Interaction of the Aryl Hydrocarbon Receptor with Several Constituents from Spinach and Komatsuna Extracts Determined Using *in Vitro* Bioassay

Yoshiaki Amakura,*,[a] Tomoaki Tsutsumi,[a] Kumiko Sasaki,[a] Masafumi Nakamura,[b] Hideyuki Ito,[c] Tsutomu Hatano,[c] Takashi Yoshida,[c] and Tamio Maitani[a]

[a]*Division of Foods, National Institute of Health Sciences, 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan,* [b]*Hiyoshi Corporation, 908 Kitanosho-cho, Omihachiman, Shiga 523–8555, Japan, and* [c]*Faculty of Pharmaceutical Sciences, Okayama University, 1–1–1 Tsushima, Okayama 700–8530, Japan*

(Received July 4, 2005; Accepted August 5, 2005; Published online August 9, 2005)

As a part of our study to clarify the interaction of food ingredients with the aryl hydrocarbon receptor (AhR), we studied the interaction of 10 constituents (phytol, *p*-coumaric acid, 5 flavonol glycosides, adenosine, guanosine and uridine), isolated from aqueous alcohol extracts of spinach and komatsuna, with the AhR by applying the AhR-based bioassay for dioxins, the Ah-Immunoassay and the AhR-dependent luciferase reporter gene assay. Among them, flavonol glycosides and phytol had slight inhibitory effects on AhR activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at high concentrations, however the effects were not comparable with those produced by kaempferol, regarded as one of the main antagonists of AhR. Also, flavonol glycosides showed weak luciferase inductions at high concentrations.

Key words ——— aryl hydrocarbon receptor, spinach, komatsuna, bioassay

*To whom correspondence should be addressed: Division of Foods, National Institute of Health Sciences, 1–18–1, Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan. Tel.: +81-3-3700-1141; Fax: +81-3-3707-6950; E-mail: amakura@nihs.go.jp

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is an inducible ligand-dependent transcription factor that mediates the biological and toxic effects of a structurally diverse range of chemicals.[1,2] Ligands of the AhR include several classes of environmental carcinogens such as dioxins representing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is an archetypal dioxin known to be the most potent congener. Therefore AhR is called a dioxin receptor, and it is indicated that AhR mediates the toxic and biological effects of dioxins.[3–5] Pollutants such as dioxins are artificial products which have only recently appeared in the environment. Therefore, AhR might have some other primary significance in the living body; for example it is considered that AhR is one of the orphan receptors. Recently, several research groups have studied this field to clarify the functional information of AhR.[2,6–13] We are also investigating this and have reported on the interaction of plant food extracts and ingredients in plant foods with AhR.[14–17] In previous reports,[14,17] we examined and reported the interaction of many food plant extracts with AhR (the inhibitory effect of AhR activation by TCDD and the induction of AhR activity) by applying the AhR-based bioassays for dioxins [Ah-Immunoassay and the chemical-activated luciferase gene expression (CALUX) assay], and green leafy vegetables such as spinach showed inhibitory effects on AhR activation by TCDD and induction of AhR activity. Therefore, it is considered that identifying the ligand ingredients of these extracts is important as a next step for elucidating the functional roles of AhR.

Based on this background, we attempted to isolate and identify AhR-bioactive substances from green leafy vegetables, spinach and komatsuna.

MATERIALS AND METHODS

Generals ——— ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR-500 instrument (500 MHz for ^{1}H and 126 MHz for ^{13}C), and dimethyl sulfoxide (DMSO)-d_6 was used as the solvent. The chemical shifts were based on those of the solvent signals (δ_H 2.49; δ_C 39.7) and given in δ (ppm) from tetramethylsilane (TMS) as an internal standard. Electrospray ionization (ESI)-MS spectra including high-resolution mass spectra were recorded on a Micromass Auto Spec OA-TOF mass spectrometer

(solvent: 50% aqueous MeOH containing 0.1% ammonium acetate). Column chromatographies were performed with MCI-gel CHP 20P (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan) and TSK gel Toyopearl HW-40F (Tosoh Corporation, Tokyo, Japan), and thin layer chromatography (TLC) was performed on precoated Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). TCDD (98%) and DMSO were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The Ah-Immunoassay kit was obtained from Kubota Corporation (Osaka, Japan). Fresh spinach and komatsuna were purchased from a supermarket in Tokyo, Japan.

Extraction and Isolation —— Fresh komatsuna (*Brassica campestris var. peruviridis*) (1.2 kg) was homogenized with 50% aqueous ethanol (5 l), filtered, and concentrated *in vaccuo*. The concentrated solution was subjected to liquid-liquid partition (each 4 l) to give four extracts: *n*-hexane (178 mg), ethyl acetate (535 mg), *n*-butanol (2.0 g), and water-soluble (22.6 g) portions. The *n*-hexane extract was applied to a silica gel column (*n*-hexane-ethyl acetate), and then further purification was achieved by preparative TLC (Silica gel, $CHCl_3$-MeOH 100 : 1) to give phytol (**1**) (4.5 mg). The ethyl acetate extract was separated using column chromatography over MCI-gel CHP 20P in a step-wise gradient of aqueous MeOH to afford isorhamnetin 3-*O*-β-D-glucoside (**2**) (7.4 mg) and kaempferol 3-*O*-β-D-sophoroside (**3**) (1.2 mg). Fresh spinach (*Spinacia oleracea* L.) (1.2 kg) was homogenized with 50% aqueous ethanol (5 l) and then partitioned with ethyl acetate (4 l), to produce dried ethyl acetate (1.3 g) and water-soluble (20.2 g) residues. The ethyl acetate extract was chromatographed over Toyopearl HW-40 and MCI-gel CHP 20P in a step-wise gradient of aqueous MeOH, and then final purification was performed by using preparative HPLC [column: YMC-Pack ODS-A (5 μm, 150 × 10 mm i.d.), solvent: acetonitrile (solvent A) and 5% acetic acid in water (solvent B) at a flow rate 2.0 ml min^{-1}, and detection at 260 nm as follows: linear gradients of A–B from 5 : 95 to 30 : 70 in 30 min, then to 95 : 5 in 5 min] to yield *p*-coumaric acid (**4**) (1.2 mg), 3,6-dimethyl-5,7,3′,4′-tetrahydroxyflavone 4′-β-D-glucuronide (**5**) (2.0 mg), 5,3′,4′-trihydroxy-3-methoxy-6 : 7-methylenedioxyflavone 4′-β-D-glucuronide (**6**) (17.0 mg), 6-methoxy-3,5,7,3′,4′-pentahydroxyflavone 3-*O*-β-D-glucosyl-(1→6)-[β-D-apiosyl-(1→2)]-β-D-glucoside (**7**) (4.5 mg), adenosine (**8**) (16.9 mg), guanosine (**9**) (8.0 mg) and uridine (**10**) (1.0 mg).

Evaluation of Inhibitory Effect on AhR Activation by TCDD and AhR Ligand Activity Using AhR-Based Bioassay —— To evaluate AhR-based activities, Ah-Immunoassay and CALUX assay described in previous papers were used.[14–17] Briefly, for Ah-Immunoassay, the cytosol was added to the sample, or DMSO was added as the control, the mixture was preincubated, and then incubated with 5 nM TCDD (the final DMSO concentration was 1% in cell culture medium). After incubation, the formation of the TCDD-AhR complex was determined with enzyme-linked immunosorbent assay (ELISA) kit and the absorbance was determined at 405 nm. The CALUX assay, which has been recently applied as a screening procedure for the identification and characterization of a novel class of AhR ligands/agonists,[18] was employed as follows. Mouse hepatoma H1L1 cells were cultured in 96-well culture plates, and samples dissolved in DMSO were added at final concentrations of 0.01–100000 nM in 8 steps (the final DMSO concentration was 1% in the cell culture medium). The plates were incubated to produce the optimal expression of luciferase activity. After the addition of luciferin as the substrate, the luciferase activity was determined under luminometer.

RESULTS AND DISCUSSION

Fresh spinach and komatsuna were extracted with 50% aqueous ethanol, and then their concentrated extracts were divided into *n*-hexane, ethyl acetate, *n*-butanol and water soluble layers in komatsuna, and ethyl acetate and water layers in spinach. After concentration of each layer, the portions' influence on the AhR-pathway induced by TCDD (each fraction was 5–500 μg/ml as a final concentration) was evaluated using the Ah-Immunoassay as an experimental model. As a result, 50% effective concentration (EC_{50}) for *n*-hexane and ethyl acetate fractions in komatsuna were 15 and 22 μg/ml, respectively, and that for *n*-butanol and water soluble fractions were > 250 μg/ml, when the concentrations producing AhR activity equal to 50% of the maximal response to TCDD in controls were expressed as the EC_{50} value. In spinach, EC_{50} for the ethyl acetate fraction and the water soluble fraction were 25 μg/ml and > 250 μg/ml, respectively. *n*-Hexane and ethyl acetate extracts from komatsuna and ethyl acetate extract from spinach showed marked inhibitory effects against TCDD-induced activation of AhR. To clarify the compounds inhibitory to AhR

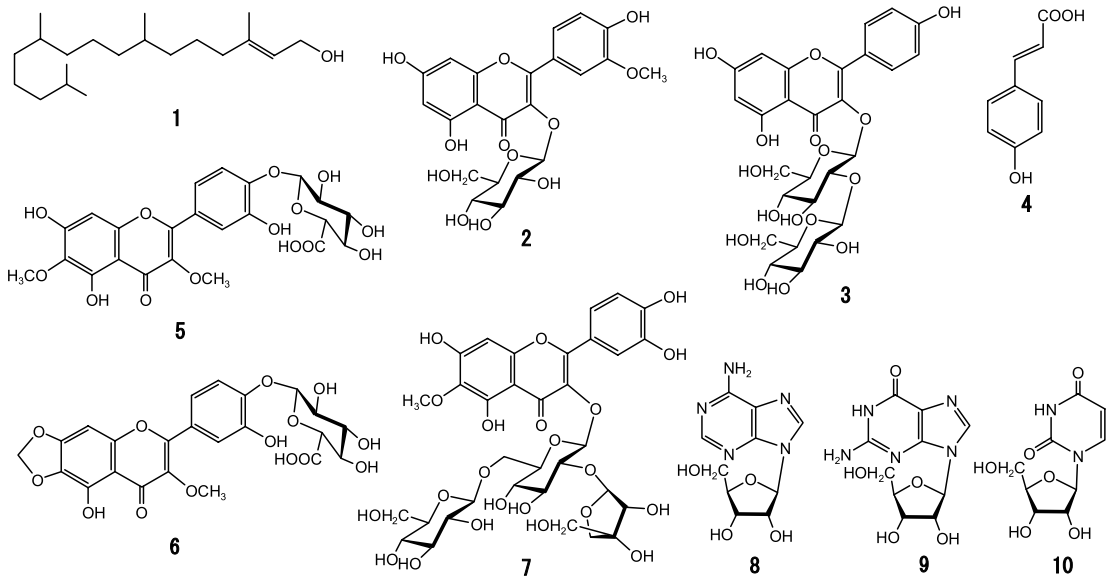

Fig. 1. Structures of Compounds **1**–**10**

activation by TCDD, *n*-hexane and ethyl acetate extracts of komatsuna (ethyl acetate extract in spinach) were subjected to a combination of chromatographies over silica gel, Toyopearl HW-40 and MCI-gel CHP 20P, to give phytol (**1**),[19] isorhamnetin 3-*O*-*β*-D-glucoside (**2**),[20] kaempferol 3-*O*-*β*-D-sophoroside (**3**)[21] from komatsuna, and *p*-coumaric acid (**4**),[22] 3,6-dimethyl-5,7,3′,4′-tetrahydroxyflavone 4′-*β*-D-glucuronide (**5**),[23] 5,3′,4′-trihydroxy-3-methoxy-6 : 7-methylenedioxyflavone 4′-*β*-D-glucuronide (**6**),[23] 6-methoxy-3,5,7,3′,4′-pentahydroxyflavone 3-*O*-*β*-D-glucosyl-(1→6)-[*β*-D-apiosyl-(1→2)]-*β*-D-glucoside (**7**),[24] adenosine (**8**), guanosine (**9**) and uridine (**10**)[25] from spinach (Fig. 1). The identification of these compounds was established by comparing the physical and spectral data with literature values or by direct comparisons with authentic specimens. Compounds **1**–**3** from komatsuna, and **8** and **9** from spinach were identified for the first time, although compounds **4**–**7** and **10** have already been isolated and identified from spinach.

Next, the inhibitory effect on TCDD-induced activation of AhR in compounds (**1**–**10**) was examined. The results are shown in Fig. 2(a) as the dose-response curves. As shown, flavonol glycosides (compounds **2**, **3** and **5**–**7**) and compound **1** showed a slight inhibitory effect on AhR activation at high concentrations around the 50 μM level (inhibitory effect of *ca*. 20%), however they had weak inhibitory effects as can be seen from comparison with that of kaempferol as shown in Fig. 2(a), and were less than the EC_{70} level even at high concentrations when the concentrations producing AhR activity equal to 70% of the maximal response to TCDD in controls were expressed as the EC_{70} value (EC_{70} of kaempferol = 2.1 μM). In previous paper,[16] we reported that the glycosides of flavonoids tend to weaken this activity. Flavonols identified in this study are also glycosides, and indicated the same tendency.

Additionally, the *in vitro* AhR-inducing potencies of compounds **1**–**10** were determined by the CALUX assay. Dose-response curves are plotted in Fig. 2(b). The response produced by TCDD was considered to be 100%. Flavonol glycosides (compounds **2**, **3**, **6** and **7**) showed slight activation of luciferase (*ca*. 10–20%) at high concentrations in the order of 10^5 nM, but it was not noteworthy as an AhR activating substance.

Although the extracts of komatsuna and spinach showed inhibitory effect on AhR activation by TCDD and AhR induction, the identified compounds showed only a low activity at high concentrations. Therefore, the presence of other compounds not isolated in this study is suggested. Further research on the other compounds, including the other fractions in komatsuna and spinach, is currently in progress.

Acknowledgements This work was supported in part by a grant from the Ministry of Health, Labour and Welfare, Japan.

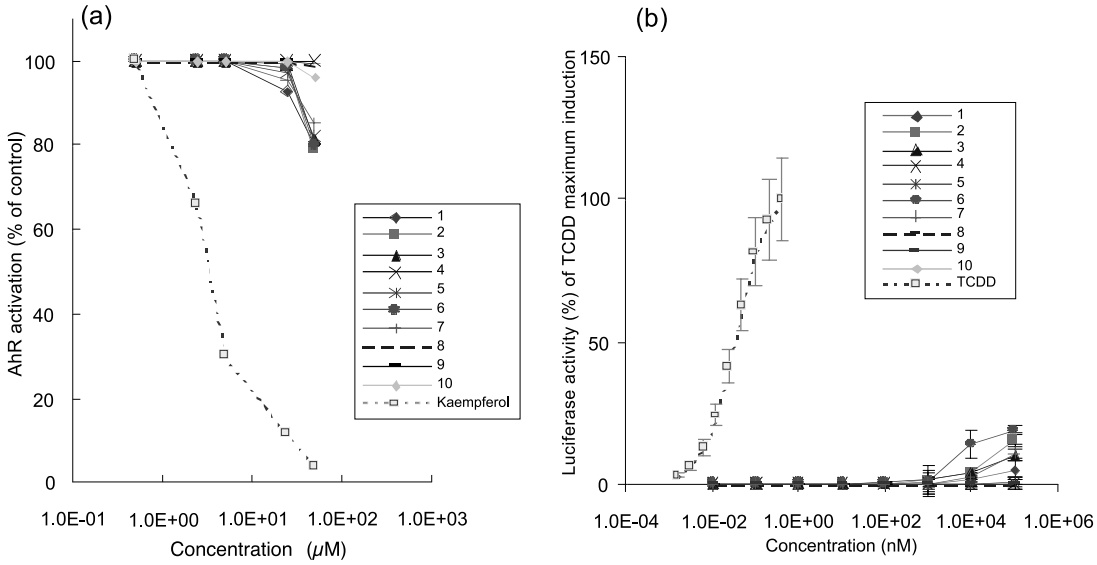

Fig. 2. Interaction of the AhR with Compounds **1**–**10**

(a) Dose–dependent inhibitory effect of compounds **1**–**10** and kaempferol on the AhR-activation induced by TCDD in the Ah-Immunoassay (each point represents the mean of two replicated analyses). (b) Concentration-response curve of compounds **1**–**10** and TCDD for the induction of luciferase activity in the CALUX assay (each point represents the mean of at least three replicated analyses, with error bars showing the standard deviation).

REFERENCES

1) Denison, M. S. and Nagy, S. R. (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol*., **43**, 309–334.
2) Denison, M. S., Pandini, A., Nagy, S. R., Baldwin, E. P. and Bonati, L. (2002) Ligand binding and activation of the Ah receptor. *Chem. Biol. Interact*., **141**, 3–24.
3) Pohjanvirta, R. and Tuomisto, J. (1994) Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals: effects, mechanisms, and animal models. *Phamacol. Rev*., **46**, 483–549.
4) Safe, S. H. (1986) Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol*., **26**, 371–399.
5) Landers, J. P. and Bunce, N. J. (1991) The Ah receptor and the mechanism of dioxin toxicity. *Biochem. J*., **276**, 273–287.
6) Ashida, H., Fukuda, I., Yamashita, T. and Kanazawa, K. (2000) Flavones and flavonols at dietary levels inhibit a transformation of aryl hydrocarbon receptor induced by dioxin. *FEBS Lett*., **476**, 213–217.
7) Loaiza-Perez, A. I., Kenney, S., Boswell, J., Hollingshead, M., Alley, M. C., Hose, C., Ciolino, H. P., Yeh, G. C., Trepel, J. B., Vistica, D. T. and Sausville, E. A. (2004) Aryl hydrocarbon receptor activation of an antitumor aminoflavone: basis of selective toxicity for MCF-7 breast tumor cells. *Mol. Cancer Ther*., **3**, 715–725.
8) MacDonald, C. J., Ciolino, H. P. and Yeh, G. C. (2004) The drug salicylamide is an antagonist of the aryl hydrocarbon receptor that inhibits signal transduction induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Cancer Res*., **64**, 429–434.
9) Ciolino, H. P., Daschner, P. J. and Yeh, G. C. (1999) Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem. J*., **340**, 715–722.
10) Ciolino, H. P. and Yeh, G. C. (1999) The flavonoid galangin is an inhibitor of CYP1A1 activity and an agonist/antagonist of the aryl hydrocarbon receptor. *Br. J. Cancer*, **79**, 1340–1346.
11) Ciolino, H. P., Daschner, P. J. and Yeh, G. C. (1998) Resveratrol inhibits transcription of CYP1A1 in vitro by preventing activation of the aryl hydrocarbon receptor. *Cancer Res*., **58**, 5707–5712.
12) Ciolino, H. P., Daschner, P. J., Wang, T. T. and Yeh, G. C. (1998) Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. *Biochem. Pharmacol*., **56**, 197–206.
13) Ciolino, H. P., Wang, T. T. and Yeh, G. C. (1998) Diosmin and diosmetin are agonists of the aryl hydrocarbon receptor that differentially affect cytochrome P450 1A1 activity. *Cancer Res*., **58**, 2754–2760.
14) Amakura, Y., Tsutsumi, T., Nakamura, M., Kitagawa, H., Fujino, J., Sasaki, K., Yoshida, T. and Toyoda,

M. (2002) Preliminary screening of the inhibitory effect of food extracts on activation of the aryl hydrocarbon receptor induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biol. Pharm. Bull.*, **25**, 272–274.
15) Amakura, Y., Tsutsumi, T., Nakamura, M., Kitagawa, H., Fujino, J., Sasaki, K., Toyoda, M., Yoshida, T. and Maitani, T. (2003) Activation of the aryl hydrocarbon receptor by some vegetable constituents determined using *in vitro* reporter gene assay. *Biol. Pharm. Bull*., **26**, 532–539.
16) Amakura, Y., Tsutsumi, T., Sasaki, K., Yoshida, T. and Maitani, T. (2003) Screening of the inhibitory effect of vegetable constituents on the aryl hydrocarbon receptor-mediated activity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biol. Pharm. Bull*., **26**, 1754–1760.
17) Amakura, Y., Tsutsumi, T., Nakamura, M., Sasaki, K., Yoshida, T. and Maitani, T. (2004) Interaction of some plant food extracts with aryl hydrocarbon receptor determined by *in vitro* reporter gene assay. *Nat. Med*., **58**, 31–33.
18) Seidel, S. D., Li, V., Winter, G. M., Rogers, W. J., Martines, E. I. and Denison, M. S. (2000) Ah receptor-based chemical screening bioassays: application and limitations for the detection of AhR receptor agonists. *Toxicol. Sci*., **55**, 107–115.
19) Sims, J. J. and Pettus, J. A., Jr. (1976) Isolation of free *cis* and *trans*-phytol from the red alga *Gracilaria andersoniana*. *Phytochemistry*, **15**, 1076–1077.
20) Bilia, A. R., Gonzalez, M., Morelli, I., Nieri, E. and Rubio, M. E. (1994) Flavonol glycosides from *Phyrus bourgaeana*. *Phytochemistry*, **35**, 1378–1380.
21) Wiedenfeld, H., Andrade-Cetto, A. and Perez-Amador, C. (2000) Flavonol glycosides from *Equisetum myriochaetim*. *Biochem. Syst. Ecol*., **28**, 395–397.
22) Bergman, M., Varshavsky, L., Gottlieb, H. E. and Grossman, S. (2001) The antioxidant activity of aqueous spinach extract: chemical identification of active fractions. *Phytochemistry*, **58**, 143–152.
23) Aritomi, M., Komori, T. and Kawasaki, T. (1986) Flavonol glycosides in leaves of *Spinacia oleracea*. *Phytochemistry*, **25**, 231–234.
24) Aritomi, M. and Kawasaki, T. (1984) Three highly oxygenated flavone glucuronides in leaves of *Spinacia oleracea*. *Phytochemistry*, **23**, 2043–2047.
25) Firl, J., Frommeyer, D. and Elstner, E. F. (1981) Isolation and identification of an oxygen reducing factor (ORF) from isolated spinach chloroplast lamellae. *Z. Naturforsch. C*, **36**, 284–294.