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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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Name

学位論文題目：
Title of Dissertation　Structure-Cytotoxic Activity Relationship of Dietary Lignans and Their Apoptosis Induction
(食物性リグナン類の構造と細胞毒性活性との関係及びアポトーシス誘導)

学位論文要約：
Dissertation Summary

Introduction

Peñalvo et al. (2008) have demonstrated the presence of lignans in 34 vegetables, 16 edible tubers and roots, 16 fruits, 4 legumes, 6 soybean-based products, and 4 cereal-based products. In plants, lignans are derived through cinnamate pathway and have been recognized for having great structural diversity. Depend on the organ and species of plant, lignans can occur as mixtures of enantiomers with various enantiomeric compositions (Suzuki et al. 2002; Williams et al. 2012).

Considering human health and utilization of food component to wider fields, the purpose of this research is to clarify the effect of natural dietary lignans along with its stereoisomers on cytotoxicity towards cancer cell lines. Effect of stereochemistry of natural dietary lignans was determined and discussed for the first time. Then, derivatives of natural dietary lignans were also prepared to construct the chemical library and have a better understanding on the structure-activity relationship. Chemical library is very crucial since new lignans are always discovered whether from plants or as metabolite of microorganism, animal, or human (Smeds et al. 2005; Struijs et al. 2009). By preparing lignan derivatives, improvement of the biological activity of natural dietary lignans was also desired in this research. Mechanism of dietary lignans and its derivatives inducing cells death was determined to ensure the safety side of dietary lignans.

Three groups of dietary lignans are focused in this research, i.e. butane-type lignans, epoxylignane, and lactone-type lignans. All natural lignans and lignan derivatives in this research were stereoselectively synthesized or obtained from the chemical library of Bioorganic Chemistry Laboratory, Faculty of Agriculture, Ehime University. No enzymatic reaction was involved in the synthesis procedures. The numbering and naming of compounds follow the nomenclature of lignans (Moss 2000).

Syntheses

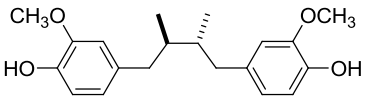
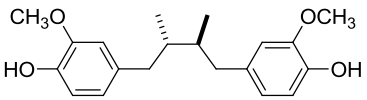
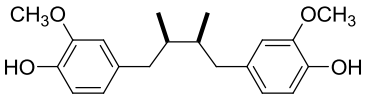
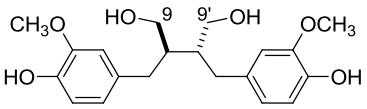
Synthetic pathway for the stereoisomers of natural dihydroguaiaretic acid, 1,7-seco-2,7′-cyclolignane, 9,9′-epoxylignane, matairesinol have been previously reported (Yamauchi et al. 2008; Kawaguchi et al. 2009; Morita et al. 2011; Yamauchi et al. 2014; Nishiwaki et al. 2014; Yamauchi et al. 2006). Modified synthetic pathways were employed to prepare the derivatives.

Result and Discussion

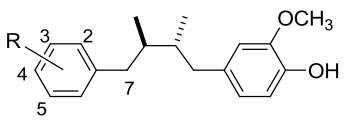
Butane-type lignan: Dihydroguaiaretic acid

Research on dihydroguaiaretic acid has been published as Wukirsari et al. (2014a). Three stereoisomers of dihydroguaiaretic acid (DGA) **1**–**3** showed the same level of cytotoxicity against HL-60. However, cytotoxicity against HeLa was stereochemistry-dependent and (–)-DGA **1** was the stronger (**Table 1**). In contrast to (–)-DGA **1**, (–)-secoisolariciresinol **4** did not show any significant activity at 100 μM; suggested that the presence of hydroxy groups on 9- and 9′-position was disadvantageous for the activity. Thus, structure of (–)-DGA **1** was chosen as lead compound for the study on structure-cytotoxic activity relationship. Moreover, synthetic cost for the preparation of (–)-DGA **1** and its derivatives was the lowest since it used cheaper L-glutamic acid as starting material.

Table 1. Cytotoxicity of three stereoisomers of dihydroguaiaretic acid (DGA) **1**–**3** and (–)-secoisolariciresinol **4** against HL-60 and HeLa cell line (IC_{50} ± SD, n = 3).

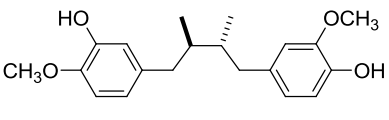
compounds	HL-60	HeLa
 (–)-DGA (**1**)	26 μM ± 0.9	22 μM ± 4.1
 (+)-DGA (**2**)	24 μM ± 1.6	32 μM ± 3.9
 *meso*-DGA (**3**)	28 μM ± 3.3	41 μM ± 1.1
 (–)-secoisolariciresinol (**4**)	>100 μM (24% inhibition at 100 μM)	>100 μM (35% inhibition at 100 μM)

As the next stage, 28 derivatives on 7-phenyl were prepared and subjected to cytotoxic assay against HL-60 and HeLa cell lines. In general, it seemed that HeLa was stronger than HL-60 because cell death induction on HeLa cells require higher sample concentration than induction on HL-60 cell line. Though it was difficult to see clear relationship between substituents on 7-phenyl and cytotoxicity, 2-hydroxy, 2-butoxy, 3-hydroxy-4-methoxy **5**, and 4-hydroxy-3,5-dimethoxy derivatives were observed having significant activity below 10 μM for both HL-60 and HeLa cell lines. It is noteworthy that switched positions of substituent on 7-phenyl moiety of (–)-DGA led to the stronger activity of 3-hydroxy-4-methoxy derivative **5**. Therefore, 3-hydroxy-4-methoxyphenyl on 7-position was fixed for further experiment on 7′-phenyl derivatives.



7-phenyl derivatives

R = OH, OCH_3, $O(CH_2)_3CH_3$, CH_3 F, Cl, or OCH_2O



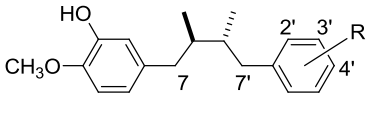
3-hydroxy-4-methoxy derivative (**5**)

IC_{50} = 8.7 μM ± 1.2 against HL-60

7.5 μM ± 0.5 againt HeLa

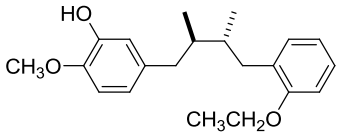
Cytotoxic activities of 13 derivatives on 7′-phenyl against HL-60 and HeLa clearly displayed notable cytotoxic activity below 10 μM for all derivatives against both HL-60 and HeLa cell line. Cytotoxicity against HL-60 was slightly higher than against HeLa cell line. 2′-Substituted derivatives hold slightly stronger activity followed by 4′- and 3′-position, respectively. The presence of hydroxy, methoxy, methyl, and fluorine were equipotent for the cytotoxic effect.

2′-Methoxy derivative did not statistically different from other 2′-substituted derivatives but it had slightly higher activity. Therefore, 2′-ethoxy, 2′-isopropoxy, and 2′-butoxy derivatives were prepared to check the effect of steric and hydrophobic properties. Cytotoxicity was elevated by the presence of ethoxy group but declined when the alkoky group was getting longer. This fact suggested that steric restriction was applied on 2′-position and more hydrophobic group was not necessarily have positive effect on cytotoxicity of (–)-DGA derivatives. 2′-Ethoxy derivative **6** was 30-fold stronger against HL-60 and 13-fold stronger against HeLa than the natural (–)-DGA. Apoptosis induction by 2′-ethoxy derivative **6** was observed using simple procedure based on cell morphology. Under light microscope, the presence of membrane blebbing and apoptotic bodies as signs of apoptosis induction were recognized on cells treated with 2′-ethoxy derivative **6**.



7'-phenyl derivatives

R = OH, OCH_3, CH_3, F, OCH_2CH_3, $OCH(CH_3)_2$, or $O(CH_2)_3CH_3$



2'-ethoxy derivative (**6**)

IC_{50} = 0.8 μM ± 0.4 against HL-60
1.7 μM ± 0.6 againt HeLa

Butane-type lignan: 1,7-Seco-2,7′-cyclolignane

Research on 1,7-seco-2,7′-cyclolignane (SCL) has been published as Wukirsari et al. (2014b). Stereisomers of SCL along with 7- and 9′-hydroxy, methoxy, and fluorine derivatives **7**–**26** did not show specific cytotoxicity over cell line (**Table 2**). Terminal methyl was more favorable than the presence of hydroxy, methoxy, or fluorine on 9′- or 7-position. It can be said that cytotoxic activity of SCL was mainly influenced by substituent rather than stereochemistry or the position of substituent. Fluorine substituted derivatives showed stereochemistry-dependent and stronger than methoxy and hydroxy derivatives.

In general, all compounds bearing 9′- or 7-hydroxy group did not exhibit notable cell growth inhibition at 100 μM. Previously, the negative effect of hydroxy group on terminal positions was also shown by (–)-secoisolariciresinol. The hydrophilic property of hydroxy group seemed holding the responsibility of the low cytotoxic effect. Hydrophilic compound is believed for having lower penetration ability through cell membranes than hydrophobic compound (van Rijt et al. 2010).

As noted that hydrophobicity played a role on the cytotoxic activity of SCL, alkyl group was chosen as a representative of functional group with high hydrophobic property. Therefore, 9′-alkyl derivatives **27**–**30** were prepared to examine the steric effect of alkyl group on the activity was explored by preparing derivatives bearing methyl, isopropyl, *n*-butyl, and heptyl on 9′-position (**Table 3**). 9′-Heptyl derivative **30** had 4-fold stronger activity than **7**, suggested that bulky group was tolerable and high hydrophobicity was favorable for the cytotoxic activity of SCL. Apoptosis induction by 9′-heptyl derivative **30** was proven by flow cytometry, western blotting, and DNA laddering analyses.

Table 2. Cytotoxic activities against HL-60 and HeLa cell lines of secocyclolignane and its hydroxy, methoxy, and fluorine derivatives (IC_{50} ± SD, *n* = 3). Ar = 4-hydroxy-3-methoxyphenyl.

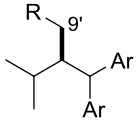
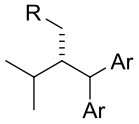
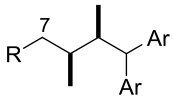
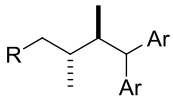
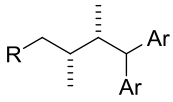
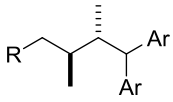
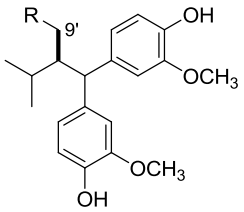
compounds		HL-60	HeLa
	**7**: R = H	17 μM ± 0.8	16 μM ± 0.5
	8: R = OH	>100 μM (10% inhibition at 100 μM)	100 μM (3% inhibition at 100 μM)
	9: R = OCH_3	72 μM ± 4.9	76 μM ± 0.6
	10: R = F	19 μM ± 0.1	19 μM ± 0.7
	**11**: R = H	20 μM ± 5.5	19 μM ± 1.8
	12: R = OH	>100 μM (18% inhibition at 100 μM)	>100 μM (2% inhibition at 100 μM)
	13: R = OCH_3	54 μM ± 4.6	69 μM ± 4.3
	14: R = F	39 μM ± 2.4	41 μM ± 6.4
	**15**: R = OH	>100 μM (7% inhibition at 100 μM)	>100 μM (3% inhibition at 100 μM)
	16: R = OCH_3	68 μM ± 2.2	77 μM ± 0.4
	17: R = F	45 μM ± 2.3	34 μM ± 4.8
	**18**: R = OH	>100 μM (23% inhibition at 100 μM)	>100 μM (0% inhibition at 100 μM)
	19: R = OCH_3	85 μM ± 4.4	>100 μM (34% inhibition at 100 μM)
	20: R = F	56 μM ± 6.0	75 μM ± 8.3
	**21**: R = OH	>100 μM (0% inhibition at 100 μM)	>100 μM (7% inhibition at 100 μM)
	22: R = OCH_3	73 μM ± 4.5	58 μM ± 6.1
	23: R = F	32 μM ± 3.1	37 μM ± 1.2
	**24**: R = OH	>100 μM (3% inhibition at 100 μM)	>100 μM (8% inhibition at 100 μM)
	25: R = OCH_3	82 μM ± 2.4	72 μM ± 1.8
	26: R = F	38 μM ± 1.2	42 μM ± 1.2

Table 3. Cytotoxic activity of 9′-alkyl derivatives of 1,7-seco-2,7′-cyclolignane (IC_{50} ± SD, *n* = 3).

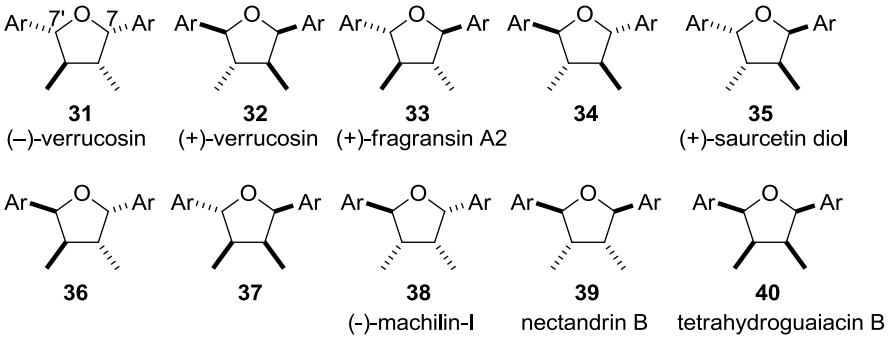
compounds	HL-60	HeLa
		
7: R = H	17 μM ± 0.8	16 μM ± 0.5
27: R = CH_3	16 μM ± 0.8	10 μM ± 0.8
28: R = $CH(CH_3)_2$	10 μM ± 0.1	8.2 μM ± 0.1
29: R = $(CH_2)_3CH_3$	10 μM ± 0.4	9.2 μM ± 0.1
30: R = $(CH_2)_6CH_3$	3.7 μM ± 0.04	3.1 μM ± 0.02

(–)-Verrucosin

High diversity in structure of epoxylignane has been recognized in human diet. In this research, di-, tri-, and tetrasubstituted tetrahydrofuran lignans along with its stereoisomers were screened for its cytotoxic activity. Stereosiomers of 9,9′-epoxylignane, lariciresinol, and (–)-pinoresinol did not have any significant activity at 100 μM whereas (+)-pinoresinol exhibited weak specific cytotoxic activity against HL-60 cell line. The presence of primary hydroxy group was disadvantageous for the cytotoxic activity of (–)- and (+)-lariciresinol. As additional support on this fact, 9-dehydroxylariciresinol without primary hydroxy group displayed higher cytotoxicity effect. 7,7′-Epoxylignane without primary hydroxy group was another potential cytotoxic agent among ether type lignans.

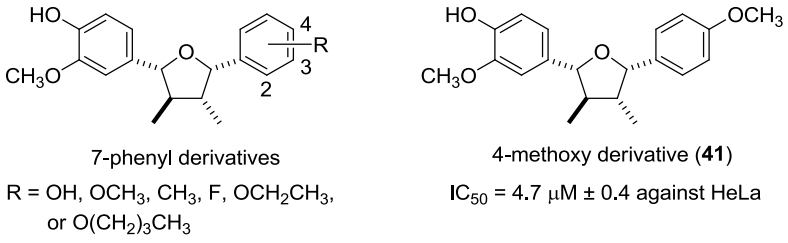
In general, nearly all stereoisomers of 7,7′-epoxyliganane showed higher cytotoxicity against HeLa than HL-60, only **32**, **36**, and **40** had identical cytotoxicity against both HL-60 and HeLa cell lines (**Table 4**). Then, as stronger active compound, the structure of (–)-verrucosin **31** was picked up as the lead compound for further research on cytotoxicity against HeLa cell lines. Effect of substituent on 7-phenyl, 7′-phenyl, and 9- and 9′-position of (–)-verrucosin **31** was studied.

Table 4. Cytotoxicity of ten stereoisomers of 7,7′-epoxylignane against HL-60 and HeLa cell lines (IC_{50} ± SD, *n* = 3). Ar = 4-hydroxy-3-methoxyphenyl

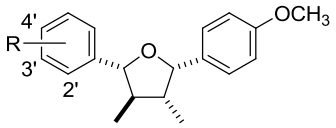


No.	HL-60	HeLa	No.	HL-60	HeLa
31	75 μM ± 4.7	6.6 μM ± 2.6	**36**	39 μM ± 2.1	38 μM ± 0.8
32	78 μM ± 1.1	80 μM ± 8.4	**37**	68 μM ± 6.7	27 μM ± 6.1
33	59 μM ± 8.2	25 μM ± 5.8	**38**	69 μM ± 7.8	18 μM ± 10
34	95 μM ± 10	48 μM ± 10	**39**	70 μM ± 6.1	33 μM ± 10
35	75 μM ± 6.5	38 μM ± 4.6	**40**	36 μM ± 6.5	32 μM ± 4.7

Among 13 derivatives on 7-phenyl of (–)-verrucosin **31**, 4-hydroxy derivative and 4-methoxy derivative **41** were noticed for having slightly stronger activity than (–)-verrucosin **31**. For this reason, 4-fluorine, 4-methyl, 4-ethoxy, 4-butoxy derivatives were prepared. Derivative having methyl as electron donating group had the same level cytotoxicity as 4-methoxy derivative. However, fluorine and longer alkoky groups on 4-position were disadvantageous for the activity.

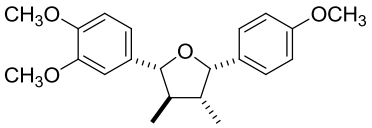


As the second stage on derivatization, the potent 7-phenyl moiety was used as the basic structure for 7′-derivatives. Then, 4-methoxyphenyl was selected over 4-hydroxyphenyl for a better stability and avoiding the cytotoxicity of phenolic group. Moreover, the absence of phenolic group means without benzyl protection and shorten the synthetic pathway. Ten derivatives bearing unsubstituted and monosubstituted phenyl on 7′-position had weak activity more than 30 μM. Therefore, cytotoxicity of 11 multisubstituted derivatives which is focused on 3′- and 4′-position was determined. Dimethoxy derivative **42** seem holding stronger cytotoxicity and gave a new hope on cytotoxicity of (–)-verrucosin derivatives. Apoptosis induction was observed under light microscope and fluorescence microscopy.



7'-phenyl derivatives

R = OH, OCH_3, F, OCH_2CH_3, $O(CH_2)_3CH_3$, or OCH_2O

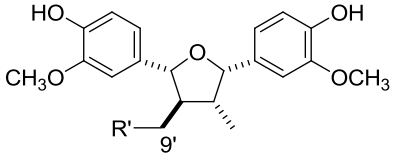


3,4-dimethoxy derivative (**42**)

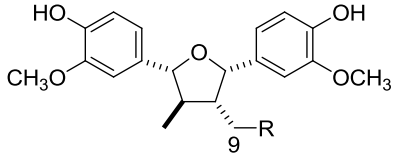
IC_{50} = 2.4 μM ± 0.1 against HeLa

The role of terminal methyl groups on cytotoxicity was investigated by introducing longer alkyl (butyl), electron withdrawing (fluorine), and hydroxy group on 9′- and 9-position. It was clarified that 9-position was more involved in cytotoxicity than 9′-position since 9-derivatives **46**–**48** had 2-fold higher activity than 9′-derivatives **43**–**45**. Butyl derivatives displayed stronger activity followed by fluorine and hydroxyl group. However, those 6 derivatives were still weaker than (–)-verrucosin **31** which indicated terminal methyl groups was more desirable on 9′- and 9-position.

Table 5. Cytotoxicity of 9′ and 9-derivatives of (–)-verrucosin against HL-60 and HeLa cell lines (IC_{50} ± SD, n = 3).



43–45

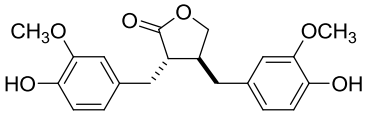


46–48

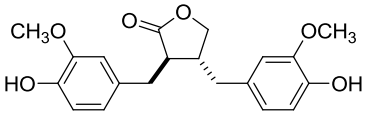
No.	R'	HeLa	No.	R	HeLa
34	9'-OH	>100 μM (0% inhibition at 100 μM)	**46**	9-OH	>100 μM (48% inhibition at 100 μM)
44	9'-F	73 μM ± 9.1	**47**	9-F	44 μM ± 2.3
45	9'-$(CH_2)_3CH_3$	33 μM ± 1.0	**48**	9-$(CH_2)_3CH_3$	18 μM ± 0.1

Matairesinol

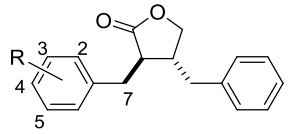
Both (+)- and (–)-matairesinol **49**–**50** did not show significant activity at 100 μM against HeLa, however, (–)-matairesinol **50** displayed cytotoxicity towards HL-60 cell line (IC_{50} = 74 μM). Therefore, 45 derivatives of (–)-matairesinol were introduced to cytotoxic assay against HL-60. 2-Butoxy, 3-butoxy, 4-butoxy, 3,4-dichloro, 2,4-dichloro, and 3,5-dichloro derivatives had higher hydrophobicity and showed stronger activity around 20 μM. However, longer alkoxy and bulkier groups declined the cytotoxicity effect as shown by heptoxy and dibutoxy derivatives. The change of HL-60 cell morphology could be recognized after treatment with 3,4-dichloro derivative. It seems that apoptotic bodies were formed as a clear sign of apoptosis induction.



(+)-matairesinol (**49**)



(–)-matairesinol (**50**)



7-phenyl derivatives

R = OH, OCH_3, $O(CH_2)_3CH_3$, $O(CH_2)_6CH_3$, OCF_3, CH_3 CF_3, F, Cl, NO_2, or OCH_2O

Conclusion

The ability of stereoisomers of dietary lignans to inhibit the proliferation of HL-60 and HeLa cell lines was clarified in this research. In general, the adverse effect of primary hydroxy groups was confirmed by the insignificant activities of secoisolariciresinol and lariciresinol at 100 μM. A weak activity was displayed by the natural lactone-type lignan (–)-matairesinol (IC_{50} = 74 μM against HL-60). On the other hand, moderate cytotoxic activity was possessed by the natural dihydroguaiaretic acid (DGA) and 1,7-seco-2,7′-cyclolignane (SCL, IC_{50} around 20 μM against both HL-60 and HeLa) while (–)-verrucosin displayed stronger activity (IC_{50} = 6.6 μM against HeLa cell).

Structure-cytotoxic activity relationship was determined by preparing derivatives of (–)-DGA, SCL, (–)-verrucosin, and (–)-matairesinol. As a result, many new compounds were discovered more potent than the natural lignans up to 30-fold. It was also understood that hydrophobicity was not always directly related to the cytotoxic effect. The advantage of hydrophobicity on cytotoxicity was only observed on SCL and (–)-matairesinol derivatives. Apoptosis induction by all active compounds was verified based on cell morphology or further analyses on flow cytometry, western blotting, DNA laddering, or fluorescence microscopy.

Considering the fact that new structure and stereoisomer of lignans are always discovered in the future, construction of chemical library of dietary lignans was a great contribution on the human health and food safety. Moreover, information on structure-activity relationship is important for further research on protein target such as biotinylation procedure. Finally, this research has broadened the application of food component as new lead compounds for cytotoxic-drug.

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