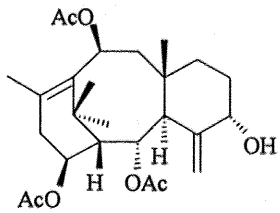
3-4 Conclusion

Compounds **23**, **25**, and **23c** showed the activity toward calcein accumulation in MDR 2780AD cells but have no cytotoxicity. These compounds are expected to be lead compounds of MDR cancer reversal agents. On the other hand, cytotoxic activity of compounds **23f** and **25a** against HepG2 is at the same level of paclitaxel. Since compounds **25a** showed moderate activity toward calcein accumulation in MDR 2780AD cells, it is expected as lead compounds of new-type anticancer agents. Compounds **22**, **23a**, **24**, and **26b** showed significant cytotoxic activity toward VA-13.

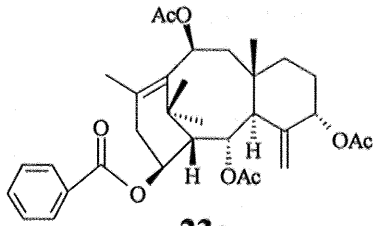
Compounds **5** showed significant cytotoxic activity toward VA-13 and HepG2 along with the strong activity toward calcein accumulation in MDR 2780AD cells. Compounds **20** showed significant cytotoxic activity toward HepG2 along with the strong activity toward calcein accumulation in MDR 2780AD cells. Compounds **26a** showed significant cytotoxic activity toward WI-13 and HepG2 along with the strongest activity in tested samples toward calcein accumulation in MDR 2780AD cells. They are expected to be lead compounds of anti-MDR cancer agents or anticancer agents.



23



25

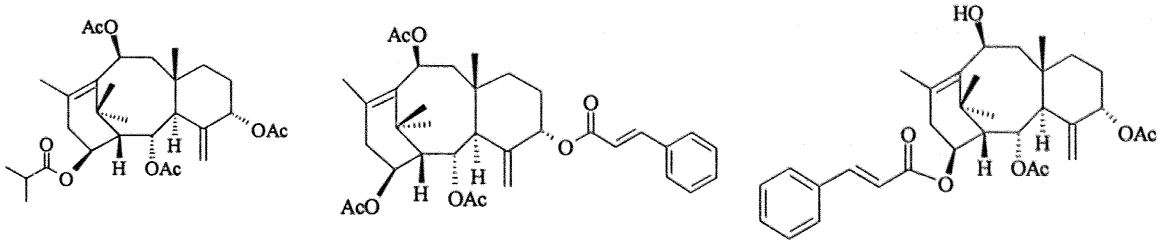


23c

Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
23	133	80	>216	148
25	149	105	183	141
23c	>176	>176	144	133

*Concentration of compounds : 25 μ g/ml

Figure 3-12. Candidates as MDR modulator

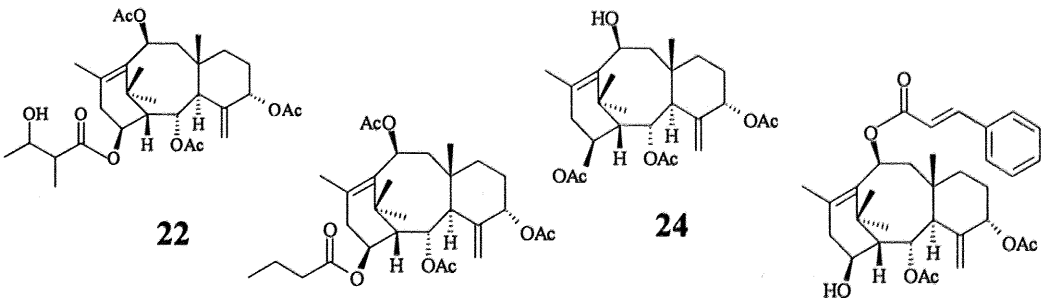


20 **25a** **26a**

Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
20	>188	90.9	28.0	148
25a	99.0	125	9.95	121
26a	13.5	37.4	15.7	190

*Concentration of compounds : 25 μ g/ml

Figure 3-13(a). Candidates as anticancer agent with MDR reversal activity



22 **24**

23a **26b**

Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
22	79.4	9.29	18.5	168
23a	73.4	17.2	18.5	114
24	105	12.6	108	111
26b	71.7	11.5	15.3	113

*Concentration of compounds : 25 μ g/ml

Figure 3-13(b). Candidates as anticancer agent with MDR reversal activity

3-5 Summary

A series of new generation taxoids bearing a bulky group on different positions such as C-2, C-5, C-7, C-9, C-10 or C-14 was obtained by chemical modifications and biotransformation of taxuyunnanine C (**18**) and its analogs, **21**, **22**, and **27**. Compounds **20**, **22**, **23**, **25**, and **26a** showed significant activity toward calcein accumulation in MDR 2780AD. The most effective compound **26a** with a cinnamoyloxy group at C-14 and a hydroxyl group at C-10 was actually efficient for the cellular accumulation of the anti-cancer agent, vincristine, in MDR 2780AD cells. The cytotoxicity (IC_{50}) of the compounds was examined against human normal cell line, WI-38 and cancer model cell lines, VA-13 and HepG2. Since compounds **23** and **25** had no cytotoxicity, they were expected to be lead compounds of MDR cancer reversal agent. On the contrary, compounds **20**, **22**, and **26a** showed cell growth inhibitory activity toward VA-13 and/or HepG2 as well as accumulation activity of calcein and/or vincristine in MDR 2780AD and they were expected to be lead compounds of new-type anticancer agents.

Part4 Structure–Activity Relationships of Some Lignans as Multidrug Resistance Modulator

4-1 Introduction

Peperomia dindygulensis Miq. (Piperaceae) is named "shi-chan-cao" in the People's Republic of China. It grows mainly in Yunnan, Guangxi, Guangdong, Fujian and Taiwan Provinces, and traditionally has been used in folk remedies to treat stomach, mammary, liver, and esophageal cancers.[56] Thirteen secolignans, including eight new ones (**31**—**38**), and five known compounds, peperomins A, B, C,[57] E (**39**—**42**), and F,[58] were isolated in the further study of chemical constituents. From a same species, five new tetrahydrofuran lignans (**43**—**47**) and four known compounds, 2-(3-phenylpropionyl)-1,3-cyclohexanedione,ergosta-6,22-diene-3β,5α,8α-triol,[59] 5-hydroxy-7,4'- dimethoxyflavone,[60] and 5-hydroxy-7,8,3',4'-tetramethoxyflavone,[61] were obtained.

Peperomia duclouxii C. DC. in Lecomte (Piperaceae) is also a folk anticancer herb in the People's Republic of China.[62] Five dibenzylbutyrolatone (**48**—**52**) and four dibenzylbutanediol lignans had been obtained from the ethyl acetate extract in our previous investigation.[63] Further work of its chemical constituents resulted in the isolation of six new dibenzylbutyrolactone (**53**—**58**) and two new dibenzylbutanediol lignans (**59**, **60**).

Although lignans seems to be the active ingredient of those herbal medicines, the structure-activity relationships of them have not been studied enough. In this part, I report the study on the structure–activity relationships of the above mentioned lignans as MDR modulator and anticancer agent.

4-2 Experimental Method

4-2-1 Isolation of the compounds

Plant Material.: The whole plant of *P. dindygulensis* and was collected from Yunnan Province, People's Republic of China, in February 2002. The plant was identified by Mr. Kaijiao Jiang, Kunming Institute of Botany. A voucher specimen (PDi-2002-2) has been deposited at Faculty of Engineering, Niigata University, Japan.

The whole plants of *P. duclouxii* were collected from Lvchun, Yunnan Province, People's Republic of China, in February 2002. The plant was identified by Mr. Kaijiao Jiang, Kunming Institute of Botany. A voucher specimen (2002-2) has been deposited at the Faculty of Engineering, Niigata University, Japan.

Extraction and Isolation.:

a) Secolignans (31—42) from *P. dindygulensis*

As shown in Scheme 4-1, the dried plant material (1.75 kg) was powdered and extracted three times (4 L/each) with MeOH at room temperature with the aid of a supersonic machine, and about 105 g of residue was obtained after evaporating the MeOH. The residue was suspended in H_2O and partitioned in sequence using hexane, EtOAc, and *n*-BuOH, respectively, to afford a hexane extract (40.7 g), an EtOAc extract (20.1 g), and an *n*-BuOH extract (15.6 g).

Scheme 4-2 shows steps for the separation of secolignans from the EtOAc extract. The EtOAc extract was separated into twelve fractions (F_1–F_{12}) by column chromatography over silica gel [7 cm i.d. column packed with silica gel (70–230 mesh, 500 g), solvent (hexane–EtOAc, gradient)]. F_6 [eluted with hexane–EtOAc (2:1), 2.30 g], F_7 [eluted with hexane–EtOAc (1:1), 3.52 g], and F_8 [eluted with hexane–EtOAc (1:5), 1.65 g] were further separated using silica gel column chromatography, normal-phase and reversed-phase HPLC methods. F_6 was subjected to silica gel column chromatography using a hexane–EtOAc gradient, yielding seven sub-fractions ($F_{6\text{-}1}$–$F_{6\text{-}7}$), and peperomins E (**42**, 5.0 mg) and A (**39**, 895.0 mg) were isolated from $F_{6\text{-}4}$ and $F_{6\text{-}5}$, respectively, by normal-phase HPLC using hexane–EtOAc as solvent. Peperomin B (**40**, 79.7 mg) and compound **34** (5.8 mg) were obtained from F_7 using silica gel column chromatography followed by normal-phase HPLC [hexane–EtOAc (55:45)].

As shown in Scheme 4-3, F_8 was divided into 8 sub-fractions ($F_{8\text{-}1}$–$F_{8\text{-}8}$) by silica gel column chromatography eluted with gradient of hexane and EtOAc. Compounds **31** (2.3 mg), **37** (3.2 mg)

and **38** (1.2 mg) were obtained from F_{8-4} using normal-phase HPLC [hexane–EtOAc (55:45)] and reversed-phase HPLC [MeOH–H_2O (7:3 and 5:5)]. Peperomin C (**41**, 38.7 mg) and compound **33** (5.5 mg) were isolated from F_{8-5} with normal-phase HPLC [hexane–EtOAc (55:45)]. Peperomin F (24.9 mg) was given from F_{8-6} using normal-phase HPLC [hexane–EtOAc (55:45)]. F_{8-7} yielded compound **36** (2.9 mg) and **35** (33.8 mg) with normal-phase HPLC [hexane–EtOAc (55:45)] and repeated reversed-phase HPLC [MeOH–H_2O (7:3)]. F_{8-8} afforded compound **32** (8.8 mg) using normal-phase [hexane–EtOAc (55:45)] and reversed-phase HPLC [MeOH–H_2O (7:3)] as solvent.

The structures of the compounds employed for the evaluation of their biological activities are shown in Figure 4-1.

b) Tetrahydrofuran lignans (**43—47**) from *P. dindygulensis*

As shown in Scheme 4-1, the dried plant material (1.75 kg) was powdered and extracted three times (4 L/each) with MeOH at room temperature with the aid of a supersonic machine, filtered, and concentrated to give residue (105g). The residue was suspended in H_2O and partitioned in sequence using hexane, EtOAc, and *n*-butanol, respectively, to afford a hexane extract (40.7 g), an EtOAc extract (20.1 g), and a *n*-butanol extract (15.6 g).

Scheme 4-4 shows steps for the separation of the tetrahydrofran lignans from the EtOAc extract. The EtOAc extract was separated into twelve fractions (F_1–F_{12}) by column chromatography over silica gel [7 cm i.d. column packed with silica gel (70–230 mesh, 500 g), solvent (hexane–EtOAc, gradient)]. F_4 [eluted with hexane–EtOAc (4 : 1), 0.45 g], F_6 [eluted with hexane–EtOAc (2 : 1), 2.30 g], and F_8 [eluted with hexane–EtOAc (1 : 5), 1.65 g] were further separated using silica gel column chromatography, normal-phase and reversed-phase HPLC methods.

5-Hydroxy-7,4'-dimethoxyflavone (3.1 mg) was obtained from F_4 using normal-phase HPLC [hexane–EtOAc (75:25)]. F_6 (2.30 g) was subjected to silica gel column chromatography using a hexane–EtOAc gradient into seven sub-fractions (F_{6-1}–F_{6-7}).

2-(3-phenylpropionyl) -1,3-cyclohexanedione (1.8 mg) and ergosta-6,22-diene-3β,5α,8α-triol (3.7 mg) were separated from F_{6-3} by normal-phase HPLC using a mixture of hexane–EtOAc (60:40 and 70:30). F_{6-5} was separated by normal phase HPLC [hexane–EtOAc (55:45)] to give 5-hydroxy-7,8,3',4'-tetramethoxyflavone (2.5 mg). Compounds **43** (33.8 mg), **44** (9.3 mg), **45** (0.6 mg), **46** (5.0 mg) and **47** (1.8 mg) were isolated from F_8 (1.65 g) using silica gel column chromatography

followed by normal-phase HPLC [hexane–EtOAc (55:45)] and reversed-phase HPLC [MeOH–H_2O (7:3)].

The structures for the compounds employed to evaluate their biological activities are shown in Figure 4-2.

c) Dibenzylbutyrolatone and Dibenzylbutanediol Lignans from *Peperomia duclouxii*

As shown in Scheme 4-5, the dried plant material (1.65 kg) was powdered and extracted four times (7.5 L/each) with methanol at room temperature with the aid of a supersonic machine, and about 100 g of a residue was obtained after evaporating the methanol. The residue was suspended in water and partitioned with hexane, ethyl acetate, and *n*-butanol, respectively, to afford a hexane extract (17.3 g), an ethyl acetate extract (29.0 g), and an *n*-butanol extract (15.0 g).

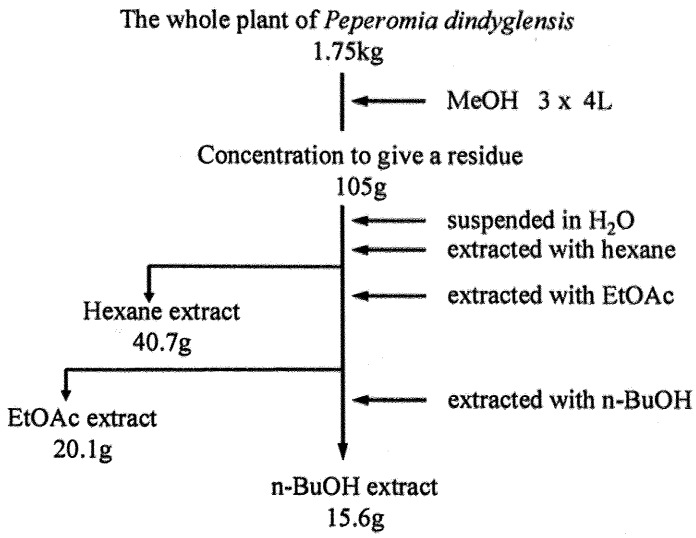
Scheme 4-6 and 4-7 show steps for the separation of the dibenzylbutyrolatone or dibenzylbutanediol lignans from the hexane and EtOAc extract, respectively.

The hexane extract was divided into four fractions (FH_1–FH_4) with silica gel column chromatography using gradient hexane and ethyl acetate as solvents. FH_4 (2.8 g) was subjected to further silica gel column chromatography to afford nine subfractions (FH_{4-1}–FH_{4-9}). Compounds **53** (10.9 mg) and **54** (10.2 mg) were obtained from FH_{4-4} with repeated normal-phase HPLC separations [hexane–ethyl acetate (85:15, 82:18, and 75:25)]. Compound **59** (2.5 mg) was isolated from FH_{4-5} by normal-phase HPLC [hexane –ethyl acetate (75:25 and 82:18)].

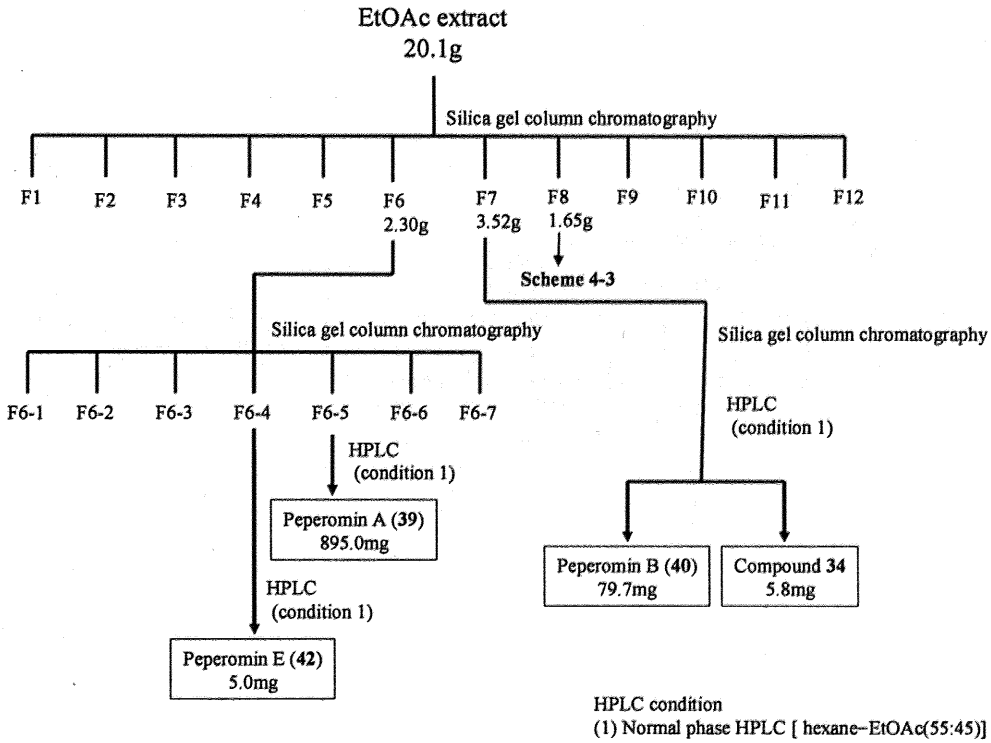
The ethyl acetate extract was chromatographed over a silica gel column eluted with hexane and ethyl acetate to give five fractions (F_1–F_5). F_3 (2.92 g) was divided into five subfractions (F_{3-1}–F_{3-5}) over silica gel column chromatography eluted with hexane and gradient mixtures of hexane and ethyl acetate of increasing polarity. F_{3-3} gave compounds **55** (1.4 mg), **56** (40 mg), and **57** (6.3 mg) with repeated normal-phase HPLC [hexane–EtOAc (65:35 and 75:25)]. F_{3-4} gave compounds **58** (1.0 mg) and **60** (38 mg) with normal-phase HPLC [hexane–EtOAc (50:50 and 65:35)].

The structures for the compounds employed to evaluate their biological activities are shown in Figure 4-3.

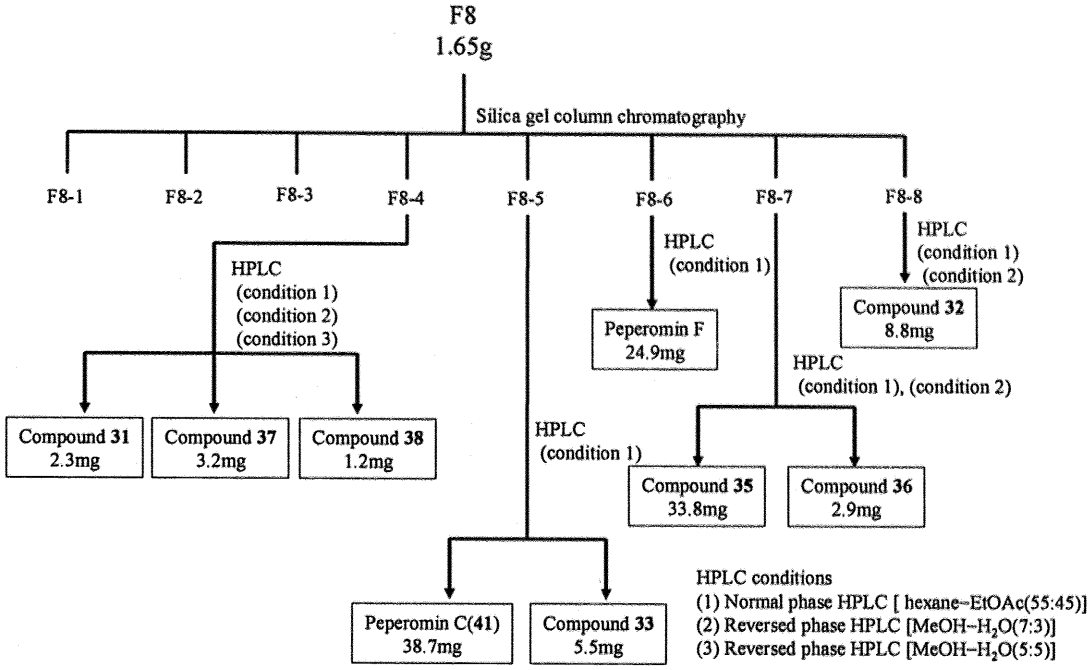
Scheme 4-1.



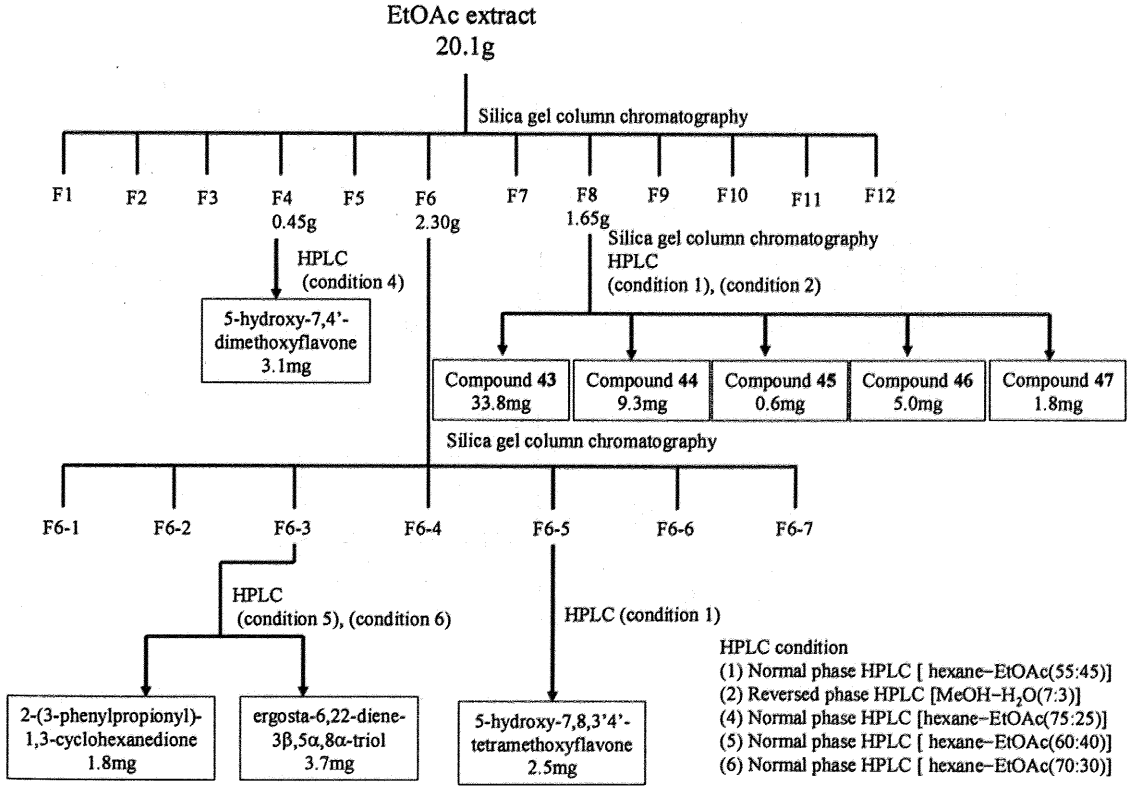
Scheme 4-2.



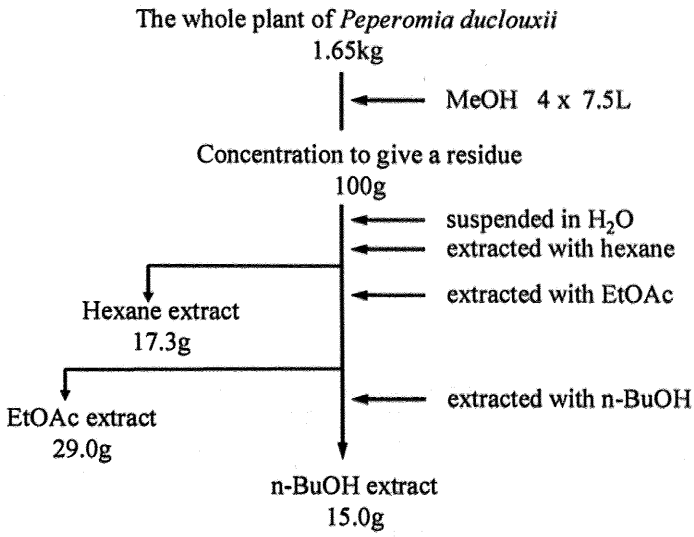
Scheme 4-3.



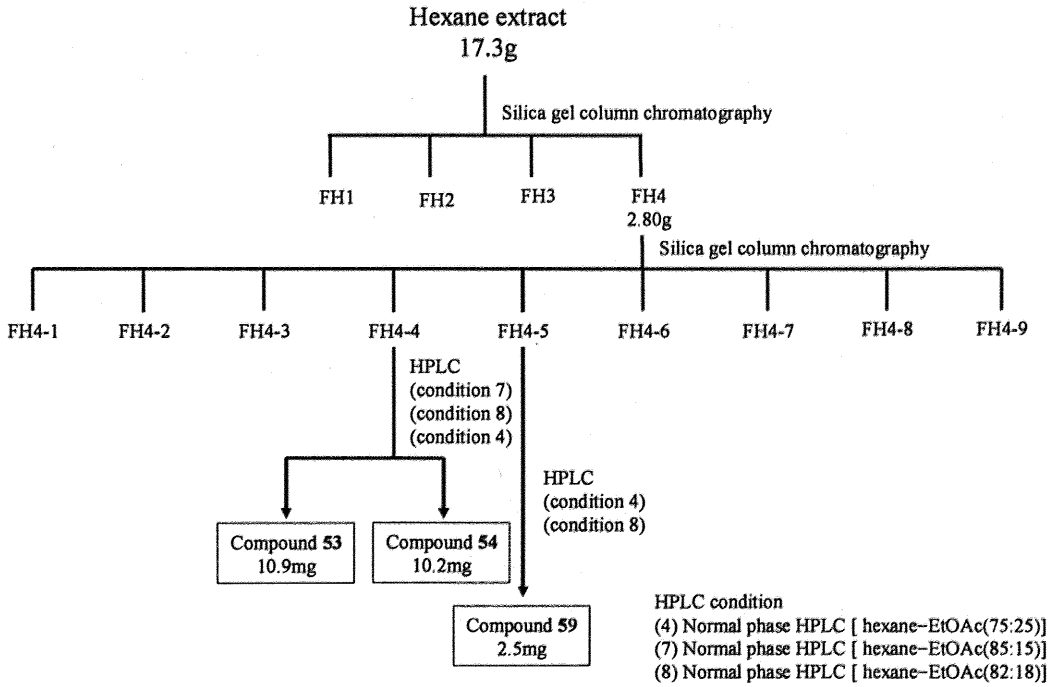
Scheme 4-4.



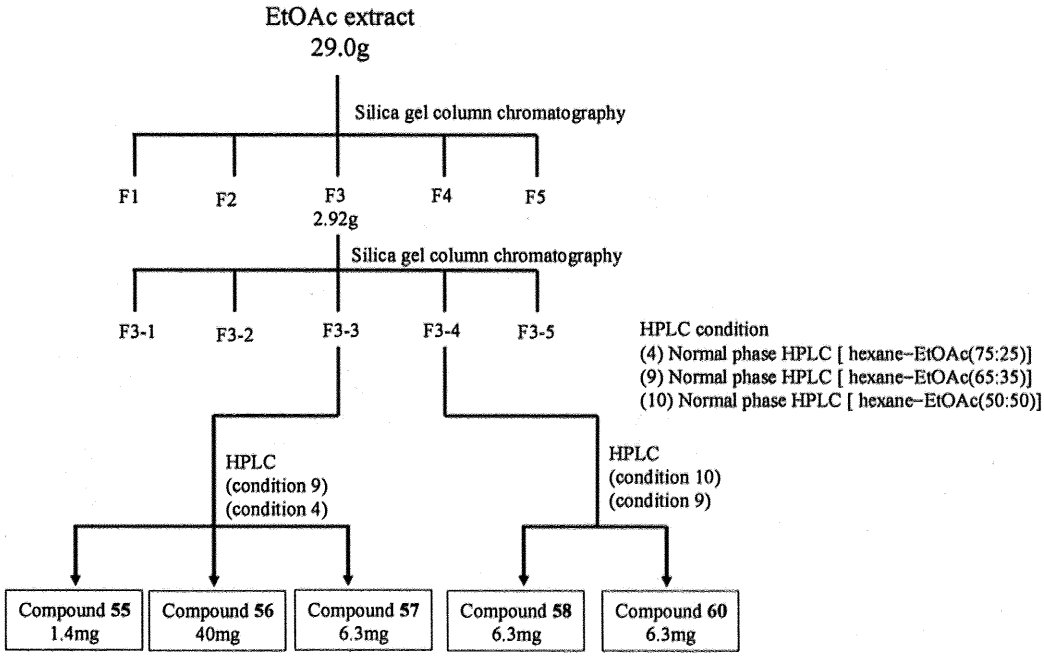
Scheme 4-5.

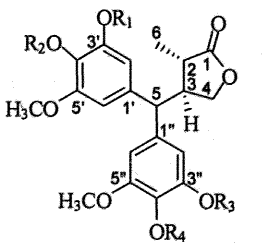


Scheme 4-6.

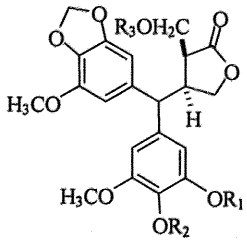


Scheme 4-7.

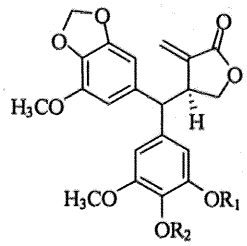

EtOAc extract
29.0g
Silica gel column chromatography
F1
F2
F3
2.92g
F4
F5
Silica gel column chromatography
F3-1
F3-2
F3-3
F3-4
F3-5
HPLC condition
(4) Normal phase HPLC [hexane–EtOAc(75:25)]
(9) Normal phase HPLC [hexane–EtOAc(65:35)]
(10) Normal phase HPLC [hexane–EtOAc(50:50)]
HPLC
(condition 9)
(condition 4)
HPLC
(condition 10)
(condition 9)
Compound 55
1.4mg
Compound 56
40mg
Compound 57
6.3mg
Compound 58
6.3mg
Compound 60
6.3mg



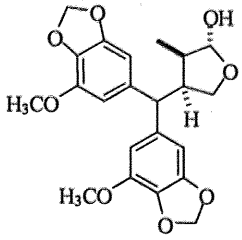
	R_1	R_2	R_3	R_4
31	CH_2		CH_3	H
32	CH_3	CH_3	CH_3	H
33	CH_3	CH_3	H	CH_3
39	CH_2		CH_2	
40	CH_2		CH_3	CH_3
41	CH_3	CH_3	CH_3	CH_3



	R_1	R_2	R_3
34	CH_2		H
35	CH_3	CH_3	H
36	CH_3	H	Ac

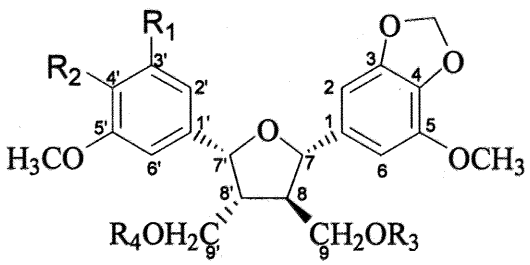


	R_1	R_2
37	CH_3	H
42	CH_2	

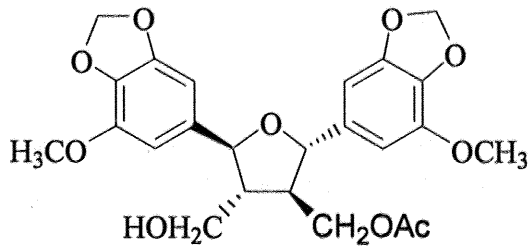


38

Figure 4-1. Structures for compounds of secolignans

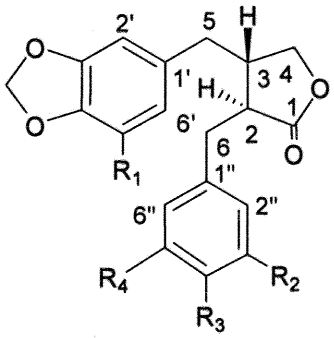


	R_1	R_2	R_3	R_4
43	OCH_2O		Ac	H
44	OCH_2O		H	Ac
46	OCH_3	OH	Ac	Ac
47	OCH_3	OCH_3	Ac	H

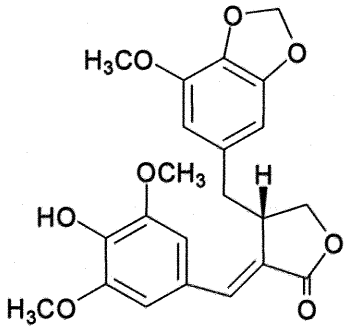


45

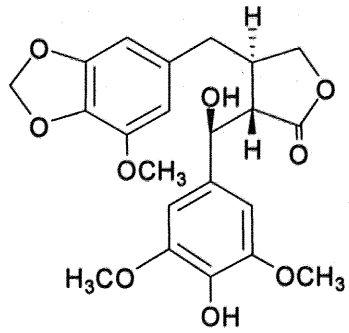
Figure 4-2. Structures for the tetrahydrofran lignans



Compound	R_1	R_2	R_3	R_4
48	OCH_3	OCH_2O		OCH_3
49	OCH_3	OCH_3	OCH_3	OCH_3
50	OCH_3	OCH_3	OH	H
51	OCH_3	OCH_3	OH	OCH_3
52	H	OCH_3	OH	OCH_3
53	OCH_3	OCH_2O		H
54	H	OCH_2O		OCH_3
55	OCH_3	OH	OH	OCH_3



56



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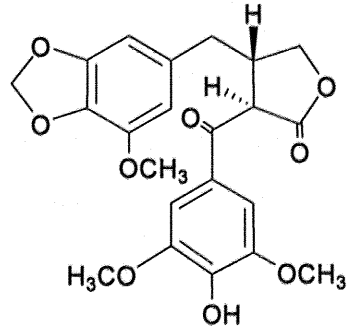
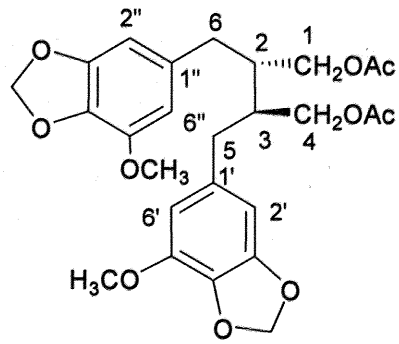
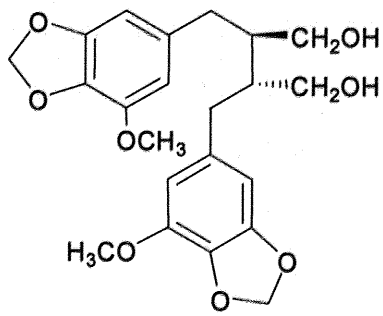


58



59



60

Figure 4-3. Structures for the dibenzylbutyrolactone and dibenzylbutanediol lignans

4-2-2 Cell Growth Inhibition

WI-38, VA-13 and HepG2 cell lines were available from the Institute of Physical and Chemical Research (RIKEN), Tukuba, Ibaraki, Japan. WI-38 and VA-13 cells were maintained in Eagle's MEM medium (Nissui Pharmaceutical Co., Tokyo, Japan) and RITC 80-7 medium (Asahi Technoglass Co., Chiba, Japan), respectively, both supplemented with 10 % (v/v) fetal bovine serum (FBS) (Filtron PTY LTD., Australia) with 80 μg/mL of kanamycin. HepG2 cells were maintained in D-MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % (v/v) FBS (Filtron PTY LTD., Australia) with 80 μg/mL of kanamycin.

Medium (100 μL) containing *ca.* 5, 000 cells (WI-38, VA-13, or HepG2) was incubated at 37 °C in humidified atmosphere of 5 % CO_2 for 24 h in a 96 well micro plate. Test samples dissolved in dimethyl sulfoxide (DMSO) were added to the medium, and incubation was continued further for 48 hours in the same conditions. Coloration substrate, WST-8 [2-(2-methyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to the medium. The resulting formazan concentration was determined by the absorption at 450 nm. Cell viability (%) was calculated as [(experimental absorbance – background absorbance) / (control absorbance – background absorbance) × 100]. Cell viability at different concentrations of compounds was plotted and 50 % inhibition of growth was calculated as IC_{50}.

4-2-3 Accumulation of the Calcein in MDR Cancer Cells

A2780AD is MDR human ovarian cancer cells derived from A2780. 2780AD cells were maintained in PRIM-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % (v/v) FBS (FILTRON PTY LTD., Australia) with 80 μg/ml of kanamycin. Medium (100 μl) containing ca. $1x10^5$cells was incubated at 37°C in humidified atmosphere of 5% CO_2 for 24h.

Test compounds were dissolved in DMSO and diluted with phosphate buffered saline PBS (–). Test samples of 50 μL were added to the medium and incubated for 15min. Then, 50μL of fluorogenic dye calcein acetoxymethyl ester [1μM in PBS (–)] was added to the medium, and incubation was continued further for 60 min. After removing the supernatant, each microplate was washed with 200 μL of cold PBS (–). The washing step was repeated two times, then 200μL of cold PBS (–) was added. Retention of resulting calcein was measured as calcein-specific fluorescence. The absorption maximum for calcein is 494 nm, and emission maximum is 517 nm. Verapamil and taxinine NN-1, the known MDR modulator were used as positive control.

4-3 Results

4-3-1 Accumulation of the Calcein in MDR Cancer Cell

a) Secolignans (**31**—**42**) from *P. dindygulensis*

The effects of the twelve kinds of compounds on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined. The experimental data are shown in Table 4-1 to 4-3 by comparison with that of verapamil, a known MDR-reversal agent, and taxinine NN-1. The results were summarized in Table 4-8(a), (b).

Compounds **41** and **42** showed significant activity toward calcein accumulation in MDR 2780AD cells. Compounds **35**, **37**, **39**, and **40** showed moderate activity. The other compounds showed weak or no activity toward calcein accumulation.

The influence of the functional groups such as hydroxyl group, and methoxyl group at 3", 4", and 5" positions were investigated. Compound **32** and **33** with two methoxyl groups and one hydroxyl group at 3", 4", or 5" positions showed week activities toward calcein accumulation, while compound **41** with three methoxyl groups at both of all three positions showed significant activity. Compund **34** with methoxyl group at 5" group, and with methylendioxy group attached to 3" and 4" positions showed no activity. On the contrary, compound **35** in which the methylendioxy groups at 3" and 4" positions were displaced by two methoxyl groups showed most effective to the calcein accumulation in MDR 2780AD cells. These results suggest that the substitution with methoxyl group at both of all 3", 4", and 5" positions is important for the expression of the activity to calcein accumulation.

Then, the influence of the functional groups at C-2 was examined. Compound **31**, **36**, and **37** possessed methyl group, acetoxymethyl group, and methylene group at C-2, respectively. The activity order of the compounds was **37** > **31** > **36**. Thus, the methylene group at C-2 was effective to the activity toward calcein acuumulation. Compound **34**, **39**, and **42** possessed hydroxymethyl group, methyl group, and methylene group at C-2, respectively. The activity order of the compounds was **42** > **39** > **34**. These results suggest a methylene group at C-2 position is important for the expression of the activity to calcein accumulation in MDR 2780AD cells.

b) Tetrahydrofran lignans (**43**—**47**) from *P. dindygulensis*

The effects of the five kinds of compounds on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined. The results were summarized in table 4-9(b), (c).

The experimental data are shown in table 4-4 and 4-5 by comparison with that of verapamil and taxinine NN-1. The results were summarized in table 4-8(b), (c).

Compounds **47** showed significant activity toward calcein accumulation in MDR 2780AD cells, while the other compounds **43**, **44**, and **46** showed week or no activity. In them, only compound **47** possessed three methoxyl groups attached to the aromatic ring. Thus, even in the case of tetrahydrofran lignans, it is suggested that the substitution with methoxyl group at both of all three positions attaced to a phenol ring is important for the expression of the activity to calcein accumulation.

c) Dibenzylbutyrolactone and dibenzylbutanediol lignans (**48—60**) from *Peperomia duclouxii*

The effects of the thirteen kinds of compounds on the cellular accumulation of calcein in MDR 2780AD cells were examined. The experimental data are shown in table 4-6 and 4-7 by comparison with that of verapamil and taxinine NN-1. The resuls were summarized in table 4-8(c), (d).

Compound **49** showed significant activity toward calcein accumulation in MDR 2780AD cells. Copmpounds **51**, **52**, **54**, **55**, and **60** showed moderate activity. Compounds **48**, **56**, and **57** showed week activity. The other compounds **50** and **53** showed no activity toward calcein accumulation.

In them, compound **49** possessed three methoxyl groups attached to the aromatic ring, and it is also suggested in the case of dibenzylbutyrolactone that the substitution with methoxyl group at both of all three positions attaced to a phenol ring is important for the expression of the activity to calcein accumulation.

Then the influence of methoxyl groups attached to aromatic ring toward the activity was investigated. Compound **48**, **49**, **53**, **54**, and **60** possessed different number of methoxyl groups at different position. The activity order of the compounds was **49** > **54** = **60** ≥ **48** > **53**. When the methoxyl groups at 3" and 4" positions were substituted with methylenedioxy group, the activity was decresed remarkably. In addition, when the methoxyl group at 5" position was lost, the activity was disappeared completely. On the contrary, the activity was not decreased when the methoxyl group at 5' position was lost. Thus, it seems that the role of the methoxyl group at 5' position for the expression of the activity is not important.

The activities of the compounds were compared among the analogues **49—53** in which methoxyl groups at 3", 4", 5", or 5' positions displaced by a hydroxyl group or hydrogen, respectively. The activity order of the compounds was **49** > **51** = **52** = **53** >> **50**. The substitution of methoxyl groups

at 3", 4", or 5" positions with hydroxyl group or hydrogen results in the disappearance of the activity. While, the substitution of methoxyl group at 5' positions with hydrogen gave no influence to the activity.

Exp. 4-1.

Table4-1(a). Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3078 ± 347
Verapamil	0.25	3273 ± 645
	2.5	3574 ± 334
	25	4632 ± 522
Taxinine NN-1	0.25	3219 ± 287
	2.5	3790 ± 445
	25	5164 ± 635
31	0.25	3240 ± 218
	2.5	3024 ± 528
	25	3466 ± 178
32	0.25	2947 ± 213
	2.5	2936 ± 99
	25	3165 ± 100
33	0.25	3094 ± 334
	2.5	3199 ± 866
	25	3228 ± 960

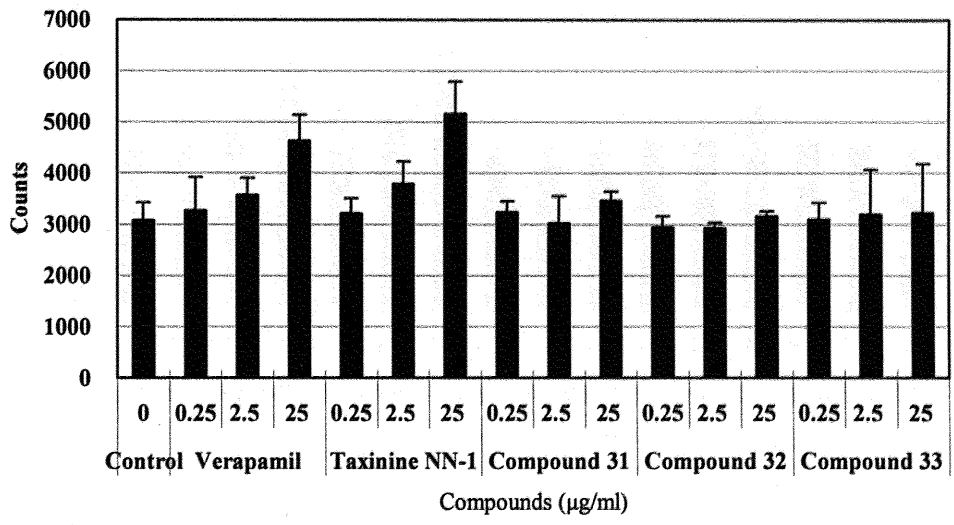


Figure 4-4(a).

Exp. 4-1.

Table4-1(b). Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3078 ± 347
Verapamil	0.25	3273 ± 645
	2.5	3574 ± 334
	25	4632 ± 522
Taxinine NN-1	0.25	3219 ± 287
	2.5	3790 ± 445
	25	5164 ± 635
34	0.25	3007 ± 193
	2.5	2929 ± 216
	25	3223 ± 378
35	0.25	3222 ± 85
	2.5	3351 ± 94
	25	3860 ± 103
36	0.25	3310 ± 244
	2.5	3192 ± 328
	25	3195 ± 334

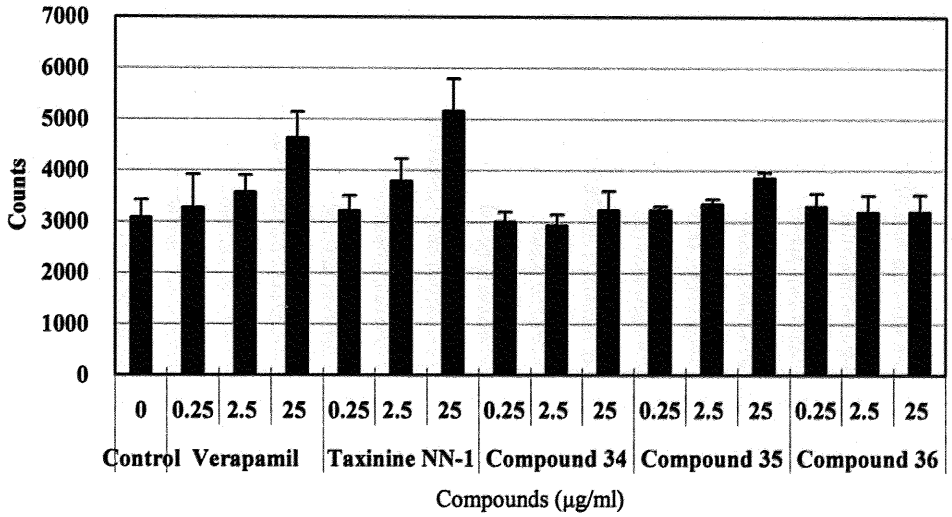


Figure 4-4(b).

Exp. 4-1.

Table4-1(c). Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3078 ± 347
Verapamil	0.25	3273 ± 645
	2.5	3574 ± 334
	25	4632 ± 522
Taxinine NN-1	0.25	3219 ± 287
	2.5	3790 ± 445
	25	5164 ± 635
37	0.25	3203 ± 213
	2.5	3032 ± 127
	25	3835 ± 256
38	0.25	3039 ± 124
	2.5	3201 ± 42
	25	3294 ± 188
42	0.25	3146 ± 266
	2.5	3133 ± 285
	25	4077 ± 838

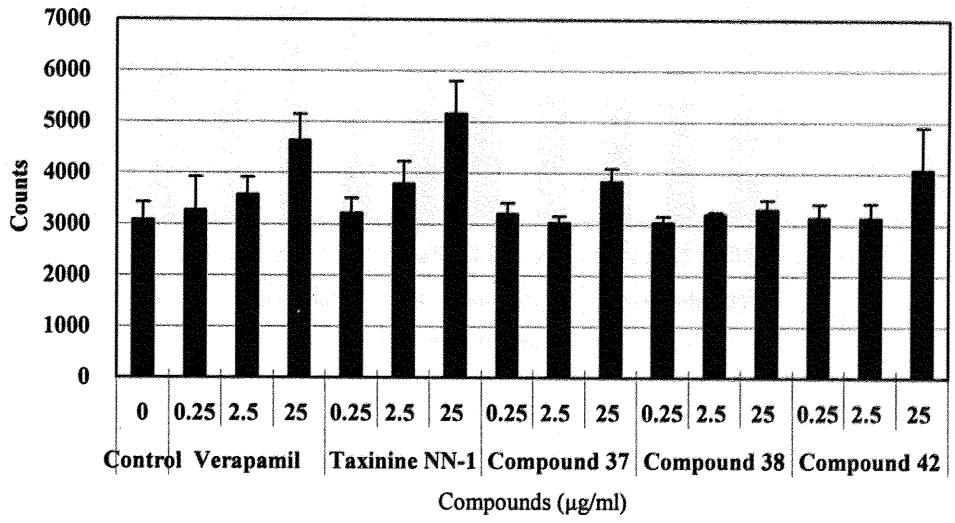


Figure 4-4(c).

Exp. 4-2.

Table4-2. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3888 ± 46
Verapamil	0.25	3690 ± 803
	2.5	4342 ± 96
	25	5615 ± 537
Taxinine NN-1	0.25	3796 ± 104
	2.5	4396 ± 530
	25	6197 ± 11
39	0.25	3312 ± 3
	2.5	3554 ± 215
	25	4658 ± 110

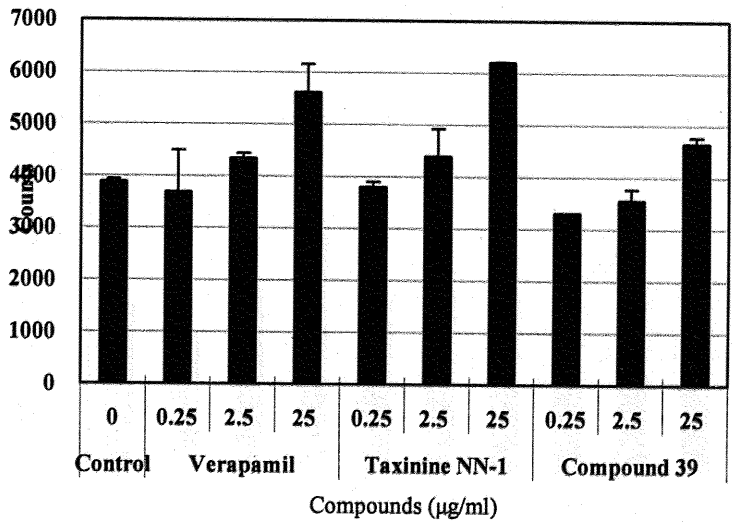


Figure 4-5.

Exp. 4-1.

Table4-3. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	2853 ± 355
Verapamil	0.25	2396 ± 389
	2.5	3317 ± 105
	25	4280 ± 405
Taxinine NN-1	0.25	2934 ± 39
	2.5	3517 ± 29
	25	4433 ± 609
40	0.25	3155 ± 59
	2.5	2966 ± 514
	25	3449 ± 320
41	0.25	3104 ± 148
	2.5	3286 ± 92
	25	3950 ± 11

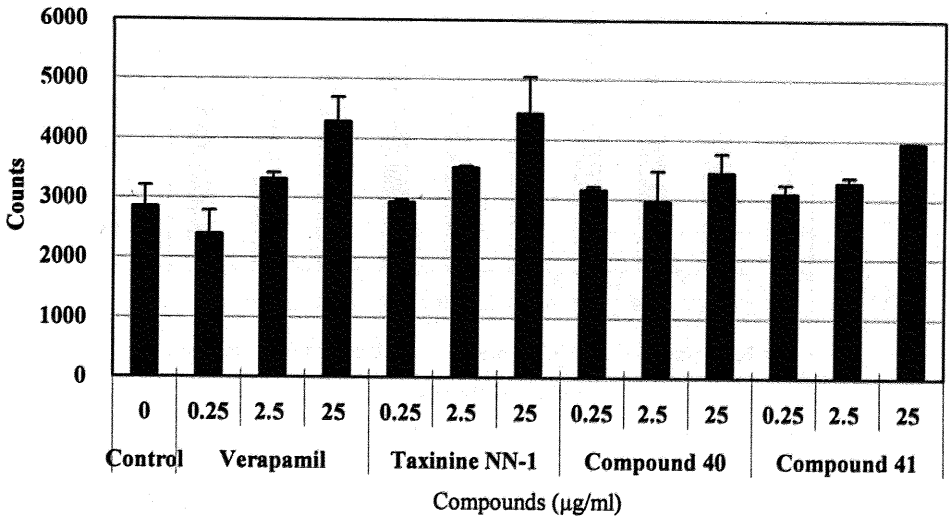


Figure 4-6.

Exp. 4-3.

Table4-4. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	3996 ± 719
Verapamil	0.25	4482 ± 463
	2.5	4994 ± 273
	25	6211 ± 445
Taxinine NN-1	0.25	4389 ± 775
	2.5	4766 ± 297
	25	7149 ± 967
43	0.25	4281 ± 546
	2.5	4322 ± 247
	25	4286 ± 639

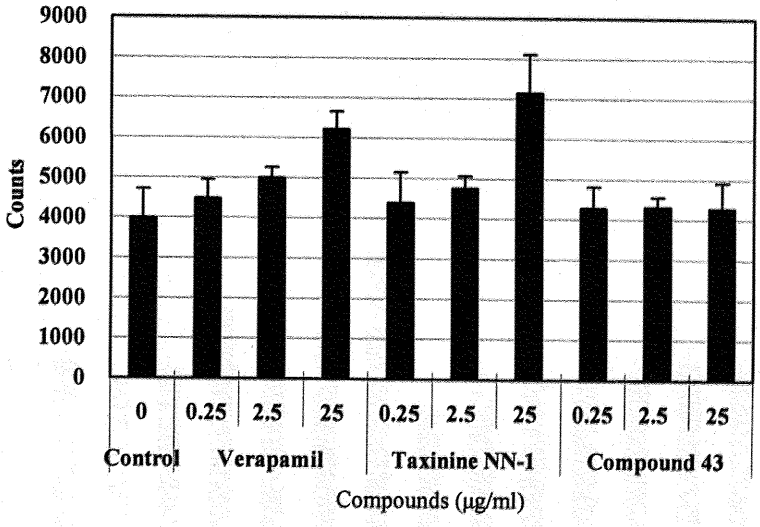


Figure 4-7.

Exp. 4-4.

Table4-5. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	4098 ± 506
Verapamil	0.25	3629 ± 113
	2.5	3909 ± 376
	25	5303 ± 300
Taxinine NN-1	0.25	3857 ± 478
	2.5	4895 ± 757
	25	6559 ± 801
44	0.25	3131 ± 47
	2.5	3496 ± 378
	25	3837 ± 241
46	0.25	3621 ± 174
	2.5	3879 ± 124
	25	4126 ± 310
47	0.25	3983 ± 170
	2.5	4317 ± 152
	25	5674 ± 778

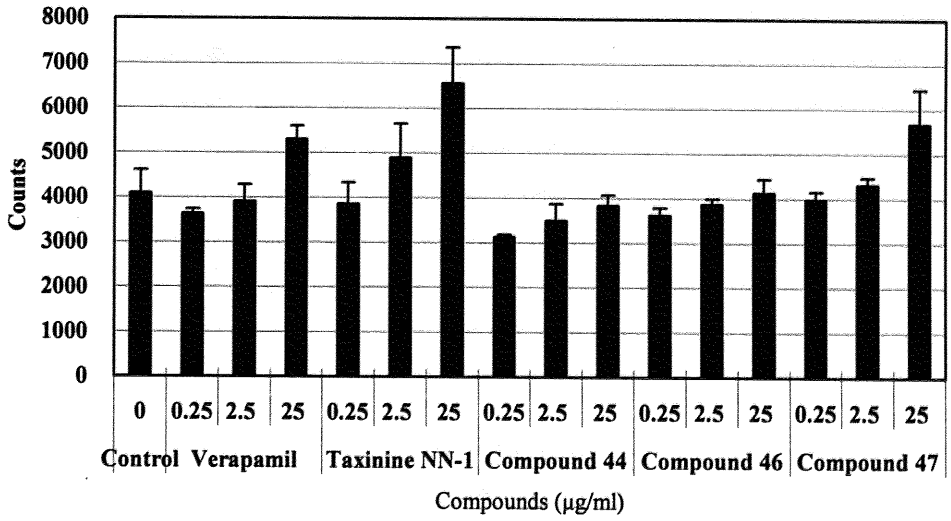


Figure 4-8.

Exp. 4-5.

Table4-6. Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	4098 ± 506
Verapamil	0.25	3629 ± 113
	2.5	3909 ± 376
	25	5303 ± 300
Taxinine NN-1	0.25	3857 ± 478
	2.5	4895 ± 757
	25	6559 ± 801
48	0.25	3131 ± 47
	2.5	3496 ± 378
	25	3837 ± 241
53	0.25	3621 ± 174
	2.5	3879 ± 124
	25	4126 ± 310
54	0.25	3983 ± 170
	2.5	4317 ± 152
	25	5674 ± 778
59	0.25	3983 ± 170
	2.5	4317 ± 152
	25	5674 ± 778

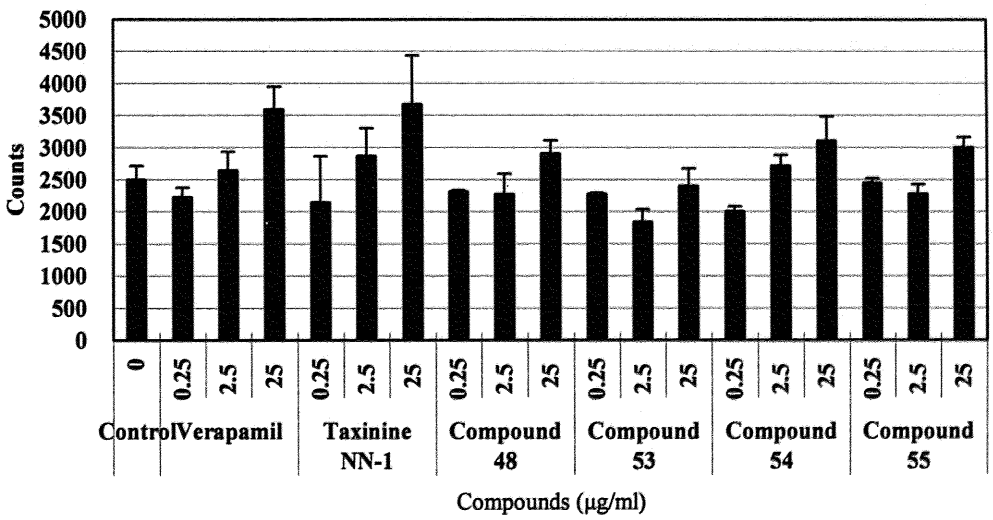


Figure 4-9.

Exp. 4-6.

Table4-7(a). Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	4098 ± 506
Verapamil	0.25	3629 ± 113
	2.5	3909 ± 376
	25	5303 ± 300
Taxinine NN-1	0.25	3857 ± 478
	2.5	4895 ± 757
	25	6559 ± 801
49	0.25	3131 ± 47
	2.5	3496 ± 378
	25	3837 ± 241
50	0.25	3621 ± 174
	2.5	3879 ± 124
	25	4126 ± 310
51	0.25	3983 ± 170
	2.5	4317 ± 152
	25	5674 ± 778
52	0.25	3983 ± 170
	2.5	4317 ± 152
	25	5674 ± 778

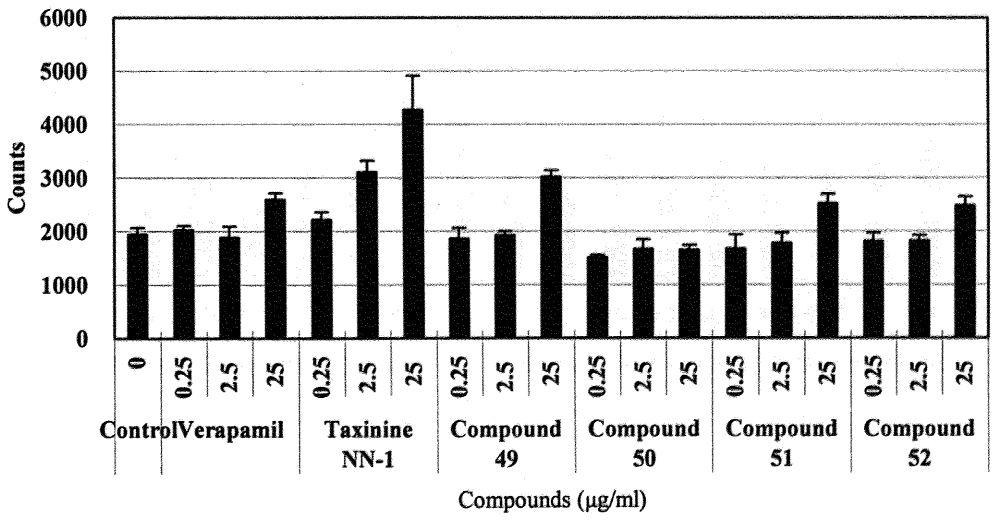


Figure 4-10(a).

Exp. 4-6.

Table4-7(b). Effect of the compounds on the accumulation of calcein in MDR 2780AD cells

Compound	concentration μg/mL	average of fluorescence/ well ± SD
Control	0	4098 ± 506
Verapamil	0.25	3629 ± 113
	2.5	3909 ± 376
	25	5303 ± 300
Taxinine NN-1	0.25	3857 ± 478
	2.5	4895 ± 757
	25	6559 ± 801
56	0.25	3131 ± 47
	2.5	3496 ± 378
	25	3837 ± 241
57	0.25	3621 ± 174
	2.5	3879 ± 124
	25	4126 ± 310
60	0.25	3983 ± 170
	2.5	4317 ± 152
	25	5674 ± 778

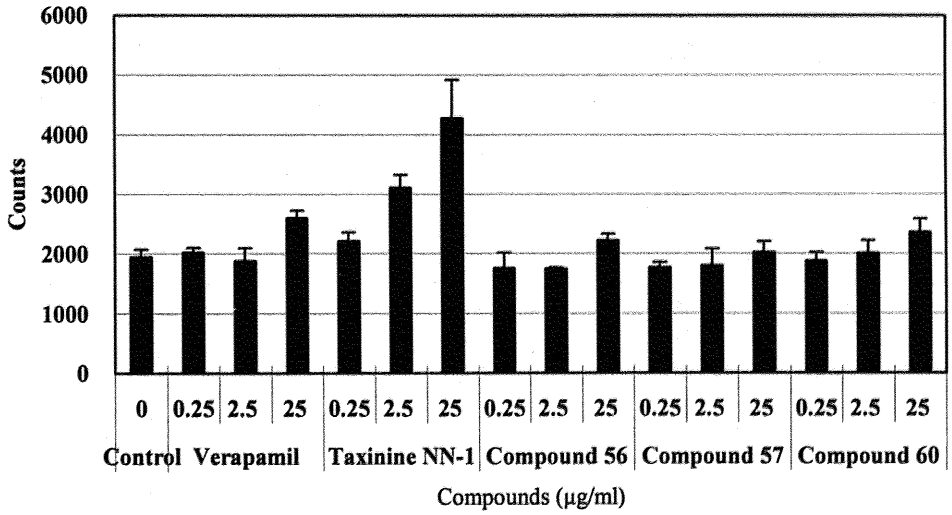
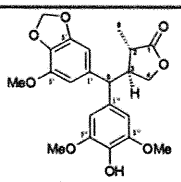
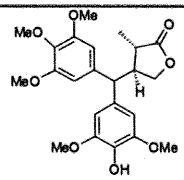
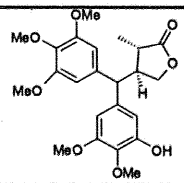
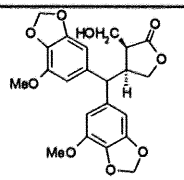
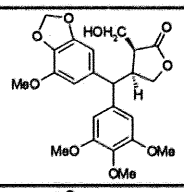
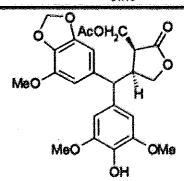
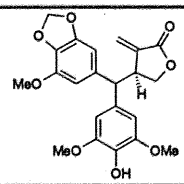
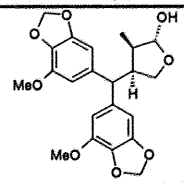


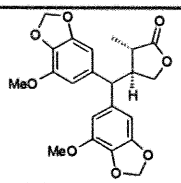
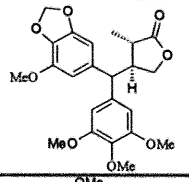
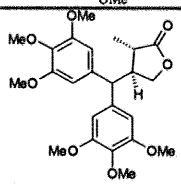
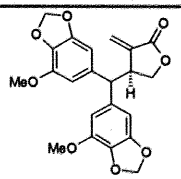
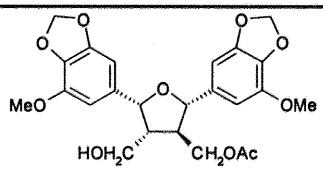
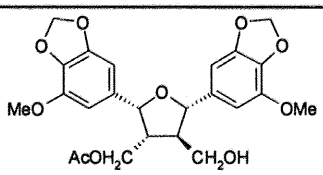
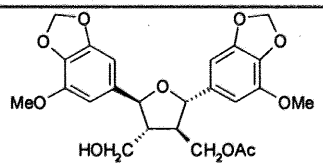
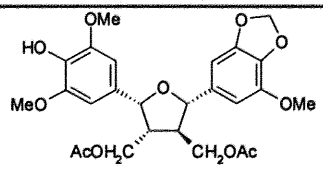
Figure 4-10(b).

Table 4-8 (a). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound			Calcein accumulation[a]		
	Structure	μg/ml	% control[b]	%verapamil	%Taxinine NN-1
31		0	100	100	100
		0.25	105	99	101
		2.5	98	85	80
		25	113	75	67
32		0	100	100	100
		0.25	96	90	92
		2.5	95	82	77
		25	103	68	61
33		0	100	100	100
		0.25	101	95	96
		2.5	104	90	84
		25	105	70	63
34		0	100	100	100
		0.25	98	92	93
		2.5	95	82	77
		25	105	70	62
35		0	100	100	100
		0.25	105	98	100
		2.5	109	94	88
		25	125	83	75
36		0	100	100	100
		0.25	108	101	103
		2.5	104	89	84
		25	104	69	62
37		0	100	100	100
		0.25	104	98	100
		2.5	98	85	80
		25	125	83	74
38		0	100	100	100
		0.25	99	93	94
		2.5	104	90	84
		25	107	71	64

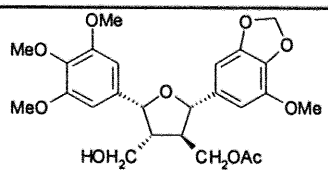
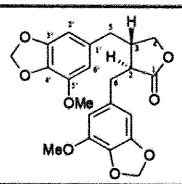
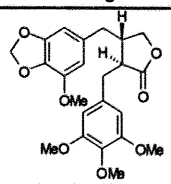
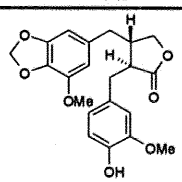
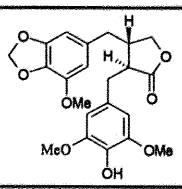
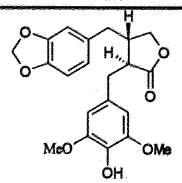
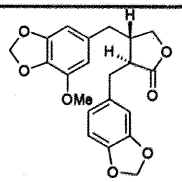
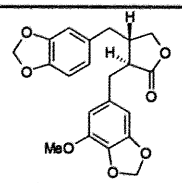
[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b]The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

Table 4-8(b). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound			Calcein accumulation[a]		
	Structure	μg/ml	% control[b]	%verapamil	%Taxinine NN-1
39		0	100	100	100
		0.25	85	90	87
		2.5	91	82	81
		25	120	83	75
40		0	100	100	100
		0.25	111	132	108
		2.5	104	89	84
		25	121	81	78
41		0	100	100	100
		0.25	109	130	106
		2.5	115	99	93
		25	138	92	89
42		0	100	100	100
		0.25	102	96	98
		2.5	102	88	83
		25	132	88	79
43		0	100	100	100
		0.25	107	96	98
		2.5	108	87	91
		25	107	69	60
44		0	100	100	100
		0.25	76	86	81
		2.5	85	89	71
		25	94	72	58
45		0	–	–	–
		0.25	–	–	–
		2.5	–	–	–
		25	–	–	–
46		0	100	100	100
		0.25	88	100	94
		2.5	95	99	79
		25	101	78	63

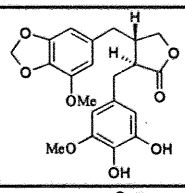
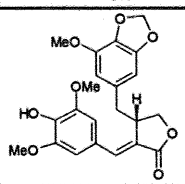
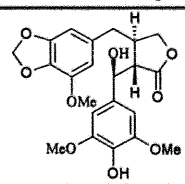
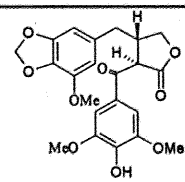
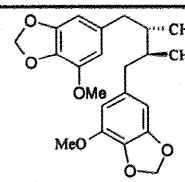
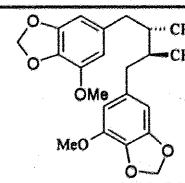
[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b]The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

Table 4-8(c). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound	Structure	μg/ml	Calcein accumulation[a] % control[b]	%verapamil	%Taxinine NN-1
47		0	100	100	100
		0.25	97	110	103
		2.5	105	110	88
		25	138	107	87
48		0	100	100	100
		0.25	93	104	108
		2.5	91	86	79
		25	116	81	79
49		0	100	100	100
		0.25	96	92	84
		2.5	99	102	62
		25	155	116	71
50		0	100	100	100
		0.25	77	74	68
		2.5	86	89	54
		25	85	64	39
51		0	100	100	100
		0.25	86	83	75
		2.5	91	94	57
		25	129	97	59
52		0	100	100	100
		0.25	93	90	82
		2.5	94	97	59
		25	127	96	58
53		0	100	100	100
		0.25	91	102	106
		2.5	74	70	64
		25	96	67	65
54		0	100	100	100
		0.25	80	90	93
		2.5	109	103	95
		25	124	86	84

[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b]The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

Table 4-8 (d). Effect of the compounds on the accumulation of calcein in MDR2780AD cells

Compound			Calcein accumulation[a]		
	Structure	μg/ml	% control[b]	%verapamil	%Taxinine NN-1
55		0	100	100	100
		0.25	98	110	114
		2.5	91	86	79
		25	120	84	82
56		0	100	100	100
		0.25	90	87	79
		2.5	90	93	56
		25	114	86	52
57		0	100	100	100
		0.25	91	88	80
		2.5	93	96	58
		25	104	78	47
58		0	–	–	–
		0.25	–	–	–
		2.5	–	–	–
		25	–	–	–
59		0	–	–	–
		0.25	–	–	–
		2.5	–	–	–
		25	–	–	–
60		0	100	100	100
		0.25	97	93	85
		2.5	103	107	65
		25	121	91	55

[a]The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5 and 25 μg/ml of test compounds , verapamil and taxinine NN-1.
[b]The values are the relative amount of calcein accumulated in the cell compared with the control experiment.The values represent means of triplicate determination .

4-3-2 Cell Growth Inhibition

a) Secolignans (**31**—**42**) from *P. dindygulensis*

The effect of the twelve kinds of compounds on the cell growth inhibitory activity against WI-38, VA-13 and HepG2 cells were examined. The results were summarized in table 4-9(a), (b).

Compound **36**, **37**, **40**, and **42** showed significant activity against WI-38 and VA-13 cells. In them, compound **37** and **42** showed significant activity against HepG2 cells, too. Compound **34** showed significant activity only against WI-38, but not against VA-13 or HepG2 cells. The other compound showed weak or no activity against the three cell lines. It seemed that there are no correlation between the cell growth inhibition activity and substitution with methoxyl group at 3", 4", and 5" positions as shown for the calcein accumulating activity.

Both Compound **37** and **42** which showed significant activity against all of three cell lines have α-mehylene γ-lactone moiety, and it is well known that α-mehylene γ-lactone shows potent cytotoxicity. On the other hand, both compound **36** and **40** which showed significant activity against WI-38 and VA-13 cells but not against HepG2 have α-acetoxymehyl γ-lactone and α-mehyl γ-lactone moiety instead of α-mehylene γ-lactone moiety, respectively.

However compound **31**, **32**, **33** and **39** have α-mehyl γ-lactone moiety, these compounds showed weak or no activity against WI-38 and VA-13 cells. It is suggested that strict structure is necessary for the expression of the cell growth inhibitory activity.

b) Tetrahydrofran lignans (**43**—**47**) from *P. dindygulensis*

The effect of the five kinds of compounds on the cell growth inhibitory activity against WI-38, VA-13 and HepG2 cells were examined. The results were summarized in table 4-9(b), (c).There were no compounds which showed significant activity.

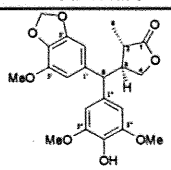
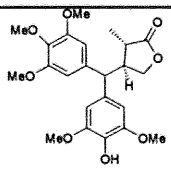
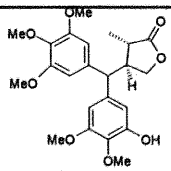
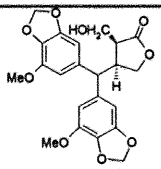
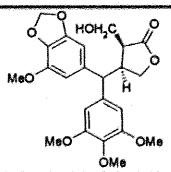
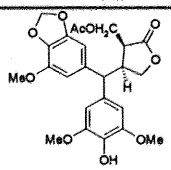
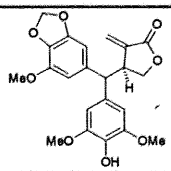
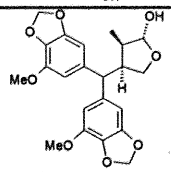
c) Dibenzylbutyrolactone and dibenzylbutanediol lignans (**48**—**60**) from *Peperomia duclouxii*

The effect of the thirteen kinds of compounds on the cell growth inhibitory activity against WI-38, VA-13 and HepG2 cells were examined. The results were summarized in table 4-9(c), (d).

Compound **49** showed moderate activity against HepG2 cells. Compound **54** showed significant activity against VA-13 cells. Compound **56** and **60** showed significant activity against WI-38 cells. The other compounds showed weak or no activity against the three cell lines. It is difficult to discus about the structure-activity relation ships for cell growth inhibition activity because there seemed to be no structural feature among those compounds.

However lignans are thought to be a major active ingredient for the anticancer activity of the plans, there was only a small kind of lingnan showing significant cell growth inhibitory activity toward the cancer cells. Cytotoxicity of the crude extracts of the plants, *P. dindygulensis* and *P. duclouxii* were evaluated (Table 4-10). The EtOAc extract of P.duclouxii showed the most significant activity against WI-38 and HepG2 cells. From these results, it can be that synergy action of MDR reversal activity and cell growth inhibitory activity of lignans enhances the pharmacologic effect of the crude extract of the plant.

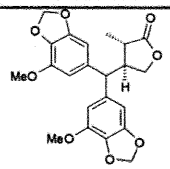
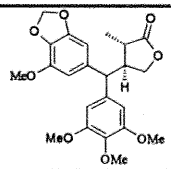
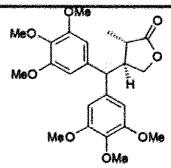
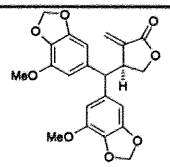
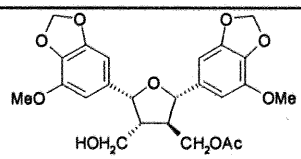
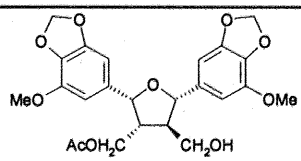
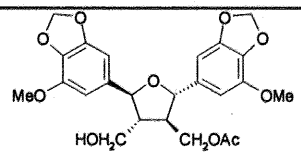
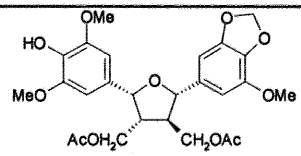
Table4-9(a). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compounds			Cell line	Cytotoxicity (IC_{50})[a]		
	Structure	M.W.		μg/ml	μM	Selectivity[b]
31		416.4	WI-38	72	174	1.00
			VA-13	47	112	1.55
			HepG2	66	159	1.10
32		432.5	WI-38	80	184	1.00
			VA-13	65	151	1.22
			HepG2	70	163	1.13
33		432.5	WI-38	>100	>231	1.00
			VA-13	98	226	>1.02
			HepG2	>100	>231	-
34		430.1	WI-38	6	13	1.00
			VA-13	49	113	0.11
			HepG2	42	98	0.13
35		446.5	WI-38	>100	>224	1.00
			VA-13	>100	>224	-
			HepG2	>100	>224	-
36		474.5	WI-38	7	15	1.00
			VA-13	7	15	0.97
			HepG2	36	76	0.19
37		414.4	WI-38	9	21	1.00
			VA-13	6	14	1.54
			HepG2	9	22	0.94
38		416.1	WI-38	79	190	1.00
			VA-13	72	174	1.09
			HepG2	54	131	1.45

[a] IC_{50} represent means of duplicate determination.

[b] The values are the ratio of IC_{50} (μM) (WI-38/VA-13, or WI-38/HepG2)

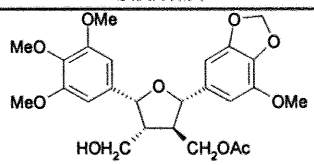
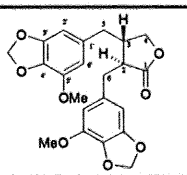
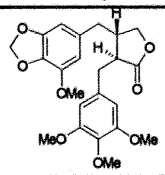
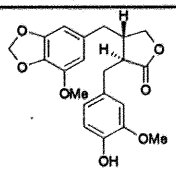
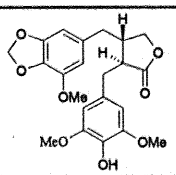
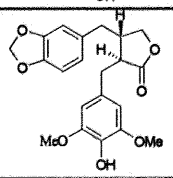
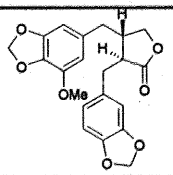
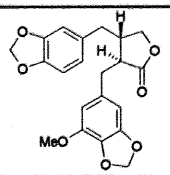
Table 4-9(b). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compounds			Cell line	Cytotoxicity (IC_{50})[a]		
	Structure	M.W.		μg/ml	μM	Selectivity[b]
39		414.1	WI-38	>100	>241	1.00
			VA-13	>100	>241	-
			HepG2	>100	>241	-
40		430.2	WI-38	4	9	1.00
			VA-13	6	14	0.65
			HepG2	52	120	0.08
41		446.5	WI-38	78	175	1.00
			VA-13	53	120	1.46
			HepG2	84	189	0.93
42		412.1	WI-38	1	1	1.00
			VA-13	1	2	0.63
			HepG2	5	12	0.10
43		474.5	WI-38	>100	>211	1.00
			VA-13	88	185	>1.14
			HepG2	>100	>211	-
44		474.5	WI-38	89	187	1.00
			VA-13	65	137	1.37
			HepG2	70	148	1.26
45		474.5	WI-38	>100	>211	1.00
			VA-13	36	76	>2.76
			HepG2	>100	>211	-
46		518.5	WI-38	71	137	1.00
			VA-13	47	91	1.50
			HepG2	61	118	1.16

[a] IC_{50} represent means of duplicate determination.

[b] The values are the ratio of IC_{50} (μM) (WI-38/VA-13, or WI-38/HepG2)

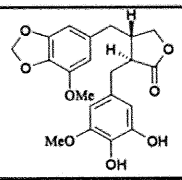
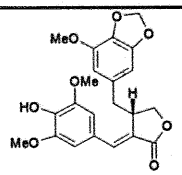
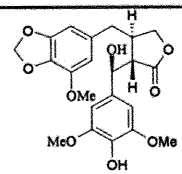
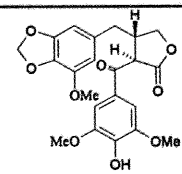
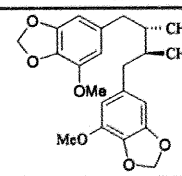
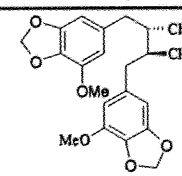
Table 4-9(c). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compounds			Cell line	Cytotoxicity (IC_{50})[a]		
	Structure	M.W.		μg/ml	μM	Selectivity[b]
47		490.5	WI-38 VA-13 HepG2	96 82 >100	195 168 >204	1.00 1.16 <0.96
48		414.1	WI-38 VA-13 HepG2	>100 >100 73	>241 >241 175	1.00 - >1.38
49		430.2	WI-38 VA-13 HepG2	>100 66 18	>232 153 43	1.00 >1.52 >5.43
50		386.1	WI-38 VA-13 HepG2	76 94 66	198 243 170	1.00 0.81 1.16
51		416.1	WI-38 VA-13 HepG2	>100 90 83	>240 216 199	1.00 >1.11 >1.21
52		386.1	WI-38 VA-13 HepG2	98 61 70	255 158 181	1.00 1.61 1.41
53		384.1	WI-38 VA-13 HepG2	>100 83 82	>260 217 212	1.00 >1.20 >1.23
54		384.1	WI-38 VA-13 HepG2	49 9 57	126 23 149	1.00 5.45 0.84

[a] IC_{50} represent means of duplicate determination.

[b] The values are the ratio of IC_{50} (μM) (WI-38/VA-13, or WI-38/HepG2)

Table 4-9(d). Cytotoxicities of compounds against WI-38, VA-13 and HepG2 cells

Compounds			Cell line	Cytotoxicity (IC_{50})[a]		
	Structure	M.W.		μg/ml	μM	Selectivity[b]
55		402.1	WI-38 VA-13 HepG2	49 46 61	122 115 151	1.00 1.06 0.80
56		414.1	WI-38 VA-13 HepG2	4 >100 63	9 >241 152	1.00 <0.04 0.06
57		432.1	WI-38 VA-13 HepG2	>100 87 77	>231 202 179	1.00 >1.15 >1.30
58		430.1	WI-38 VA-13 HepG2	60 88 71	139 204 166	1.00 0.68 0.84
59		502.5	WI-38 VA-13 HepG2	>100 >100 >100	>199 >199 >199	- - -
60		418.2	WI-38 VA-13 HepG2	5 >100 62	12 >239 148	1.00 <0.05 0.08

[a] IC_{50} represent means of duplicate determination.

[b] The values are the ratio of IC_{50} (μM) (WI-38/VA-13, or WI-38/HepG2)

4-4 Conclusion

As shown in figure 4-10 to 4-12, compounds **35**, **39**, **47**, **49**, **51**, and **52** showed the activity toward calcein accumulation in MDR 2780AD cells but have no cytotoxicity. In them, compounds **35**, **39**, and **41** were secolignans from *P. dindygulensis*. Compound **47** is tetrahydrofran lignans from *P. dindygulensis*. Compound **49**, **51**, and **52** were dibenzylbutylolactones from *P.duclouxii*. These compounds are expected to be lead compounds of MDR cancer reversal agents. On the other hand, since compounds **37**, **40**, **and 42** showed moderate to significant cytotoxic activity toward VA-13, or HepG2 cells, it is expected as lead compounds of new-type anticancer agents.

Table 4-10. Cytotoxicity of the crude extract from the plants

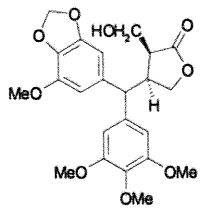
Cell line	IC50(μ g/ml)					
	1-a	1-b	1-c	2-a	2-b	2-c
WI-38	57.3	65.5	>100	>100	8.60	39.8
VA-13	61.8	47.1	>100	>100	33.5	46.3
HepG2	58.1	64.3	>100	>100	8.20	39.8

1)*Peperomia dindygulensis*

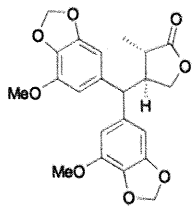
1-a) Hexane ext.
1-b) EtOAc ext.
1-c) n-BuOH ext.

2)*Peperomia duclouxii*

2-a) Hexane ext.
2-b) EtOAc ext.
2-c) n-BuOH ext.



35



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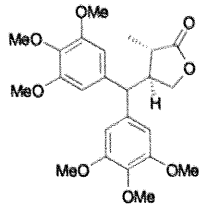
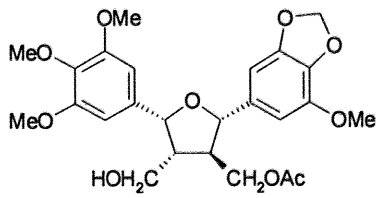


41

Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
35	>224	>224	>224	125
39	>239	>239	>239	120
41	175	120	189	138

*Concentration of compounds : 25 μ g/ml

Figure 4-10. Candidates as MDR modulator

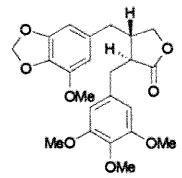


47

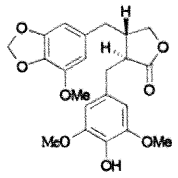
Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
47	195	168	>204	138

*Concentration of compounds : 25 μ g/ml

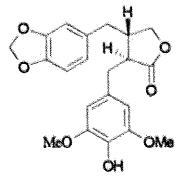
Figure 4-11. Candidate as MDR modulator



49



51

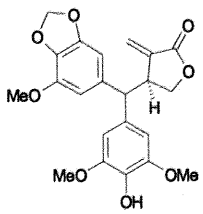


52

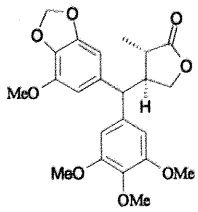
Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
49	>232	117	42.8	155
51	195	130	205	129
52	>259	183	>260	127

*Concentration of compounds : 25 μ g/ml

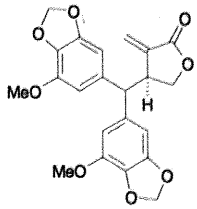
Figure 4-12. Candidates as MDR modulator



37



40



42

Compounds	Cytotoxicity IC_{50}(μ M)			Calcein accumulation
	WI-38	VA-13	HepG2	(% control)*
37	20.9	13.5	22.3	125
40	9.1	13.9	119	121
42	1.2	1.9	12.1	132

*Concentration of compounds : 25 μ g/ml

Figure 4-13. Candidates as anticancer agent with MDR reversal activity

4-5 Summary

Twelve kinds of secolignans (**31**—**42**) and four kinds of tetrahydrofran lignans (**43**—**47**) were isolated from the EtOAc extract of *Peperomia dindygulensis*. In addition, thirteen kind of dibenzylbutyrolactone and dibenzylbutanediol (**48**—**60**) were isolated from *Peperomia duclouxii*.

Effect of the compounds on the accumulation of calcein in MDR 2780AD cells and cytotoxicity against WI-38, VA-13, and HepG2 were examined.

Compounds **35**, **39**, **47**, **49**, **51**, and **52** showed the activity toward calcein accumulation in MDR 2780AD cells but have no cytotoxicity. These compounds are expected to be lead compounds of MDR cancer reversal agents. On the other hand, since compounds **37**, **40**, **and 42** showed moderate to significant cytotoxic activity toward VA-13, or HepG2 cells, it is expected as lead compounds of new-type anticancer agents.

However lignans are thought to be a major active ingredient for the anticancer activity of the plans, there was only a small kind of lingnan showing significant cell growth inhibitory activity toward the cancer cells. It may be that synergy action of MDR reversal activity and cell growth inhibitory activity of lignans enhances the pharmacologic effect of the crude extract of the plant.

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Acknowledgements

I would like to express my sincere appreciation to Professor Masayoshi Ando for his valuable guidance, helpful suggestions and firm encouragement throughout this work and the preparation of this thesis.

I wish to express my thanks to Professor Hisahiro Hagiwara, Professor Toshio Suzuki, Professor Masaki Kamata, and Professor Masayuki Taniguchi for their valuable discussions and suggestions.

I wish to express my thanks to Professor Takashi Tsuruo and Professor Akihiro Tomida in Japanese Foundation for Cancer Research for their valuable suggestions.

I extend my grateful acknowledgements to all member of Ando laboratory.

I also wish to express my gratitude to Dr. Masami Orisaku for his kind support and encouragement, and to the people involved in this work in Mitsubishi Gas Chemical Company, INC.

Finally, I express my deep appreciation to my wife and my son, who is Yuki Hasegawa and Shunpei Hasegawa for their kind support.