薬物代謝工学分野 Division of Metabolic Engineering

教　授	服部　征雄	Professor	Masao Hattori (Ph. D.)
助教授	横澤　隆子	Associate Professor	Takako Yokozawa (Ph. D.)
助　手	宮代　博継	Assistant Professor	Hirotsugu Miyashiro (Ph. D.)
技　官	中村　憲夫	Research Associate	Norio Nakamura (Ph. D.)

薬物代謝工学分野は和漢薬の薬効，毒性発現に関与する代謝系の分子生物学的研究を発展させることを設置目的とし，① 和漢薬の薬効発現に関与する腸内細菌の役割，② 薬物代謝に関する腸内細菌遺伝子の解明，③ 腎毒性物質産生機構の分子生物学的解明とその制御に関する研究を課題として取りあげ，和漢薬の薬効発現機構、生体へのレスポンスなどの基礎的研究を通じて，和漢薬の科学的評価や臨床応用をはかることを目指している。主な研究題目を以下に示す。

1．天然物のバイオトランスフォーメイション
2．和漢薬の薬効発現に関与する腸内細菌，酵素 および その遺伝子の解析
3．AIDS，C型肝炎の予防 および 治療薬の開発
4．腎疾患における病態の解明と腎臓病治療薬の開発
5．糖尿病性腎症の治療戦略

本年度の主な研究を列挙すると：

1．ヒト腸内細菌による杜仲の樹皮に多量に含まれる pinoresinol diglucoside のエストロゲン様物質（エンテロジオール，エンテロラクトン）への変換を検討し，この化合物が加水分解，エーテル環の開裂，脱メチル化，脱水酸基化反応を経て変換されることを明らかにした。また，これらの代謝物のエストロゲン様作用，抗エストロゲン様作用を検討した。
2．霊芝に含まれる苦味成分の HPLC による分析法を確立し，霊芝製品の品質評価を行なった。また 台湾産樟芝から 5 種の新規化合物を単離し，その細胞毒性を検討した。
3．中国少数民族薬物の C 型肝炎ウイルス由来の RNA ポリメラーゼ阻害活性を探索した。
4．蛋白・糖修飾を surrogate マーカーとして利用し，腎不全並びに糖尿病性腎症における病態把握に着手した。また新たな治療手段を探索するために，温脾湯，epicatechin 3-*O*-gallate，緑茶ポリフェノール，桂枝茯苓丸，八味地黄丸，芥子菜，韓国産生薬を中心に検討した。

◇著　書　Book

1）楊秀偉，郝美荣，服部征雄．：『中葯成分代謝分析』中国医葯科技出版．，北京，(2003).

2）横澤隆子：『長寿科学事典』医学書院，東京，2003, pp. 919-922.

◇原　著　Original paper

1）**Abdel-Hafez A. A., Hosny A. H. E., Jo M., Kurokawa M., Shiraki K., Kawahata T., Otake T., Nakamura N., and Hattori M.: Synthesis and evaluation of anti-HIV-1 and anti-HSV-1 activities of 4H-[1,2,4]-triazolo[1,5-a]pyrimidin-5-one derivatives.** ***Arzneim. Forsch. Drug. Res.,*** **52:833-839, 2002.**

In a one pot procedure, 18 compounds of 7-(substituted phenyl)-2-substituted-6,7-dihydro-4H- [1,2,4] triazolo [1,5-a] pyrimidin-5-one derivatives (**16-33**) have been synthesized. 3(5)-Amino-5(3)-substituted-1,2,4-triazole derivatives (**7-12**) were used as synthones which were cyclocondensed by fusion with substituted methyl cinnamate esters (**13-15**) to afford the target compounds (**16-33**). In an effort to develop new non-nucleoside antiviral agents, compounds **16-33** were evaluated for their anti-HIV-1 and anti-HSV-1 activities. Complete inhibition of the proliferation of HIV-1 viruses was achieved by compounds **22**, **23** and **24** at concentrations of 25, 25 and 50 μg/ml, respectively. 7-Phenyl-2-(*n*-pentyl)-6,7-dihydro-4H-[1,2,4]triazolo[1,5-a]pyrimidin-5-one (**19**) exhibited potential activity against HSV-1 with 88% reduction in the viral plaques. The suggested marked specificity of this class of compounds as anti-HIV-1 agents is discussed.

2）**Takahashi K., Ouyang X., Komatsu K., Nakamura N., Hattori M., Baba A., and Azuma J.: Sodium tanshinone IIA sulfonate derived from Danshen (*Salvia miltiorrhiza*) attenuates hypertrophy induced by angiotensin II in cultured neonantal rat cardiac cells.** ***Biochem. Pharm.,*** **64:745-750, 2002.**

和漢薬研究所年報 29巻（2002年）p.88 参照.

3）**Kuboyama K., Tohda C., Zhao J., Nakamura N., Hattori M., and Komatsu K.: Axon- or dendrite-predominant outgrowth induced by constituents from Ashwagandha.** ***Neourochemistry,*** **13:1715-1720, 2002.**

和漢薬研究所年報 29巻（2002年）p.87 参照.

4）**Xie L., Ahn E., Akao T., Abdel-Hafez A. A., Nakamura N., and Hattori M.: Transformation of arctiin to estrogenic and antiestrogenic substances by human intestinal bacteria.** ***Chem. Pharm. Bull.,*** **51: 378-384, 2003.**

After anaerobic incubation of arctiin (**1**) from the seeds of *Arctium lappa* with a human fecal suspension, six metabolites were formed, and their structures were identified as (-)-arctigenin (**2**), (2*R*,3*R*)-2-(3',4'-dihydroxybenzyl)-3-(3",4"-dimethoxybenzyl)butyrolactone (**3**), (2*R*,3*R*)-2-(3'-hydroxybenzyl)-3-(3",4"-dimethoxybenzyl)butyrolactone (**4**), (2*R*,3*R*)-2-(3'-hydroxybenzyl)-3-(3"-hydroxy-4"-methoxybenzyl)butyrolactone (**5**), (2*R*,3*R*)-2-(3'-hydroxybenzyl)-3-(3",4"-dihydroxybenzyl)butyrolactone (**6**), and (-)-enterolactone (**7**) by various spectroscopic means including two dimensional (2D)-NMR, mass spectrometry, and circular dichroism. A possible metabolic pathway was proposed on the basis of their structures and the time course of the transformation. Enterolactones obtained from the biotransformation of arctiin and secoisolariciresinol diglucoside (SDG, from the seeds of *Linum usitatissium*) by human intestinal bacteria were proved to be enantiomers, with the (-)-(2*R*,3*R*) and (+)-(2*S*,3*S*) configurations, respectively. Compound **6** showed the most potent proliferative effect on the growth of MCF-7 human breast cancer cells in culture among **1** and six metabolites, while it showed inhibitory activity on estradiol-mediated proliferation of MCF-7 cells at a concentration of 10 μM. These results indicate that the transformation of **1** by intestinal flora might

be essential for the manifestation of the estrogenic and antiestrogenic activity of **1**.

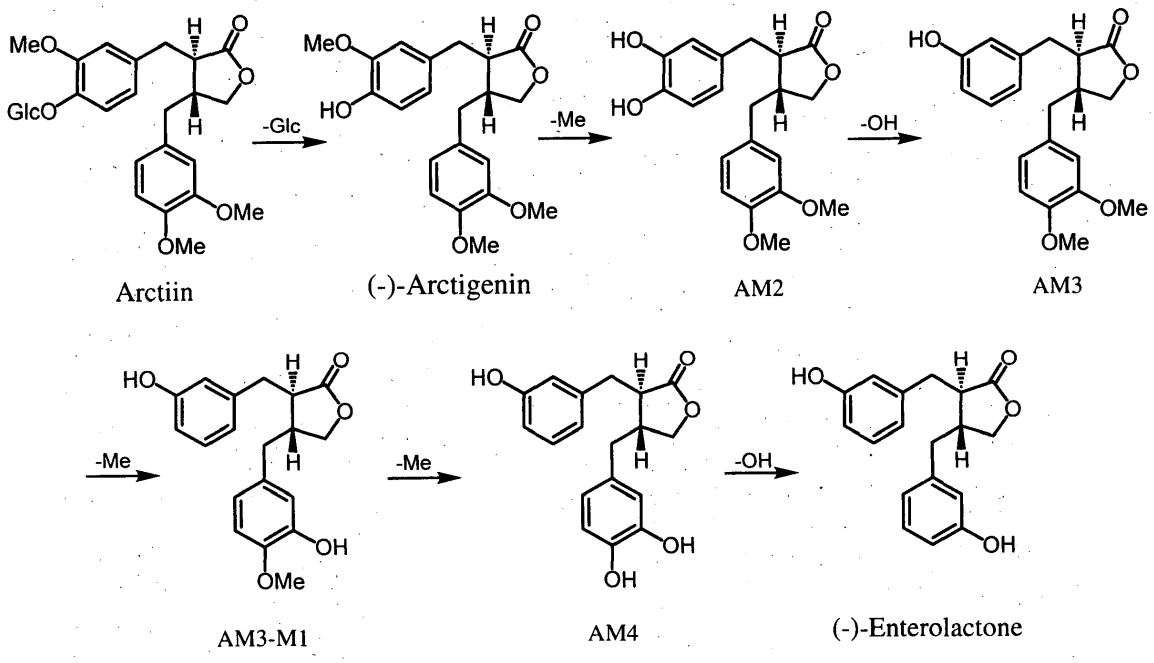

Fig. 1

5) **Tewtrakul S., Miyashiro H., Nakamura N., Hattori M., Kawahata T., Otake T., Yoshinaga T., Fujiwara T., Supavita T., Yuenyongawad S., Rattanasuwon P., and Dej-Adisai S.: HIV-integrase inhibitory substances from *Coleus parvifolius*. *Phytother. Res.*, 17:232-239, 2003.**

For the purpose of discovering anti-HIV-1 agents from natural sources, water and EtOH extracts of 50 Thai plants were screened for their inhibitory activity against HIV-1 integrase (IN), an enzyme essential for viral replication. Of these plants, an EtOH extract of *Coleus parvifolius Benth.* (aerial parts) showed potent activity against HIV-1 IN with an IC_{50} value of 9.2 μg/mL. From this extract, 11 compounds were isolated and identified as luteolin 5-*O*-β-D-glucopyranoside (**1**), luteolin (**2**), luteolin 7-methyl ether (**3**), luteolin 5-*O*-β-D-glucuronide (**4**), 5-*O*-β-D-glucopyranosyl-luteolin 7-methyl ether (**5**), rosmarinic acid (**6**), rosmarinic acid methyl ester (**7**), daucosterol (**8**), a mixture of α- and β-amyrins (**9**, **10**) and phytol (**11**). Of these compounds, rosmarinic acid methyl ester (**7**), rosmarinic acid (**6**), luteolin (**2**) and luteolin 7-methyl ether (**3**) exhibited inhibitory activities against HIV-1 IN with IC_{50} values of 3.1, 5.0, 11.0 and 11.0 μM, respectively. Among rosmarinic acid derivatives, the HIV-1 IN inhibitory activity increased in turn for a dimer (IC_{50} = 5.0 μM), a trimer (IC_{50} = 1.4 μM), and a tetramer (IC_{50} = 1.0 μM).

6) **Xie L., Akao T., Hamasaki K., Deyama T., and Hattori M.: Biotransformation of pinoresinol diglucoside to mammalian lignans by human intestinal microflora, and isolation of *Enterococcus faecalis* strain PDG-1 responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol. *Chem. Pharm. Bull.*, 51: 508-515, 2003.**

By anaerobic incubation of pinoresinol diglucoside (**1**) from the bark of *Eucommia ulmoides* with a fecal suspension of humans, eleven metabolites were formed, and their structures were identified as (+)-pinoresinol (**2**),(+)-lariciresinol (**3**), 3'-demethyl-(+)-lariciresinol (**4**), (-)-secoisolariciresinol (**5**), (-)-3-(3", 4"-dihydroxybenzyl)-2-(4'-hydroxy-3'-methoxybenzyl)butane-1,4-diol (**6**), 2-(3',4'-dihydroxybenzyl)-3-(3",4"-dihydroxybenzyl)butane-1,4-diol (**7**), 3-(3"-hydroxybenzyl)-2-(4'-hydroxy-3'-methoxybenzyl)butane-1,4-diol (**8**), 2-(3',4'-dihydroxybenzyl)-3-(3"-hydroxybenzyl)butane-1,4-diol (**9**), (-)-enterodiol (**10**), (-)-(2*R*,3*R*)-3-(3",4"-dihydroxybenzyl)-2-(4'-hydroxy-3'-methoxybenzyl)-butyrolactone (**11**), (-)-(2*R*,3*R*)-2-(3',4'-dihydroxybenzyl)-3-(3",4"-dihydroxybenzyl)butyrolactone (**12**), (-)-(2*R*,3*R*)-3-(3"-hydroxybenzyl)-2-(4'-hydroxy-3'-methoxybenzyl)butyrolactone (**13**), 2-(3',4'-dihydroxybenzyl)-

3-(3"-hydroxybenzyl)butyrolactone (**14**), 2-(3'-hydroxybenzyl)-3-(3",4"-dihydroxybenzyl)butyrolactone (**15**) and (-)-(2*R*,3*R*)-enterolactone (**16**) by various spectroscopic means, including two dimensional (2D)-NMR, mass spectrometry and circular dichroism. A possible metabolic pathway was proposed on the basis of their structures and time course experiments monitored by thin-layer chromatography. Furthermore, a bacterial strain responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol was isolated from a human fecal suspension and identified as *Enterococcus faecalis* strain PDG-1.

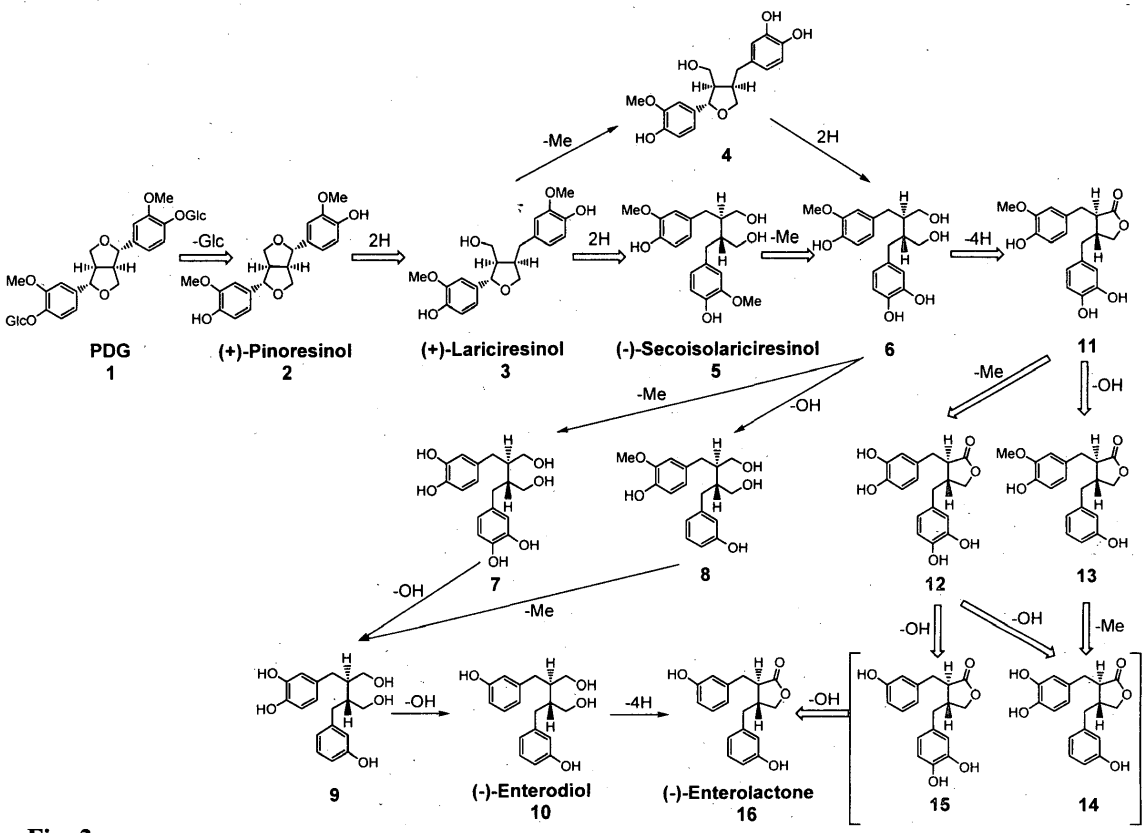

Fig. 2

7) **Tanaka N., Sekiya N., Hattori M., Goto H., Shibahara N., Shimada Y., and Terasawa K.: Measurement of plasma procyanidin B-2 and procyanidin B-3 levels after oral administration in rat. *Phytomedicine,* 10:122-126, 2003.**

Using a high-performance liquid chromatographic method and mass spectrometry analysis, we successfully measured the absorption of orally administered procyanidin B-2 and procyanidin B-3 isolated from Cinnamonoimi Cortex (the bark of *Cinnamomum cassia* Blume) in the rat plasma. This method used a TSK-GEL ODS-80TS column, two solvents (A: 0.01% acetic acid; B: methanol with 0.01% acetic acid) in a linear gradient at a flow-rate of 1.0 ml/min, and fluorescence detection at excitation and emission wavelengths of 220 and 327 nm.

8) **Min B., Lee S., Kim J., Lee J., Kim T., Kim D., Kim Y., Joung H., Lee H., Nakamura N., Miyashiro H., and Hattori M.: Anti-complement activity of constituents from the stem-bark of *Juglans mandshurica. Biol. Pharm. Bull.,* 26:1042-1044, 2003.**

Four known flavonoids and two galloyl glucoses isolated from the stem-bark of *Juglans mandshurica* (Juglandaceae), namely taxifolin (**1**), afzelin (**2**), quercitrin (**3**), myricitrin (**4**), 1,2,6-trigalloylglucose (**5**), and 1,2,3,6-tetragalloylglucose (**6**), were evaluated for their anti-complement activity against a complement system. Afzelin (**2**) and quercitrin (**3**) showed inhibitory activity against the complement system with 50% inhibitory concentration (IC_{50}) values of 258 and 440 μM. 1,2,6-Trigalloylglucose (**5**) and 1,2,3,6-tetragalloylglucose (**6**) exhibited anti-complement activity with IC_{50} values of 136 and 34 μM. In terms of the evaluation of the structure-activity

relationship of 3,5,7-trihydroxyflavone, compounds **2**, **3**, and **4** were hydrolyzed with naringinase to give kaempferol (**2a**), quercetin (**3a**), and myricetin (**4a**) as their aglycones, and these were also tested for their anti-complement activity. Of the three aglycones, kaempferol (**2a**) exhibited weak anti-complement activity with an IC_{50} value of 730 μM, while quercetin (**3a**) and myricetin (**4a**) were inactive in this assay system. Among the compounds tested, 1,2,3,6-tetragalloylglucose (**6**) showed the most potent anticomplement activity (IC_{50}, 34 μM).

9) **Tazawa T., Zhao H., Li Y., Meselhy M. R., Nakamura N., Akao T., and Hattori M.: A new enzyme immunoassay for aconitine and its application to quantitative determination of aconitine levels in plasma. *Biol. Pharm. Bull.*, 26:1289-1294, 2003.**

A reliable enzyme immunoassay (EIA) method was developed for quantitative determination of aconitine with high sensitivity and specificity. The bovine serum albumin (BSA)- and β-galactosidase (β-Gal) conjugates as immunogens and enzyme-labeled antigens were prepared by coupling of their proteins with succinic acid (short chain length; n=2, where n represents the number of methylene units) and hexadecanedioic acid (long chain length; n=14) hemiesters of benzoylaconine through the respective *N*-hydroxysuccinimide esters as intermediates. Two types of the BSA-conjuagates with short and long chains were repeatedly injected into rabbits to obtain anti-aconitine antisera (As1 and As2, respectively). All combinations of β-Gal-labeled antigens LAg1 (n=2) and LAg2 (n=14) with antisera As1 (n=2) and As2 (n=14) showed high sensitivity to aconitine in a range of 0.1-1.0 ng. Although the combination of LAg2 (n=14) with antiserum As1 (n=2) showed high specificity to aconitine, the combination of LAg2 (n=14) and As2 (n=14) was highly specific to both aconitine and mesaconitine. When aconitine was intravenously administered to rats, the aconitine concentration in their plasma remarkably decreased within the first 60 min, and then gradually declined, suggesting a two-compartment pharmacokinetic model in (V_C 0.41±0.09 l/kg, V_{dss} 1.7±0.4 l/kg, CL_{tot} 10±2 ml/min•kg, $AUC_{0\text{-}4800}$ 2055±294.3 ng•min/ml). Following oral administration of aconitine to rats at two doses of 0.1 and 1.0 mg/kg b.w., the maximum plasma concentrations (C_{max}) were 0.73±0.08 and 3.3±0.6 ng/ml at times of 45±9 and 150±52 min, respectively, and the $AUC_{0\text{-}1440}$ values were 130±4 and 1600±270 ng•min/ml. The bioavailavility (F) of aconitine was determined to be 0.013, where only 1.3% of the aconitine administered orally was absorbed into the body fluid.

10) **Zhao J., Sun X., Nakamura N., Zuo F., Yang X., Akao T., and Hattori M.: Development of an enzyme immunoassay system for mesaconitine and its application to the disposition study on mesaconitine. *J. Trad. Med.*, 20:201-207, 2003.**

For the purpose of quantitative determination of mesaconitine, an extremely toxic and major alkaloid in aconite tuber, a highly sensitive enzyme immunoassay (EIA) was developed as follows; Firstly, a hapten [*N*-desethylaconitine *N*-glutarate (DEAG)] was synthesized by introduction of a glutaryl group to the nitrogen atom of aconitine after elimination of an *N*-ethyl group. Subsequently it was coupled with bovine serum albumin (BSA) and β-galactosidase (β-Gal) to give an immunogen (DEAG-BSA) and a labeled antigen (DEAG-β-Gal), respectively. After immunization of female albino rabbits with DEAG-BSA for six months to elicit a polyclonal antiserum (As-DEAG), the experimental conditions were optimized for a highly sensitive EIA. At 10^3-fold dilution of DEAG-β-Gal and 2.5×10^4-fold dilution of As-DEAG, the assay exhibited a linear range of 0.005-5 ng/tube for mesaconitine. Under the same conditions, the antiserum had a 24.4% cross-reaction with aconitine and virtually no reaction with benzoylmesaconine.

Next, the EIA method was applied to the disposition study on mesaconitine to monitor the plasma concentration after oral administration of mesaconitine at 1 mg/kg to rats, its low bioavailability being suggested from C_{max} and AUC values obtained.

11) **Ahn E., Nakamura N., Akao T., Komatsu K., Qui M., and Hattori M.: Prenylated flavonoids from *Moghania philippinensis*. *Phytochemistry*, 64:1389-1394, 2003.**

Five prenylated flavonoids, 8-(1,1-dimethylallyl)genistein (**1**), 5,7,3',4'-tetrahydroxy-2',5'-di(3-methylbut-2-enyl) -isoflavone (**2**), 5,7,3'-trihydroxy-2'-(3-methylbut-2-enyl)-4',5'-(3,3-dimethylpyrano)isoflavone (**3**), (2*R*)-5,2',4'-trihydroxy-8,5'-di(3-methylbut-2-enyl)-6,7-(3,3-dimethylpyrano)flavanone (**4a**) and (2*S*)-5,2',4'-trihydroxy-8,5'-di(3-methylbut-2-enyl)-6,7-(3,3-dimethylpyrano) flavanone (**4b**), were isolated from the roots of *Moghania philippinensis*. The structures of these compounds were determined on the basis of spectroscopic and chemical means.

12) **Takako K., Jyo M., Nakamura N., Komatsu K., Hattori M., Shimotohono K., Shimotohono K., and Kakiuchi N.: Inhibitory effect of Tibetan medicinal plants on viral polymerases. *J. Trad. Med.*, 20:243-250, 2003.**

For the purpose of development of novel anti-virus agents from ethnical drugs, we examined 76 traditional Tibetan medicines for inhibitory effect on two viral enzymes, reverse transcriptase (RTase) of HIV and RNA dependent RNA polymerase (RdRp) of HCV. Although 28 methanol extracts inhibited RTase more than 70% at a concentration of 100 μg/ml, only 3 samples, T42(藏青果, *Terminalia chebula* RETZ.), T46 (檳榔, *Areca catechu* L.) and T61 (紅檳榔), were found still inhibitory after eliminating the effect of tannins by addition of BSA in the enzyme reaction mixture. In the case of the RdRp, 7 extracts (IC_{50} values of less than 10 μg/ml) contained less than 20% tannins. The extract of *Rhodiola sacra,* whose IC_{50} for RTase was 25.9 μg/ml, was subject to phytochemical investigation. Out of 8 compounds isolated from the extract, daucosterol was found effective for RTase.

13) **Lipipun V., Kurokawa M., Suttisri R., Taweehotipatr P., Pramyothin P., Hattori M., and Shiraki K.: Efficacy of Thai medicinal plant extracts against herpes simplex virus type 1 infection *in vitro* and *in vivo*. *Antiviral Research*, 60:175-180, 2003.**

Twenty Thai medicinal plant extracts were evaluated for anti-herpes simplex virus type 1 (HSV-1) activity. Eleven of them inhibited plaque formation of HSV-1 more than 50% at 100 μg/ml in a plaque reduction assay. *Aglaia odorata, Moringa oleifera,* and *Ventilago denticulate* among the 11 were also effective against thymidine kinase-deficient HSV-1 and phosphonoacetate-resistant HSV-1 strains. These therapeutic efficacies were characterized using a cutaneous HSV-1 infection in mice. The extract of *M. oleifera* at a dose of 750 mg/kg per day significantly delayed the development of skin lesions, prolonged the mean survival times and reduced the mortality of HSV-1 infected mice as compared with 2% DMSO in distilled water ($P < 0.05$). The extracts of *A. odorata* and *V. denticulate* were also significantly effective in limiting the development of skin lesions ($P < 0.05$). There were no significant difference between acyclovir and these three plant extracts in the delay of the development of skin lesions and no significant difference between acyclovir and *M. oleifera* in mean survival times. Toxicity of these plant extracts were not observed in treated mice. Thus, these three plant extracts may be possible candidates of anti-HSV-1 agents.

14) **Park J. C., Miyashiro H., and Hattori M.: Inhibitory effects of methanol extracts from Korean medicinal plants against HIV-1 protease activity. *Korean J. Med. Crop Sci.*, 11:264-267, 2003.**

Korean medicinal plants were screened for their inhibitory activity against HIV-1 protease. The inhibitory activity of protease was determined by incubating the extracts in reaction mixtures containing protease and substrate His-Lys-Ala-Arg-Val-Leu-(*p*-NO_2-Phe)-Glu-Ala-Nle-Ser-NH_2 to perform proteolytic cleavage reactions. In this study the twenty six extracts from medicinal plants were investigated. Of the extracts tested, the extracts from the stem of *Morus alba* exhibited the strongest activity with inhibition of 81% at a concentration of 100 μg/ml. The extracts of the flower of *Saxifraga stolonifera,* and stems of *Euonymus japonica* and *Castamea crenata* showed appreciable inhibitory activity (>50%) against HIV-1 protease at the same concentration.

15) **Yokozawa T., Rhyu D.Y., Cho E.J., Aoyagi K.: Protective Activity of (-)-Epicatechin 3-*O*-gallate against Peroxynitrite-mediated Renal Damage.** ***Free Radic. Res.,*** **37:561-571, 2003.**

The protective effect of (-)-epicatechin 3-*O*-gallate (ECg) against peroxynitrite ($ONOO^-$)-mediated damage was examined using an animal model and a cell culture system. In rats subjected to lipopolysaccharide (LPS) administration plus ischemia-reperfusion, the plasma 3-nitrotyrosine level, an indicator of $ONOO^-$ production *in vivo*, was elevated, whereas it declined significantly and dose-dependently after the oral administration of ECg at doses of 10 and 20 mmoles/kg body weight/day for 20 days prior to the process. Moreover, oral administration of ECg significantly enhanced the activities of the antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase, and the antioxidant glutathione, showing enhancement of the biological defense system against the damage induced by $ONOO^-$. In addition, the significant increase in the renal mitochondrial thiobarbituric acid-reactive substance level of LPS and ischemic-reperfused control rats was attenuated in rats given ECg. Furthermore, the elevations in the plasma urea nitrogen and creatinine (Cr) levels and the urinary methylguanidine/Cr ratio induced by the procedure were attenuated markedly after oral administration of ECg, implying amelioration of renal impairment. The addition of ECg (25 or 125 mM) prior to 3-morpholinosydnonimine (SIN-1, 800 mM) exposure reduced $ONOO^-$ formation and increased the viability of cultured renal epithelial ($LLC\text{-}PK_1$) cells in a dose-dependent manner. In particular, ECg inhibited $ONOO^-$-mediated apoptotic cell death, which was confirmed by decreases in the DNA fragmentation rate and the presence of apoptotic morphological changes, i.e., small nuclei and nuclear fragmentation. Furthermore, adding ECg before SIN-1 treatment regulated the cell cycle by enhancing G_2/M phase arrest. This study provides evidence that ECg has protective activity against the renal damage induced by excessive $ONOO^-$ in cellular and *in vivo* systems.

16) **Yokozawa T., Ishida A., Cho E.J., Nakagawa T.: The effects of Coptidis Rhizoma extract on A hypercholesterolemic animal model.** ***Phytomedicine,*** **10:17-22, 2003.**

The serum cholesterol (total, free, esterified, low density lipoprotein (LDL) and oxidized LDL) levels of rats fed a diet containing, by weight, 1% cholesterol and 0.5% cholic acid increased compared with those of rats fed a normal diet. However, the levels, especially, of total cholesterol, LDL and oxidized LDL, were significantly reduced, in a dose-dependent manner, in rats given Coptidis Rhizoma extract orally at doses of 50 and 100 mg/kg body weight/day for 30 days. This result indicates that Coptidis Rhizoma extract is effective in reducing the pathological damage caused by hypercholesterolemia through lowering serum cholesterol levels. In addition, Coptidis Rhizoma extract reduced the level of liver cholesterol, whereas it did not reduce that of fecal cholesterol, suggesting that the cholesterol level-lowering effect resulted from the reduction of cholesterol synthesis, not the enhancement of its excretion. Furthermore, the serum thiobarbituric acid-reactive substance level decreased after oral administration of Coptidis Rhizoma extract, indicating that Coptidis Rhizoma could prevent hypercholesterolemic disease through reducing lipid peroxidation. This study demonstrates that Coptidis Rhizoma may be a useful therapy for hypercholesterolemia by reducing oxidative stress and cholesterol levels.

17) **Yokozawa T., Cho E.J., Nakagawa T.: Influence of green tea polyphenol in rats with arginine-induced renal failure.** ***J. Agric. Food Chem.,*** **51:2421-2425, 2003.**

To determine whether green tea polyphenol ameliorates the pathological conditions induced by excessive dietary arginine, green tea polyphenol was administered to rats at a daily dose of 50 or 100 mg/kg body weight for 30 days with a 2% w/w arginine diet. In arginine-fed control rats, urinary and/or serum levels of guanidino compounds, nitric oxide (NO), urea, protein and glucose increased significantly, while the renal activities of the oxygen species-scavenging enzymes superoxide dismutase (SOD) and catalase decreased, compared with casein-fed rats. However, rats given green tea polyphenol showed significant and dose-dependent decreases in serum levels of creatinine (Cr) and urea nitrogen and urinary excretion of Cr, and they exerted a slight reduction of nitrite plus nitrate, indicating

that green tea polyphenol reduced the production of uremic toxins and NO. In addition, in arginine-fed rats the urinary urea, protein and glucose level increases were reversed by the administration of green tea polyphenol. Moreover, in rats given green tea polyphenol the SOD and catalase activities suppressed by excessive arginine administration increased dose-dependently, implying the biological defense system was augmented as a result of free radical scavenging. These results suggest that green tea polyphenol would ameliorate renal failure induced by excessive dietary arginine by decreasing uremic toxin, and NO production and increasing radical-scavenging enzyme activity.

18) **Nakagawa T., Yokozawa T., Terasawa K., Nakanishi K.: Therapeutic usefulness of Keishi-bukuryo-gan for diabetic nephropathy. *J. Pharm. Pharmacol.*, 55:219-227, 2003.**

Keishi-bukuryo-gan is a traditional herbal medicine, which is used clinically as a vascular system disorder-eliminating drug. In this study, its effects on the progression of diabetic nephropathy in experimental rats were investigated. The diabetic nephropathy model used in this study shows functional and morphological changes of the kidney resembling those seen in patients with diabetic nephropathy. Increased proteinuria and serum urea nitrogen and creatinine levels and decreased creatinine clearance, which are important parameters of renal function, were observed in rats with diabetic nephropathy. Pathological examination of the kidney revealed diffuse, nodular and exudative lesions and arteriolar hyalinosis. The deterioration of renal function was ameliorated in rats treated with Keishi-bukuryo-gan for 15 weeks and these results agreed with the renal histological findings. In addition, metabolic abnormalities mediated by persistent hyperglycemia (the glycation reaction, excessive polyol pathway activity, oxidative stress and lipid metabolic abnormalities) were also observed. However, Keishi-bukuryo-gan reduced accumulation of advanced glycation end-products, determined by measuring fluorescence, and serum lipid peroxidation, triglyceride and total cholesterol levels dose-dependently. Thus, this study indicates the potential therapeutic usefulness of Keishi-bukuryo-gan for retarding the progression of renal damage and suggests that its beneficial effects were due to its ability to improve metabolic abnormalities associated with diabetes.

19) **Yokozawa T., Rhyu D.Y., Chen C.P.: Protective effects of Acanthopanax Radix extract against endotoxemia induced by lipopolysaccharide. *Phytother. Res.*, 17:353-357, 2003.**

Endotoxemia causes an enhanced production of reactive oxygen radicals, which contribute to multiple organ dysfunction. When rats were given intravenous lipopolysaccharide and tested 6 h later we found that the activities of catalase and glutathione peroxidase (GSH-Px) in kidney, were acutely suppressed while in serum the levels of nitric oxide (NO), lipid peroxidation, urea nitrogen and creatinine were significantly increased, indicating the excessive production of reactive oxygen radicals and the presence of renal injury. Pretreatment of rats with Acanthopanax Radix extract administered orally for 30 days reduced the NO and lipid peroxidation levels, increased the activities of catalase and GSH-Px, and attenuated the renal dysfunction. These results suggested that scavenging of reactive oxygen radicals is part of the mechanism by which Acanthopanax Radix extract works as an effective agent in preventing multiple organ dysfunction.

20) **Kim H.Y., Yokozawa T., Cho E.J., Cheigh H.S., Choi J.S., Chung H.Y.: *In Vitro* and *in Vivo* Antioxidant effects of mustard leaf (*Brassica Juncea*). *Phytother. Res.*, 17:465-471, 2003.**

To investigate the antioxidant activity of mustard leaf (*Brassica juncea*), we prepared four fractions (CH_2Cl_2, EtOAc, BuOH and H_2O fractions) and examined their radical scavenging activities *in vitro* and *in vivo*. Based on the *in vitro* results of spin trapping and 1,1-diphenyl-2-picrylhydrazyl radical, we carried out an *in vivo* study with the BuOH fraction to investigate its effect on oxidative stress in rats with streptozotocin-induced diabetes. We found that in comparison with untreated diabetic control rats, oral administration of the BuOH fraction (100 or 200 mg/kg body weight/day for 10 days) induced a significant decrease in serum glucose and glycosylated protein, which is

glycosylated with hemoglobin as an indicator of oxidative stress. Moreover, administration of the BuOH fraction also effectively reduced the serum superoxide and nitrite/nitrate levels. Furthermore, the levels of thiobarbituric acid-reactive substances in serum and liver were also significantly lower than in the control group. These results indicate that the BuOH fraction of mustard leaf controls glucose metabolism and reduces lipid peroxidation as well as the level of oxygen radicals, ameliorating the damage caused by oxidative stress in diabetes.

21) **Yokozawa T., Kim H.Y., Cho E.J., Yamabe N., Choi J.S.: Protective effects of mustard leaf (*Brassica juncea*) against diabetic oxidative stress. *J. Nutr. Sci. Vitaminol.*, 49:87-93, 2003.**

Of four fractions (CH_2Cl_2, ethyl acetate (EtOAc), butanol (BuOH) and H_2O) from mustard leaf (*Brassica juncea*), the EtOAc fraction showed the strongest inhibitory effects, which were concentration-dependent, on the formation of advanced glycation end products and free radical-mediated protein damage in an *in vitro* system, indicating a potential protective role of this fraction against diabetes and/or its complications. Based on these results, we carried out an *in vivo* study to determine whether the EtOAc fraction protected against diabetic oxidative stress induced by streptozotocin. Oral administration of the EtOAc fraction at doses of 50 and 200 mg/kg body weight/day for 10 days reduced serum levels of glucose and glycosylated protein, implying that the impaired glucose metabolism due to diabetes had been ameliorated. In addition, the EtOAc fraction significantly reduced the thiobarbituric acid-reactive substance levels of serum and hepatic and renal mitochondria. Furthermore, the elevated levels of superoxide and nitrite/nitrate were reduced, in a dose-dependent manner, by oral administration of the EtOAc fraction. These findings suggest that the EtOAc fraction from mustard leaf might be beneficial in attenuating the damage caused by oxidative stress involved in diabetes and its complications.

22) **Cho E.J., Yokozawa T., Rhyu D.Y., Kim S.C., Shibahara N., Park J.C.: Study on the inhibitory effects of Korean medicinal plants and their main compounds on the 1,1-diphenyl-2-picrylhydrazyl radical. *Phytomedinine,* 10:544-551, 2003.**

A 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-generating system was used to evaluate the antioxidant properties of Korean medicinal plants, which have been widely used as folk medicines for several disorders, and compounds isolated from them. Among the Rosaceae, *Rosa rugosa* and *Rosa davurica* showed strong DPPH radical-scavenging activity. The most effective medicinal plant of the families other than Rosaceae was *Cedrela sinensis,* followed in order by *Nelumbo nucifera, Eucommia ulmoides, Zanthoxylum piperitum, Cudrania tricuspidata* and *Houttuynia cordata.* These results serve as a good index of the free radical-scavenging activities of Korean medicinal plants. Furthermore, the polyphenols isolated from these plants, procyanidin B-3, (+)-catechin, gallic acid, methyl gallate, quercetin, quercetin 3-*O*-β-D-glucoside, quercetin 3-*O*-β-D-galactoside, quercetin 3-*O*-rutinose and kaempferol, exerted strong DPPH radical-scavenging activity. These results suggest that the Korean medicinal plants and polyphenols isolated from them that exhibited effective radical-scavenging activity may be promising agents for scavenging free radicals and treating diseases associated with excess free radicals.

23) **Nakagawa T., Yokozawa T., Oowada S., Goto H., Shibahara N., Shimada Y., Terasawa K.: Amelioration of kidney damage in spontaneously diabetic WBN/Kob rats after treatment with Keishi-bukuryo-gan. *J. Trad. Med.*, 20:156-164, 2003.**

In this study, we investigated whether Keishi-bukuryo-gan can retard the occurrence and progression of diabetic nephropathy in spontaneously diabetic WBN/Kob rats. Administration of Keishi-bukuryo-gan did not affect body weight loss or blood glucose levels but effectively lowered urinary protein excretion and serum creatinine levels, and ameliorated glomerular, vascular and tubulointerstitial lesions. In addition, treatment of the diabetic rats with Keishi-bukuryo-gan reduced renal levels of thiobarbituric acid reactive substances and advanced glycation end products significantly and elevated renal superoxide dismutase activity significantly. These results suggest that Keishi-bukuryo-

gan exerts antioxidant effects in the kidneys of diabetics and may prove that the herbal medicine is useful for inhibiting the progression of diabetic kidney disease.

24) **Yokozawa T., Rhyu D.Y., Cho E.J.: Protection by the Chinese prescription We-Pi-Tang against renal tubular LLC-PK1 cell damage induced by 3-morpholinosydnonimine. *J. Pharm. Pharmacol.*, 55:1405-1412, 2003.**

We investigated the effects of Wen-Pi-Tang extract on the protective mechanisms of renal tubular LLC-PK_1 cells, as renal tubular cells are the most vulnerable renal tissue to oxidative stress. Exposure to 800 μM 3-morpholinosydnonimine (SIN-1) resulted in a marked increase in cellular peroxynitrite ($ONOO^-$) that converted nonfluorescent dihydrorhodamine 123 to fluorescent rhodamine 123, a detectable probe for the long-lived $ONOO^-$. In addition, it resulted in apoptotic cell death, assessed by a DNA fragmentation assay. However, treatment with Wen-Pi-Tang extract, at concentrations of 50 and 100 μg/ml, together with SIN-1 protected renal tubular cells against $ONOO^-$ through scavenging $ONOO^-$ and inhibiting apoptotic cell death in a dose-dependent manner. Moreover, treatment with Wen-Pi-Tang extract both before and after exposure to SIN-1 was also protective: it reduced cellular $ONOO^-$ levels, increased cell viability and decreased the DNA fragmentation rate. These results suggest that Wen-Pi-Tang would be expected to have protective activity against $ONOO^-$-induced renal tubular injury through the inhibition of $ONOO^-$ production and apoptotic cell death by both preventing and treating renal injury. Furthermore, morphological characteristics of apoptosis were observed in SIN-1 treated tubular cells, while the addition of Wen-Pi-Tang extract with SIN-1 attenuated these morphological changes. $ONOO^-$ generated by SIN-1 also disturbed the cell cycle by decreasing the cellular G_2/M phase ratio, while Wen-Pi-Tang extract regulated the cell cycle by G_2/M phase arrest.

25) **Soung D.Y., Rhee S.H., Kim J.S., Lee J.Y., Yang H.S., Choi J.S., Yokozawa T., Han Y.N., Chung H.Y.: Peroxynitrite scavenging activity of lithospermate B from *Salvia miltiorrhiza*. *J. Pharm. Pharmacol.*, 55:1427-1432, 2003.**

Peroxynitrite ($ONOO^-$) is produced by the reaction of superoxide (O_2^-) with nitric oxide. $ONOO^-$ damages proteins through nitration or oxidation. For protection from $ONOO^-$ induced protein modifications, $ONOO^-$ scavengers should be supplemented. Evidence was obtained that lithospermate B extracted from *Salvia miltiorrhiza* showed the strongest scavenging activity among its constituents. Its $ONOO^-$ scavenging activity is via an electron donation mechanism. A dihydroxyl group and a double bond seem to be essential structure requirements. The data from the experiments further confirmed a protective effect of lithospermate B on bovine serum albumin and low-density lipoprotein against $ONOO^-$. This study demonstrated that lithospermate B with hydroxyl groups and double bonds exerts an anti-nitration effect by scavenging $ONOO^-$.

26) **Yokozawa T., Rhyu D.Y., Cho E.J., Satoh A., Hattori M.: Novel pharmacological potential of Rhei Rhizoma against peroxynitrite-induced oxidative renal injury. *J. Trad. Med.*, 20: 235-242, 2003.**

This study was focused on the protective role of Rhei Rhizoma extract from peroxynitrite ($ONOO^-$)-induced renal damages. The employed *in vivo* $ONOO^-$-generation model of lipopolysaccharide plus ischemia-reperfusion resulted in the elevations of plasma 3-nitrotyrosine level and renal myeloperoxidase (MPO) activity as the indicators of *in vivo* $ONOO^-$ generation. However, the administration of Rhei Rhizoma extract at doses of 30 and 60 mg/kg body weight/day for 30 days significantly decreased the concentrations of 3-nitrotyrosine and MPO activity. In addition, the rats given Rhei Rhizoma extract inhibited xanthine oxidase (XOD) activity but not inducible nitric oxide synthase activity. It suggests that Rhei Rhizoma extract might play the role as a superoxide anion scavenger through the inhibition of XOD activity as well as direct $ONOO^-$ scavenger. The protective role of Rhei Rhizoma extract was

also shown in the increase of antioxidant, glutathione, and the reduction in the lipid peroxidation in renal mitochondria. Moreover, the *in vivo* $ONOO^-$ generation system resulted in the renal functional impairment assessed by the increase in plasma levels of urea nitrogen and creatinine, whereas the rats administered Rhei Rhizoma extract decreased the levels significantly, implying the alleviation of renal dysfunction induced by $ONOO^-$. This study suggests that Rhei Rhizoma would play the role as an effective therapeutic potential for the oxidative stress-induced renal failure.

27) **Cho E.J., Yokozawa T., Rhyu D.Y., Kim H.Y., Shibahara N., Park J.C.: The inhibitory effects of Korean medicinal plants and their component compounds on lipid peroxidation. *Am. J. Chin. Med.*, 31:907-917, 2003.**

The antioxidative activities of twelve medicinal plants and compounds isolated from them were investigated using the thiocyanate method to evaluate inhibitory effects on lipid peroxidation in the linoleic acid system. The peroxide levels gradually increased during incubation in the presence of linoleic acid over 3 days and most of the plants inhibited lipid peroxidation. In particular, of the plants tested, *Cudrania tricuspidata, Zanthoxylum piperitum, Houttuynia cordata* and *Ulmus parvifolia* reduced lipid peroxidation more effectively as lipid peroxidation progressed, resulting in inhibition of about 80% relative to the control value by the 3rd day of incubation. In addition, the polyphenols isolated from the plants also showed marked and dose-dependent inhibitory effects on lipid peroxidation. The compounds with the strongest activities were 3,4-dihydroxylbenzoic acid, quercetin, the quercetin glycosides quercetin 3-*O*-β-D-galactoside, quercetin 3-*O*-α-L-rhamnoside, quercetin 3-*O*-β-D-glucoside and quercetin 3-*O*-rutinose, catechin, gallic acid, methyl gallate and rosamultin isolated from *Zanthoxylum piperitum, Houttuynia cordata, Rosa rugosa* and *Cedrela sinensis*. Moreover, quercetin glycosides showed stronger activity than quercetin, suggesting that glycosylation increases the antioxidative activity of quercetin. Our results indicate that the medicinal plants and their polyphenols show promise as therapeutic agents for various disorders involving free radical reactions.

◇総　説　Review paper

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14) 中村憲夫 : 抗 HIV 薬の開発研究. 研究所セミナー（資源開発大部門制発足記念), 2003, 12, 15, 富山医科薬科大学.

◇**共同研究**　Co-operative research

1）下遠野邦忠（京都大学ウイルス研究所），下遠野久美子（共立薬科大学），垣内信子（金沢大学薬学部）:「C型肝炎RNAポリメラーゼ阻害活性を指標とした抗HCV剤の開発研究」

2）白木公康（富山医科薬科大学医学部）:「抗ヘルペスウイルス活性を有するタイ薬用植物の探索」

3）木谷健一（国立長寿医療センター）:「抗老化薬に関する研究」

4）青柳一正（筑波技術短期大学），鄭　海泳 (国立釜山大学校薬学大学)，田中　隆（長崎大学薬学部），柏田良樹（新潟薬科大学）:「抗酸化に関する研究」

◇**非常勤講師**　Part-time lecturer

1）服部征雄：千葉大学薬学研究科，4月～9月

2）横澤隆子：富山大学，4月～8月

◇**研究費取得状況**　Acquisition of research funds

1）日本科学協会 平成15年度笹川科学研究助成（高江静代表）50万円.

2）富山県受託研究「和漢薬・バイオテクノロジー研究」（継続，服部分担）39万円.

3）島根県川本農林振興センター受託研究「邑智霊芝の苦味成分の分析（継続，服部代表）30万円.

4）つくし奨学・研究基金「加齢過程におけるフリーラジカルの役割と紅花の効果」（継続，横澤代表）120万円.

5）財団法人富山県新世紀産業機構平成15年度さきがけ研究開発助成金「柿ポリフェノールの低分子化による健康食品素材の研究開発」（新規，横澤分担）300万円.

6）平成15年度研究拠点形成費補助金（COE）「東洋の知に立脚した個の医療の創生」（服部分担）250万円.

◇**受賞**　Award

1）横澤隆子：第28回（2003年度）漢方研究イスクラ奨励賞「漢方薬から老化抑制の可能性を探る」.

◇**学位および論文名**　Academic degrees and theses

課程博士（2003年3月）

謝　麗華：Biotransformation of plant lignans (arctiin and pinoresinol diglucoside) to mammalian lignans by human intestinal microflora

柳　東泳：A novel activity of Chinese prescription Wen-Pi-Tang and its component (-)-epicatechin 3-*O*-gallate against peroxynitrite-induced renal injury

修　士（2003年3月）

石田 あい：酸化ストレスにおける黄連の役割とその活性成分の探索

土屋 真澄：ヒト腸内細菌を利用した(-)-enterolactone, (-)-enterodiolの簡易合成法の開発

条 美智子：中国少数民族の単純ヘルペスウイルス及びC型肝炎ウイルスポリメラーゼ阻害活性について

学　士（2003年3月）

西畑 友尋：ヒト腸内嫌気性細菌によるイソフラボン類の変換反応

◇**研究室在籍者**　Research member

4年次学生：近藤直子，和田江美子

大学院前期1年：西畑友尋

大学院前期2年：平川暁子，山辺典子

大学院後期1年：条 美智子

大学院後期 2 年 : 佐藤亜希子, Kanjana Sangul
大学院後期 3 年 : 高　江静, 安　恩美
外国人客員研究員 : 朴　鐘喆（博士）, Park, Hye Jin（博士）, 趙　宇峰, 于　超, 左　風（博士）,
　宋　陞赫, Gafur, Md. Abdul（博士）, Khunkitti, Watcharee
機関研究員 : 趙　静（博士）
受託研究員 : 山下範子
事務補佐員 : 黒岩純子