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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation　Studies on anti-cancer activity of a new dimer sesquiterpene lactone from yacon leaves
(ヤーコン葉に含まれる新規ダイマーセスキテルペンラクトンの抗がん活性に関する研究)

学位論文要約：
Dissertation Summary

Yacon (*Smallanthus sonchifolius*) belongs to the Asteraceae family, a perennial plant with 1.5 to 3 m height, producing tubers roots. It is native to the Andes and has been widely distributed in many countries in Europe, America, and Asia such as China and Japan. In 1984, the first cultivar of yacon which is originally from Peru was introduced into Japan via New Zealand. Thereafter, some of other cultivar's have been introduced from Bolivia and Ecuador. (Sugiura et al, 2007) In Japan, there are four varieties of yacon (sarada otome, sarada okame, andesu no yuki and andesu no otome), which are hybridized with the original yacon lines. (Fujino et al, 2008 and Sugiura et al, 2014) Those yacon cultivars have been produced in Kagawa, Hiroshima, Tsukuba, Hokkaido and so on. Yacon tea has been used as a traditional folk medicine, and consumed in local markets (Sugahara et al., 2015). Melampolide-type SLs, enhydrin and uvedalin, were the main components in yacon leaf. (Mercado et al., 2014) In recent study, the aqueous extracts of yacon leaf which contained enhydrin was investigated for its safety assessment in rat and maximum doses for safety with the hypoglycemic effect. (Barcellina et al., 2012)

Sesquiterpene lactones (SLs) are colorless and bitter substances, classified as plant secondary metabolites. SLs which consist of three isoprene units with 15 carbon atoms will be biosynthesized via farnesyl pyrophosphate (FPP). (Ghantous et al., 2010) Almost all of SLs are modified from germacranolide which is a category of cyclic sesuquiterpene compounds that are derivered from germacradiene. There are 4 major

categories, depending on the carbon skeleton. Germacranolides have a 10 membered ring. Guaianolides have fused 7-membered and 5-membered rings, and a methyl group at C-4. Eudesmanolides have fused two 6 membered rings. Pseudoguaianolides have fused 7-membered and 5-membered rings and a methyl group at C-5. (Chadwick et al., 2013) Melampolide type SLs, can be categorized as a kind of germacranolides, which have the 10-membered ring that adopts a distorted chair-boat conformation. It has been reported that melampolides can be found in *Enhydra*, *Smallanthus* and *Polymnia*. (Stefani and Costa, 2006)

So far, more than 5000 SLs have been identified. Recently, 60 compounds out of them are known as rare dimer SLs composed by two SLs isolated from the Asteraceae family such as *Helianthus annuus*, *Scorzonera austriaca* and *Artemisia annua*. SLs isolated from a variety of medicinal plants are used as traditional medicines for treatment of inflammatory disease and cancer. As other interest therapeutic activities, antimicrobial, antiviral and antimalaria have been demonstrated recently. (Kreuger et al., 2012) The major chemical properties of SLs can be attributed to α, β-unsaturated-γ-lactone ring, side chains and lipophilicity. The α-methylene-γ-lactone moiety in SLs acts as an alkylation center which is considered to be the most important site for the biological activity.

SLs have been demonstrated the potentials for anticancer activity due to induction of apoptosis through downstream effect including activation of p53 and inhibition of NF-κB activity, which could be candidates in cancer treatment near further. (Shanmugan et al., 2011) Investigation of effective novel substances may lead to the creation of new reagents with the increase of various biological activities.

In this study, we firstly report and evaluate anti-cancer activity of a novel dimer sesquiterpene lactone (SL), uvedafolin, newly isolated from yacon (*S. sonchifolius*) leaf. The initial approach of the study is to isolate dimer SLs, uvedafolin (**1**) and enhydrofolin (**2**), from yacon leaf and evaluate their cytotoxic activity on HeLa (cervical cancer), HL-60 (leukemia) and B16-F10 melanoma (skin melanoma) cell lines.

The stereostructure of the novel dimer SL, uvedafolin (**1**) was elucidated by 1D/2D NMR, ESI-TOF-MS and single-crystal X-ray diffraction.

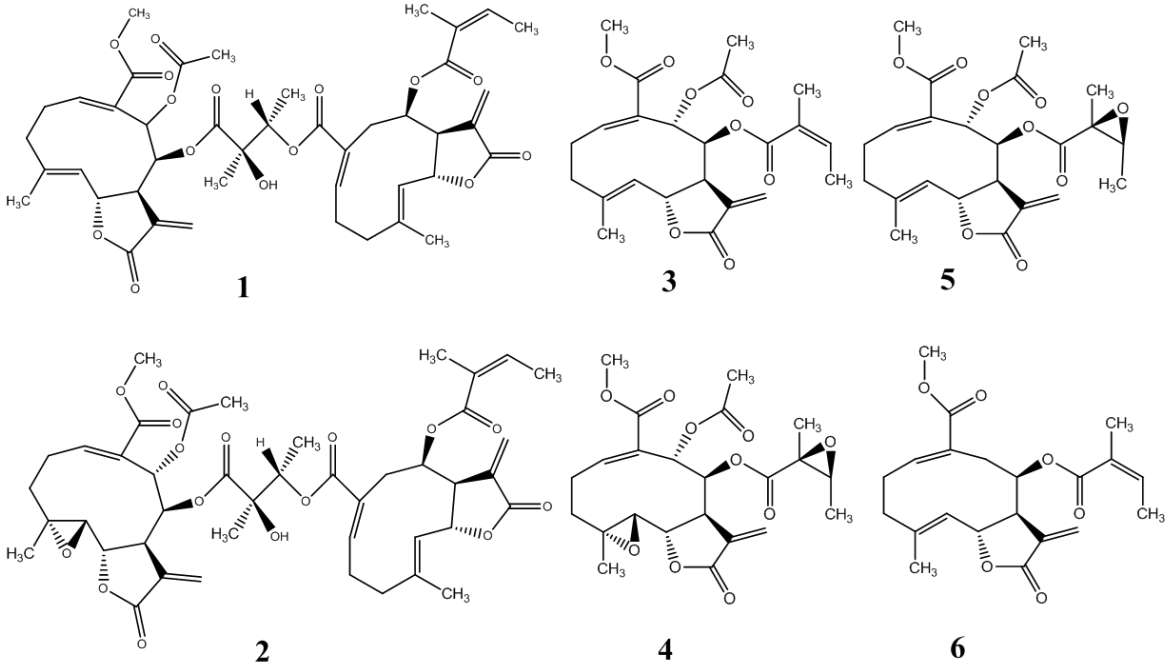


Figure 1. Chemical structures of sesquiterpene lactones from yacon leaves.
(**1**) Uvedafolin. (**2**) Enhydrofolin. (**3**) PolymatinB. (**4**) Enhydrin. (**5**) Uvedalin. (**6**) Sonchifolin.

After that we further carried out quantification of the valuable dimer SLs in eight varieties of yacon leaves that cultivated and hybridized in the Western Region Agricultural Research Center at Zentsuji city (National Agriculture and Food Research Organization). Among the eight varieties, the newly identified dimer, **1**, was found only in 'Sarada otome' (0.064 mg/g fresh weight) and 'Sarada okame' (0.045 mg/g fresh weight) cultivars and the related varieties.

The cytotoxicity of two dimer SLs was determined against typical cancer cell lines such as HeLa (cervical cancer), HL-60 (leukemia) and B16-F10 melanoma (skin melanoma) cells. The dimers, **1** and **2**, had the most effective IC_{50} values against the three tumor cell lines in a range from 0.2 to 1.9 μM, compared with those of monomers, **3**-**6** (0.7 - 9.9 μM). Moreover, 1:1 mixtures of enhydrin (**4**) + sonchifolin (**6**), and uvedalin (**5**) + sonchifolin (**6**) did not show high cytotoxicity as **1** and **2** did. Therefore, a cross-linkage of both monomer SLs can be concluded as an important factor for the

great cytotoxicity, which may be due to higher lipophilicity and disorder of functional structure of the cell membranes.

The second approach in study is to clarify the *in vitro* cytotoxic mechanism of dimer SL, uvedafolin (**1**) and the related monomer SLs by using HeLa cell line and NIH/3T3 cell line as the normal cell line (to see the toxicity against normal cell line). Apoptotic pathway by compound **1** was in-depth investigated by various kinds of assay systems such as cell cycle analysis, DAPI stain, caspase activity, JC-1 stain and cytochrome c release.

IC_{50} of compound **1** against NIH/3T3 cell line at 24 h was 4.98 μM, meaning that it is 1.68-times safer than that of HeLa cells. Cell cycle analysis after treatment of compound **1** showed a significant increase in the percentage of G_2/M phase from 14.0% to 22.9% for 24 h and to 38.1% for 48 h time-dependently when compared with control. Furthermore, the percentage of G_1 phase decreased significantly from 61.2% to 40.1% at 48 h. Moreover, an increase in a sub-G_1 peak, a parameter of fragmented DNA caused by apoptosis, was observed from 1.9% to 5.1% for 24 h and to 10.0% for 48 h after treatment of compound **1**.

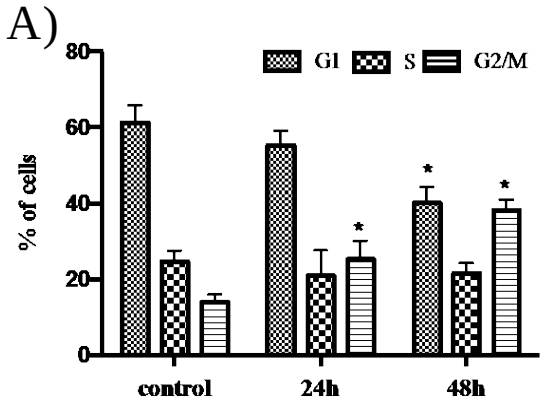


Figure 3. Effect of uvedafolin on HeLa cell cycle distribution. Cells were exposed to uvedafolin at 3μM for the indicated time and stained with PI. DNA content was analyzed by flow cytometry. A) Percentage of cell cycle distribution after treatment with compound **1**.

Morphological change in nuclear caused by apoptosis was observed by using DAPI. The increases of caspase activities by compound **1**, were observed in the following

order; caspase-9 > caspase-3/7 > caspase-8, meaning mitochondria pathway was expected to be the major apoptotic pathway. The photos and red fluorescence intensities (RFI) of untreated cells (control), etoposide treated cells (positive control) and cells treated by compound **1** were recorded and compared among them. Control showed high intensity of RFI (294), indicates keeping healthy cell whereas the etoposide treated cells (48 h) decreased red fluorescence (red fluorescence intensity: 17.56). Treated cells with compound **1** (24 h and 48 h) had decreased red fluorescence (red fluorescence intensity: 111.72 for 24 h and 15.57 for 48 h), indicating time-dependent apoptosis for the majority of cells.

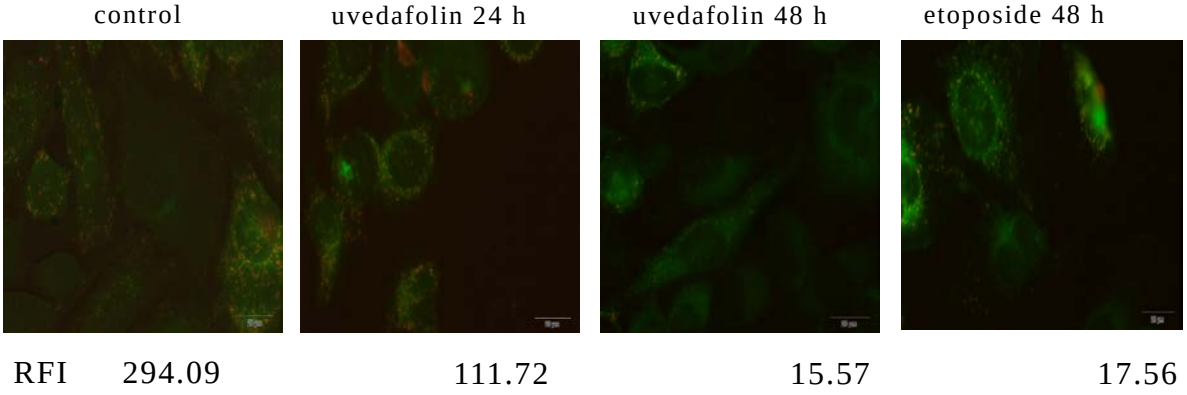
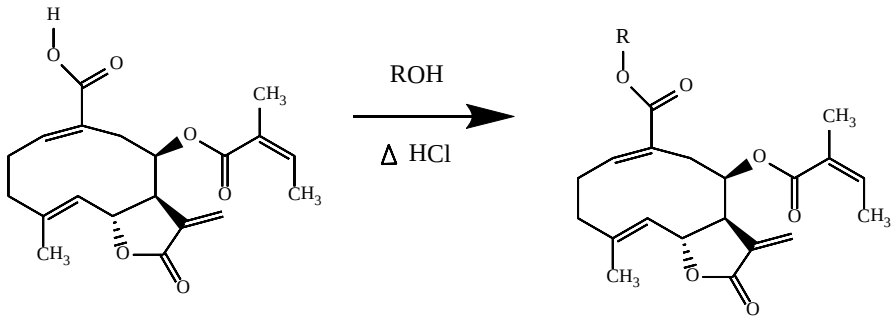


Figure 6. Fluorescent microscopy images to see the effect of uvedafolin on mitochondrial membrane potential of HeLa cells. Cells were treated with 3 μM uvedafolin and 10 μM etoposide. Fluorescent microscopy images were obtained by excitation at 520 nm and emission at 595 nm. The red fluorescence intensity (RFI) was described the values at the bottom of images.

Mitochondria membrane potential change and cytochrome c releasing were observed after treatment of **1**. Therefore, we can conclude that **1** surely induces apoptosis via mitochondria pathway more than cell death receptor pathway. Compound **1** would have a significant potential as one of leading compounds in anticancer drugs, since it has not been elucidated for the cytotoxic mechanism of the dimer SL previously.

The third experiment is to expand the possibility of creation of new drugs by

isolating sonchifolinic acid (**7**) from the yacon leaf and synthesizing its derivatives. In order to understand the effect of lipophilicity of the esterified derivatives, sonchifolinic acid (**7**) and its synthetic ester derivatives (Scheme-1) such as propyl, butyl and pentyl esters were first of all synthesized and examined for the cytotoxicity and structure-activity relationship (SAR).



Scheme-1 Esterification of sonchifolinic acid

Consequently, sonchifolinic acid (**7**) did not show strong cytotoxic activity (IC_{50} 47.12 μM). Insdead, the ester derivatives exhibited the moderate activity as following order: butyl (**7b**, 5.29 μM) > propyl (**7a**, 5.39 μM) > pentyl (**7c**, 8.94 μM) > sonchifolin (**6**, 15.11 μM) > 2-Methoxy-ethyl sonchifolin (**7d**, 21.07 μM). The contribution of methoxyethyl group of 2-methoxyethyl ester of sonchifolinic acid (**7d**) was surprisingly weak for the activity. Therefore, we could conclude that hydrophobicity of alkyl chains should be more effective to the cytotoxicity than the length of carbon chain. The activity of dimer SLs (**1** and **2**) demonstrated 1.7 times greater (IC_{50} values 2.83 μM for **1** and 2.97 μM for **2**) than the monomer SLs, including the monomeric derivatives (**7a** - **7c**) even though these alkyl esters have the similar log *P* values with compounds **1** and **2**. From our results, it can be concluded that esterification at C-14 is important to enhance the biological activity. Thus, not only length of alky chains but also other structural features such as alkylation number, linkage group and conformational space would contribute to cytotoxicity. This kind of chemical modification may accelerate further

investigation of new anticancer drug creation by the modification of compound **7** and other portions of the complex sesquiterpene lactones, including dimer SLs as potent lead compounds through semi-synthetic strategy.

In conclusion, a novel dimer SL, uvedafolin (**1**) newly isolated from the yacon leaf could has potential for an anti-cancer drug and lead compound for further investigation, contributing to cancer drug designing.

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