Activation of the Aryl Hydrocarbon Receptor by Methyl Yellow and Related Congeners: Structure–Activity Relationships in Halogenated Derivatives

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates the biological action of many environmental compounds. Methyl yellow (4-dimethylaminoazobenzene; MY) is a principal azo-dye, and structurally related compounds were subjected to analysis of structure–activity relationships as AhR ligands by using a yeast AhR signaling assay. The effects of halogen-substitution among 23 halogenated MYs on the AhR ligand activity can be summarized as follows: enhancement by halogen-substitution at the *ortho*-position (2′- and 6′-position), and reduction by substitution at the *para*-position (4′-position). The greatest enhancement of the ligand activity was observed in 2′,6′-dichlorinated MY (13.5-fold of MY), and its AhR ligand activity was very close to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the present assay system. In the study of compounds structurally related to MY, benzanilide (BA) showed almost the same AhR ligand activity as azobenzene and *trans*-stilbene. Furthermore, 4′-chlorobenzanilide, in which the length of the molecule is similar to that of MY, enhanced the AhR ligand activity by *ortho*(2′)-chlorine-substitution, and the AhR ligand activity of 2′,4′-dichlorobenzanilide was similar to that of 2′-chloro-MY. These results suggest that the amide bond is equivalent to the –N=N– or –CH=CH– double bond for recognition as the ligand by AhR in 1,2-diphenyl-1,2-ene derivatives.

Key words aryl hydrocarbon receptor; azocompound; structure–activity relationship; methyl yellow; halogenated derivative

The aryl hydrocarbon receptor (AhR), also called the dioxin receptor, is a transcriptional factor that mediates the toxicity of a variety of compounds, most notably, of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated PAHs.[1—3] This receptor is a member of the basic helix-loop-helix superfamily of DNA-binding proteins.[4] Upon ligand-binding, the AhR is transformed and dissociates from a heat shock protein dimer with which it is complexed in the cyotsol, that it enters the nucleus where it joins with a nuclear protein, the AhR nuclear translocator (ARNT). Interaction of this AhR/ARNT heterodimer with specific DNA sequences, called dioxin response elements (DREs), in the regulatory regions of responsive genes can result in activation of a certain group of genes, most of which are involved primarily in xenobiotic metabolism, including CYP1A1, CYP1A2, CYP1B1, the glutathione S-transferase Ya subunit, quinone oxidoreductase, UDP-glucuronosyltransferase, and aldehyde-3-dehydrogenase.[5—11]

Azocompounds are used extensively as dyes in a variety of industries, including the cosmetics, food, leather, paper and textile industries. However, some azocompounds manifest carcinogenicity through highly reactive metabolic intermediates that can interact covalently with DNA and cause mutations.[12] Methyl yellow (4-dimethylaminoazobenzene; MY) is a basic structure of those azo-dyes which have been used as dyes in food, but the use of MY is now prohibited because of its carcinogenic activity in rodents. It was suggested that MY is metabolically converted to a procarcinogen, *N*-hydroxy-*N*-methyl-4-aminoazobenzene, by demethylation followed by *N*-hydroxylation.[13—15] In the case of 4-aminoazobenzene (AAB), metabolic activation may proceed through *N*-hydroxylation by the microsomal cytochrome P450-dependent mixed-function oxidase and flavin monooxygenase system.[16,17] The *N*-hydroxylation of AAB appears to be catalyzed in most cases by the CYP1A family, and especially by the CYP1A2 protein.[16—18] The CYP1A2 protein is expressed in the liver of both animals and humans, and is the primary tumor site of most azocompounds.[19] It has been reported that AAB derivatives induce CYP1A2.[20] The induction of CYP1A proteins is regulated by AhR.

Numerous studies of the structure–activity correlation of ligands for AhR have been reported.[21] The effects of halogen-substitution, especially chlorine-substitution, on the AhR ligand activity have been reported. However, most of these reports are on dibenzodioxin, dibenzofuran and biphenyl, and there but few reports on other aromatic nuclei.

Therefore, in order to explore the characteristics of the general structure of AhR ligands, we investigated the AhR ligand activities of MY and its halogen-substituted analogs using a yeast AhR signaling assay.[22—25] Additionally, structural analogs of MY, azobenzenes, stilbenes, and benzanilides were also subjected to the analysis of their AhR ligand activities.

MATERIALS AND METHODS

Materials TCDD was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). MY, azobenzene, 4-aminoazobenzene, benzanilide, *p*-dimethylaminostilbene, *trans*-stilbene, *cis*-stilbene, halogenated anilines and *N*,*N*-dimethylaniline were purchased from Aldrich. 2′-Fluoro-4-dimethylaminoazobenzene (2′-F-MY, CAS Registry No. 331-91-9), 3′-fluoro-4-dimethylaminoazobenzene (3′-F-MY, 332-54-7), 4′-fluoro-4-dimethylaminoazobenzene (4′-F-MY, 150-74-3), 2′,3′,4′,5′,6′-pentafluoro-4-dimethylaminoazobenzene (2′,3′,4′,5′,6′-penta-F-MY, 970-150-0), 2′-chloro-4-dimethylaminoazobenzene (2′-Cl-MY, 3010-47-7), 3′-chloro-4-dimethylaminoazobenzene (3′-Cl-MY, 3789-77-3), 4′-chloro-4-dimethylaminoazobenzene (4′-Cl-MY, 2491-76-1), 2′,4′-

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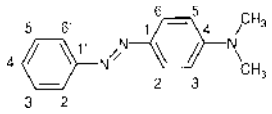
Table 1. [1]H-NMR Chemical Shifts and UV Absorbance of MY and Its Halogenated Derivatives Examined

Compounds	[1]H-MMR chemical shifts (ppm)								UV absorbance	
	N-CH_3	H-2, 6	H-3, 5	H-2′	H-3′	H-4′	H-5′	H-6′	λ_{max} (nm)	ε
MY	3.08	7.88	6.75	7.83	7.47	7.37	7.47	7.83	406.2	29700
										
Monofluorinated derivatives										
2′-F-	3.09	7.90	6.75	F	7.14—7.25	7.33	7.14—7.25	7.73	415.8	28320
3′-F-	3.09	7.87	6.75	7.53	F	7.60	7.42	7.65	416.0	24600
4′-F-	3.08	7.86	6.76	7.84	7.14	F	7.14	7.84	406.2	25500
Difluorinated derivatives										
2′,4′-diF-	3.09	7.88	6.74	F	6.88—7.00	F	6.88—7.00	7.77	415.8	28740
2′,5′-diF-	3.11	7.90	6.74	F	7.17	7.01	F	7.47	429.0	25440
2′,6′-diF-	3.10	7.89	6.75	F	6.99	7.20	6.99	F	409.2	22980
3′,5′-diF-	3.13	7.87	6.75	7.38	F	6.81	F	7.38	426.0	28380
Polyfluorinated derivatives										
2′,3′,4′,5′,6′-pentaF-	3.13	7.87	6.74	F	F	F	F	F	432.6	29700
Monochlorinated derivatives										
2′-Cl-	3.10	7.93	6.74	Cl	7.50	7.28 or 7.30		7.67	421.4	28440
3′-Cl-	3.10	7.88	6.76	7.83	Cl	7.34	7.40	7.74	421.4	28440
4′-Cl-	3.10	7.86	6.76	7.78	7.43	Cl	7.43	7.78	416.6	30480
Dichlorinated derivatives										
2′,3′-diCl-	3.11	7.93	6.76	Cl	Cl	7.45	7.24	7.56	431.2	26133
2′,4′-diCl-	3.11	7.95	6.75	Cl	7.52	Cl	7.27	7.52	433.8	31860
2′,6′-diCl-	3.12	7.92	7.37	Cl	7.37	7.11	7.37	Cl	397.0	21840
3′,5′-diCl-	3.12	7.87	6.75	7.73	Cl	7.34	Cl	7.73	432.2	30600
2,3′-diCl-	3.10	7.79 (H-6)	6.79 (H-3) 6.62 (H-5)	7.87	Cl	7.35	7.41	7.78	419.6	31620
Polychlorinated derivatives										
2′,4′,5′-triCl-	3.12	7.91	6.75	Cl	7.62	Cl	Cl	7.83	449.4	16680
3′,4′,5′-triCl-	3.12	7.86	6.76	7.88	Cl	Cl	Cl	7.88	445.0	29520
2′,3′,5′,6′-tetraCl-	3.13	7.91	6.77	Cl	Cl	7.50	Cl	Cl	410.2	22333
2′,3′,4′,5′,6′-pentaCl-	3.13	7.90	6.76	Cl	Cl	Cl	Cl	Cl	414.8	20800
Monobrominated derivatives										
2′-Br-	3.13	7.93	6.76	Br	7.64	7.35	7.21	7.70	421.6	29340
3′-Br-	3.10	7.78	6.76	7.98	Br	7.49	7.34	7.78	419.4	30360
4′-Br-	3.09	7.86	6.72	7.72	7.59	Br	7.59	7.72	418.4	33420
Dibrominated derivatives										
2′,4′-diBr-	3.11	7.92	6.75	Br	7.86	Br	7.55	7.47	419.2	32580
2′,6′-diBr-	3.12	7.94	6.78	Br	7.60	6.98	7.60	Br	400.2	24780

dichloro-4-dimethylaminoazobenzene (2′,4′-diCl-MY, 10286-76-7), 2′-bromo-4-dimethylaminoazobenzene (2′-Br-MY, 17128-10-8), 3′-bromo-4-dimethylaminoazobenzene (3′-Br-MY, 17576-88-4) and 4′-bromo-4-dimethylaminoazobenzene (4′-Br-MY, 3805-65-0) were synthesized from 4-dimethylaniline and the corresponding halogenated anilines by the diaza coupling reaction. 2′-Chlorobenzanilide (1020-39-9), 4′-chlorobenzanilide (2866-82-2), 2′-methylbenzanilide (584-70-3) and 2′-ethylbenzanilide (78987-16-3) were synthesized from benzoic anhydride and the corresponding chlorinated or alkylated anilines. Melting points were determined with a Yamato MP-500D micro melting point apparatus without correction. Mass spectra were measured with a JEOL AX505HA spectrometer. [1]H-NMR spectra were recorded with a JEOL JNM-GSX 400 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard. [1]H-NMR chemical shifts and the UV absorbance of MY and its halogenated derivatives examined are summarized in Table 1.

The following compounds were newly synthesized for this study.

2′,4′-Difluoro-4-dimethylaminoazobenzene (2′,4′-diF-MY): 2,4-Difluoroaniline (1.42 g) was dissolved in 20 ml of 1 N HCl, and sodium nitrite (954 mg) was added to this solution at 0 °C. After stirring for 5 min, *N*,*N*-dimethylaniline (1.40 ml, 1 eq) was added. The resulting precipitate was washed with water and dried. 2′,4′-DiF-MY was obtained by recrystallization from methanol as brown plates in 50% yield. mp 135—137 °C. MS *m*/*z*: 261 (M^+). *Anal.* Calcd for $C_{14}H_{13}F_2N_3$: C, 64.36; H, 5.02; N, 16.08. Found: C, 64.31; H, 4.96; N, 15.85.

3′,5′-Difluoro-4-dimethylaminoazobenzene (3′,5′-diF-MY): 3,5-Difluoroaniline (227 mg) and *N*,*N*-dimethylaniline (2.15 g) were dissolved in 30 ml of methanol, and 5 ml of c-HCl was added to this solution. Sodium nitrite aq. (4.31 g of sodium nitrite in 10 ml of water) was added to the solution at 0 °C. After stirring for 15 min at room temperature, the reaction mixture was poured into water and extracted with $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. Purification of the extract by column chromatography (silica gel, $CHCl_3$: *n*-hexane=2 : 3) and recrystallization from methanol yielded 3′,5′-diF-MY as red brown plates in 32% yield. mp 114—116 °C. MS *m*/*z*: 261

(M^+). High resolution (HR)-MS *m/z*: 261.1077, Calcd for $C_{14}H_{13}F_2N_3$: 261.1078.

2′,5′-Difluoro-4-dimethylaminoazobenzene (2′,5′-diF-MY): 2′,5′-DiF-MY was synthesized from 2,5-difluoroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 2′,5′-DiF-MY was obtained by recrystallization from methanol as brown plates in 70% yield. mp 137—139 °C. MS *m/z*: 261 (M^+). *Anal.* Calcd for $C_{14}H_{13}F_2N_3$: C, 64.36; H, 5.02; N, 16.08. Found: C, 64.12; H, 4.86; N, 16.06.

2′,6′-Difluoro-4-dimethylaminoazobenzene (2′,6′-diF-MY): 2′,6′-DiF-MY was synthesized from 2,6-difluoroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 2′,6′-DiF-MY was obtained by recrystallization from methanol as brown needles in 56% yield. mp 96—97 °C. MS *m/z*: 261 (M^+). HR-MS *m/z*: 261.1074, Calcd for $C_{14}H_{13}F_2N_3$: 261.1078.

2′,3′-Dichloro-4-dimethylaminoazobenzene (2′,3′-diCl-MY): 2′,3′-DiCl-MY was synthesized from 2,3-dichloroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 2′,3′-DiCl-MY was obtained by recrystallization from methanol as brown plates in 35% yield. mp 200—209 °C. MS *m/z*: 294 (M^+). *Anal.* Calcd for $C_{14}H_{13}Cl_2N_3$: C, 57.16; H, 4.45; N, 14.28. Found: C, 56.99; H, 4.39; N, 14.02.

2′,6′-Dichloro-4-dimethylaminoazobenzene (2′,6′-diCl-MY): 2′,6′-DiCl-MY was synthesized from 2,6-dichloroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 2′,6′-DiCl-MY was obtained by recrystallization from methanol as brown needles in 59% yield. mp 134—137 °C. MS *m/z*: 294 (M^+). HR-MS *m/z*: 293.0490, Calcd for $C_{14}H_{13}Cl_2N_3$: 293.0487.

3′,5′-Dichloro-4-dimethylaminoazobenzene (3′,5′-diCl-MY): 3′,5′-DiCl-MY was synthesized from 3,5-dichloroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 3′,5′-DiCl-MY was obtained by recrystallization from methanol as brown needles in 64% yield. mp 134—137 °C. MS *m/z*: 294 (M^+). HR-MS *m/z*: 293.0490, Calcd for $C_{14}H_{13}Cl_2N_3$: 293.0487.

2,3′-Dichloro-4-dimethylaminoazobenzene (2,3′-diCl-MY): *m*-Chloroaniline (2.96 g) was dissolved in 2.3 ml of methanol, and 60% H_2SO_4 was added to this solution. After keeping the solution at 200 °C for 16 h, the reaction mixture was poured into water and neutralized with Na_2CO_3, then extracted with $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. Purification of the extract by column chromatography (silica gel, *n*-hexane→*n*-hexane : AcOEt=19 : 1→*n*-hexane : AcOEt=1 : 1) yielded *m*-chloro-*N*,*N*-dimethylaniline as a colorless oil in 77% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.94 (s, 6H, $N(CH_3)_2$), 6.58 (ddd, 1H, H-6), 6.66 (m, 2H, H-2, H-4), 7.13 (dd, 1H, H-5); $J_{5\text{—}6}$=8.4 Hz. HR-MS *m/z*: 155.0491, Calcd for $C_8H_{10}ClN$: 155.0502. 2,3′-DiCl-MY was synthesized from *m*-chloroaniline and *m*-chloro-*N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 2,3′-DiCl-MY was obtained by recrystallization from methanol as red brown columns in 59% yield. mp 93—94 °C. MS *m/z*: 294 (M^+). *Anal.* Calcd for $C_{14}H_{13}Cl_2N_3$: C, 57.16; H, 4.45; N, 14.28. Found: C, 57.44; H, 4.48; N, 14.38.

2′,4′,5′-Trichloro-4-dimethylaminoazobenzene (2′,4′,5′-triCl-MY): 2′,4′,5′-TriCl-MY was synthesized from 2,4,5-trichloroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 2′,4′,5′-TriCl-MY was obtained by recrystallization from methanol as brown needles in 26% yield. mp 190—193 °C. MS *m/z*: 327 (M^+), 329 (M^++2). HR-MS *m/z*: 327.0086, Calcd for $C_{14}H_{12}Cl_3N_3$: 327.0097.

3′,4′,5′-Trichloro-4-dimethylaminoazobenzene (3′,4′,5′-triCl-MY): 3′,4′,5′-TriCl-MY was synthesized from 3,4,5-trichloroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 3′,5′-diF-MY. 3′,4′,5′-TriCl-MY was obtained by recrystallization from methanol as red brown needles in 67% yield. mp 163—167 °C. HR-MS *m/z*: 327.0106, Calcd for $C_{14}H_{12}Cl_3N_3$: 327.0097.

2′,3′,5′,6′-Tetrachloro-4-dimethylaminoazobenzene (2′,3′,5′,6′-tetraCl-MY): 2′,3′,5′,6′-TetraCl-MY was synthesized from 2,3,5,6-tetrachloroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 2′,3′,5′,6′-TetraCl-MY was obtained by recrystallization from methanol as red brown needles in 38% yield. mp 247—249 °C. HR-MS *m/z*: 360.9709, Calcd for $C_{14}H_{11}Cl_4N_3$: 360.9707.

2′,3′,4′,5′,6′-Pentachloro-4-dimethylaminoazobenzene (2′,3′,4′,5′,6′-pentaCl-MY): 2′,3′,4′,5′,6′-PentaCl-MY was synthesized from pentachloroaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 3′,5′-diF-MY. 2′,3′,4′,5′,6′-PentaCl-MY was obtained by recrystallization from methanol as red brown needles in 15% yield. mp 224—228 °C. HR-MS *m/z*: 394.9317, Calcd for $C_{14}H_{10}Cl_4N_3$: 394.9317.

2′,4′-Dibromo-4-dimethylaminoazobenzene (2′,4′-diBr-MY): 2′,4′-DiBr-MY was synthesized from 2,4-dibromoaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 2′,4′-diF-MY. 2′,4′-DiBr-MY was obtained by recrystallization from methanol as red brown prisms in 54% yield. mp 127—128 °C. *Anal.* Calcd for $C_{14}H_{13}Br_2N_3$: C, 43.89; H, 3.42; N, 10.97. Found: C, 43.92; H, 3.48; N, 11.01.

2′,6′-Dibromo-4-dimethylaminoazobenzene (2′,6′-diBr-MY): 2′,6′-DiBr-MY was synthesized from 2,6-dibromoaniline and *N*,*N*-dimethylaniline by the same method as for the synthesis of 3′,5′-diF-MY. 2′,6′-DiBr-MY was obtained by recrystallization from methanol as red brown prisms in 46% yield. mp 127—128 °C. MS *m/z*: 381 (M^+), 383 (M^++2), 385 (M^++4). *Anal.* Calcd for $C_{14}H_{13}Br_2N_3$: C, 43.89; H, 3.42; N, 10.97. Found: C, 44.01; H, 3.50; N, 11.08.

2′,4′-Dichlorobenzanilide (2′,4′-diCl-BA): 2,4-Dichloroaniline (685 mg) was dissolved in 30 ml of pyridine, and benzoic anhydride (1.90 g) was added to this solution. After stirring for 3 h at 80 °C, the solvent was evaporated. The resultant residue was dissolved in $CHCl_3$, and this solution was washed with water and 5% Na_2CO_3. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. Purification of the extract by recrystallization (*n*-hexane–acetone) yielded 2′,4′-dichlorobenzanilide as white needles in 58% yield. mp 120—121 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.32 (dd, 1H, H-5′), 7.44 (d, 1H, H-3′), 7.53 (t, 2H, H-3, H-5), 7.60 (t, 1H, H-4), 7.91 (d, 2H, H-2, H-6), 8.39 (br, 1H, NH), 8.55 (d, 1H, H-6); $J_{3'\text{—}5'}$=2.1, $J_{5'\text{—}6'}$=8.9, $J_{2\text{—}3}$=$J_{3\text{—}4}$=$J_{4\text{—}5}$=$J_{5\text{—}6}$=7.2 Hz. *Anal.* Calcd for $C_{13}H_9Cl_2NO$: C, 58.67; H, 3.41; N, 5.26. Found: C, 58.60; H, 3.44; N, 5.20. HR-MS *m/z*: 265.0056, Calcd for $C_{13}H_9Cl_2NO$: 265.0061.

2-Chlorobenzanilide (2-Cl-BA): Aniline (208 mg) was

added to 10 ml of pyridine, and *m*-chlorobenzoyl chloride (339 μl) was added to this solution. After stirring for 3 h at 80 °C, the solvent was evaporated. The resultant residue was dissolved in $CHCl_3$ and this solution was washed with water and 5% Na_2CO_3. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. Purification of the extract by column chromatography ($CHCl_3$: *n*-hexane=1 : 4→2 : 3) and recrystallization from *n*-hexane–acetone yielded 2-chlorobenzanilide as white needles in 64% yield. mp 121—124 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.18 (t, 1H, H-5), 7.37—7.48 (m, 5H, H-3, -4, -3′, -4′, H-5′), 7.65 (d, 2H, H-2′, H-6′), 7.78 (d, 1H, H-6), 7.87 (br, 1H, NH); $J_{2'—3'}=J_{5'—6'}=7.8$, $J_{4—6}=2.2$, $J_{5—6}=7.1$ Hz. *Anal.* Calcd for $C_{13}H_{10}ClNO$: C, 67.40; H, 4.35; N, 6.05. Found: C, 67.48; H, 4.44; N, 6.03. HR-MS *m/z*: 231.0448, Calcd for $C_{13}H_{10}NO$: 231.0451.

2′-*tert*-Butylbenzanilide (2′-*tert*-Bu-BA): 2′-*tert*-Butylbenzanilide was synthesized from *o*-*tert*-butylaniline and benzoic anhydride by the same method as for the synthesis of 2′,4′-dichlorobenzanilide. 2′-*tert*-Bu-BA was obtained by recrystallization from methanol as white needles in 50% yield. mp 194—196 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (s, 9H, $C(CH_3)_3$), 7.29 (dt, 1H, H-4′ or H-5′), 7.21 (dt, 1H, H-4′ or H-5′), 7.45 (dd, 1H, H-3′), 7.20 (dt, 2H, H-3, H-5), 7.58 (dt, 1H, H-4), 7.75 (d, 1H, H-6′), 7.88 (br, 1H, NH), 7.92 (dd, 2H, H-2, H-6); $J_{3'—5'}=2.1$, $J_{5'—6'}=8.9$, $J_{2—3}=J_{3—4}=J_{4—5}=J_{5—6}=7.2$ Hz. *Anal.* Calcd for $C_{17}H_{19}NO$: C, 80.60; H, 7.56; N, 5.53. Found: C, 80.60; H, 7.47; N, 5.58. HR-MS *m/z*: 253.1471, Calcd for $C_{17}H_{19}NO$: 253.1467.

Yeast Assays for AhR Ligand Activity In the yeast strain YCM3,[22] the human AhR and ARNT genes are integrated into chromosome III. AhR and ARNT are expressed from the galactose-regulated GAL 1,10 promoter. Transcriptional activation mediated by the AhR/ARNT heterodimer is assessed by β-galactosidase activity. Expression of the LacZ reporter plasmid, pTXRE5-Z, is directed by AhR-ARNT complex binding to five response elements in the promoter region.

The assay procedure was essentially the same as described by Miller *et al.*[22—25] The yeast strain YCM3 was grown overnight at 30 °C in a synthetic glucose medium lacking tryptophan. One microliter of test chemicals (dissolved in DMSO), 20 μl of the overnight culture and 1 ml of the synthetic medium containing 2% galactose were mixed and incubated for 18 h at 30 °C. Cell density was determined by reading the absorbance at 595 nm. Ten microliters of each cell suspension was added to 700 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol and 0.2% sarcosyl, adjusted to pH 7), and the reaction was started by adding 200 μl of *o*-nitrophenol-β-D-galactopyranoside (4 mg/ml solution in Z-buffer), with subsequent incubation for 60 min at 37 °C. Absorbances of the β-galactosidase assay were read at 405 nm. β-Galactosidase activity (referred to as *lacZ* units) was calculated by the following formula: absorbance at 405 nm×1000/(absorbance at 595 nm×ml cell suspension added×min reaction time).

RESULTS

AhR ligand activities of the test compounds were assessed with the *lacZ* reporter gene assay using *Saccharomyces cerevisiae* strain YCM3, in which the human AhR and ARNT genes are co-expressed, as previously reported.[22—25]

TCDD, an exogenous potent AhR ligand, showed an appreciable increase in the *lacZ* unit in a dose-dependent manner in this assay system, and the EC_{50} value of TCDD was determined to be 70 nM from the dose-response curve (Fig.

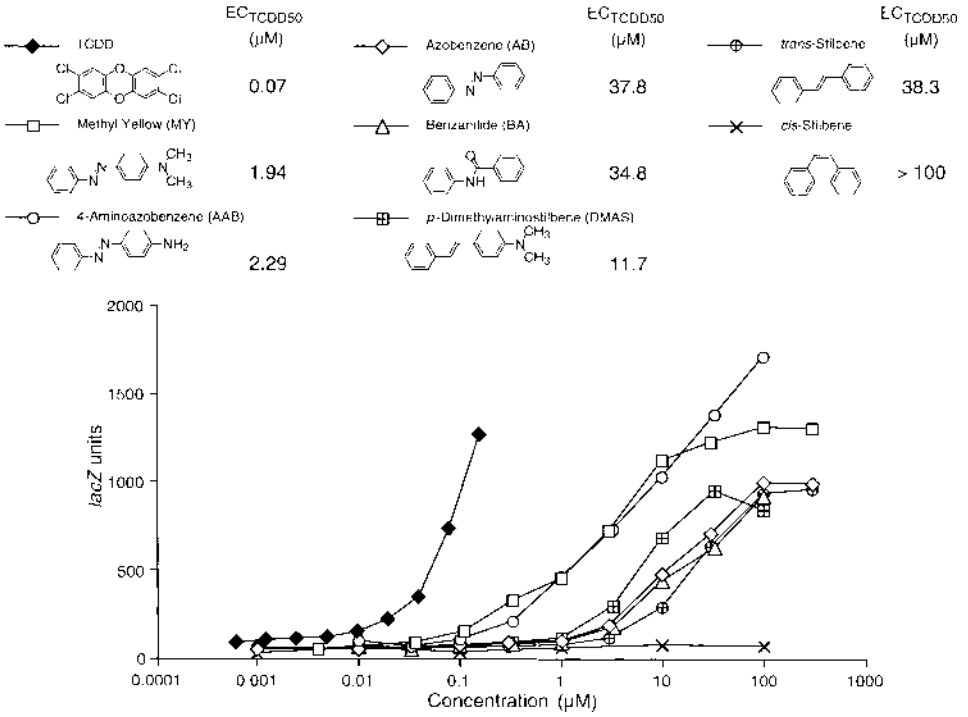


Fig. 1. AhR Ligand Activities of TCDD, MY, and Its Structural Analogs

AhR ligand activities of TCDD (closed diamonds), Methyl Yellow (open squares), and MY structural analogs were assessed with the reporter gene assay using *Saccharomyces cerevisiae* strain YCM3, in which the human AhR and ARNT genes were co-expressed. Each point represents the mean of at least three analyses. Concentrations were plotted on a log scale. EC_{TCDD50} is the concentration producing *lacZ* units equal to 50% of the maximal response to TCDD.

1). To compare the activation potency of MY and related congeners with TCDD, the concentrations of test compounds producing *lacZ* units equivalent to 50% of the maximal *lacZ* units produced by TCDD were calculated from the dose-response curve, and expressed as EC_{TCDD50} values, according to the previously reported method.[26,27] Furthermore, to evaluate the relative activity of each compound, the value of *lacZ* units per μM of the compound was also calculated from the slope of the initial linear portion of each dose-response curve because a few compounds showed levels of maximal induction of *lacZ* units unsatisfactory to calculate the EC_{TCDD50} value.

Table 2 shows the AhR ligand activities of the MY derivatives. Among all the monohalogenated derivatives, 2′-halogenated MYs (2′-F, 2′-Cl, and 2′-Br-MY) showed higher ligand activities than that of MY, and the halogen-substitution effects on the ligand activity (relative activity) decreased in the following order, Cl (7.8)>Br (5.0)>F (3.1). On the other hand, all the 3′- and 4′-halogenated MYs showed lower ligand activities than that of MY, in which the relative activity ranged from 0.09- to 0.9-fold. In the case of 16 di- and polyhalogenated derivatives, only 2′,6′-dihalogenated MYs showed more than 10-fold enhancement of the ligand activity, and the enhancing effects decreased in the following order, diCl (13.5)>diBr (11.1)>diF (10.8). The EC_{TCDD50} value of 2′,6′-diCl-MY (0.12 μM), which showed the highest AhR ligand activity among the halogenated MYs tested in this study, was close to that of TCDD (0.07 μM).

Table 2. AhR Activation Potencies of MY and Its Halogenated Derivatives

Chemicals	EC_{TCDD50}[a] (μM)	*lacZ* units/μM[b]	Relative activity[c]
TCDD	0.07	6373.5	—
MY	1.94	898.4	1.00
Monofluorinated derivatives			
2′-F-	0.63	2755.8	3.07
3′-F-	4.87	323.9	0.36
4′-F-	2.72	817.6	0.91
Difluorinated derivatives			
2′,4′-diF-	1.95	353.6	0.39
2′,5′-diF-	1.29	1239.8	1.38
2′,6′-diF-	0.34	9674.9	10.77
3′,5′-diF-	25.40	41.4	0.05
Polyfluorinated derivatives			
2′,3′,4′,5′,6′-pentaF-	1.99	1031.2	1.15
Monochlorinated derivatives			
2′-Cl-	0.15	6997.6	7.79
3′-Cl-	4.41	591.5	0.66
4′-Cl-	6.71	189.5	0.21
Dichlorinated derivatives			
2′,3′-diCl-	1.50	3661.3	4.08
2′,4′-diCl-	4.09	338.2	0.38
2′,6′-diCl-	0.12	12079.5	13.45
3′,5′-diCl-	3.02	216.1	0.24
2,3′-diCl-	21.30	43.2	0.05
Polychlorinated derivatives			
2′,4′,5′-triCl-	44.23	259.1	0.29
3′,4′,5′-triCl-	1.80	432.7	0.48
2′,30019,5′,6′-tetraCl-	>10	325.3	0.36
2′,3′,4′,5′,6′-pentaCl-	>10	237.7	0.26
Monobrominated derivatives			
2′-Br-	0.25	4530.5	5.04
3′-Br-	3.00	597.0	0.66
4′-Br-	>30	80.0	0.09
Dibrominated derivatives			
2′,4′-diBr-	4.53	200.2	0.22
2′,6′-diBr-	0.36	9987.6	11.12

Each value is the mean of at least three analyses. *a*) Concentrations producing *lacZ* units equal to 50% of the maximal response to TCDD. *b*) Calculated from the slope of the linear portion of each dose-response curve near the origin. *c*) Relative values of *lacZ* units/μM of each compound compared to MY.

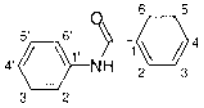
As shown in Fig. 1, the AhR ligand activities of structural analogs of MY decreased in the following order: MY≈AAB>DMAS>BA≈AB≈*trans*-stilbene≫*cis*-stilbene. BA, AB and *trans*-stilbene, which have the same skeleton, *i.e.*, two phenyl groups connected with two linker atoms, showed almost the same activity, but showed lower activities than those of MY, AAB and DMAS, in which the amino or dimethylamino group is located at the *para*-position of one phenyl moiety. *cis*-Stilbene, in which two phenyl groups are not in the same plane because of steric hindrance by each *ortho*-proton, did not show the ligand activity up to 100 μM.

Chlorine- and alkyl-substitution effects on the AhR ligand activity were investigated in BA to give the results shown in Table 3. The ligand activity of benzanilide was increased by *ortho*- or *para*-chlorine substitution, and the relative activity ranged from 2.4- to 100-fold. With regard to *ortho*-alkyl-substitution on BA, methyl and ethyl group substitution showed a slight decrease in the AhR ligand activity. However, *tert*-butyl group substitution on the *ortho*-position of BA was devoid of AhR ligand activity in the whole dose range examined (up to 300 μM).

DISCUSSION

We measured the AhR ligand activity of 24 MYs, 6 BAs, 3 stilbenes, and 2 azobenzenes, which have the same skeleton in the molecule, *i.e.*, two benzene moieties connected with two linker atoms, using the yeast AhR signaling assay to investigate their structure–activity relationships as an AhR ligand. MY was about two orders less active as an AhR ligand than TCDD, but showed the same ligand activity as benzo[*a*]pyrene in the present study (EC_{50} of TCDD= 0.07 μM, EC_{TCDD50} of MY=1.94 μM, and EC_{TCDD50} of BaP=1.81 μM). This result indicates that MY may be a moderate AhR ligand having the capacity to alter AhR-mediated gene expression, such as induction of the CYP1A family. In

Table 3. AhR Activation Potencies of BA and Its Derivatives

Chemicals	EC_{TCDD50}[a] (μM)	*lacZ* units/μM[b]	Relative activity[c]
Benzanilide	34.80	34.2	1.00
			
Chlorine-substituted derivatives			
2′-Cl-BA	2.30	536.0	15.67
4′-Cl-BA	2.12	695.0	20.32
2′,4′-diCl-BA	0.38	3427.0	100.20
2-Cl-BA	11.40	82.7	2.42
Alkyl-substituted derivatives			
2′-Me-BA	52.90	24.7	0.72
2′-Et-BA	52.90	12.7	0.37
2′-*tert*-Bu-BA	>300	Negative	—

Each value is the mean of at least three analyses. *a*) Concentrations producing *lacZ* units equal to 50% of the maximal response to TCDD. *b*) Calculated from the slope of the linear portion of each curve near the origin. *c*) Relative values of *lacZ* units/μM of each compound compared to BA.

fact, Cheung *et al.* reported that 4-aminoazobenzene (4-AAB) derivatives induced CYP1A2.[20] Considering that most of the MYs tested in this study showed higher ligand activities than that of 4-AAB, MYs may act as inducers of the CYP1A family.

The effect of halogen-substitution in monohalogenated MYs on the AhR ligand activity can be summarized as follows: enhancement by substitution at position 2′, and reduction by substitution at position 3′ and 4′. Gillner *et al.* suggested that all potent AhR ligands could fit into a rectangle of 6.8×13.7 Å, on the basis of a ball-and-stick model.[28,29] The length of the MY molecule is about 14 Å and the MY molecule fits into this rectangle. However, the length of the MY molecule will exceed 14 Å by halogen substitution at position 4′, in particular with Cl and Br. It might be possible to explain the decrease in the AhR ligand activity of MY by deviation from the suitable size of a potent AhR ligand molecule due to halogen substitution at position 4′ and 3′. Substitution position-specific effects were similarly shown in polyhalogenated MYs. All the compounds with very high activities ($EC_{TCDD50}<1\ \mu M$ and relative activity>3) had a halogen atom at position-2′, and all the compounds having a Cl or Br atom at position-4′ showed lower AhR ligand activities than that of MY.

BA showed almost the same AhR ligand activity as those of AB and *trans*-stilbene (EC_{TCDD50} were 34.8, 37.8, and 38.3 μM, respectively). This result suggests that the amide bond is equivalent to a –N=N– or –CH=CH– double bond in recognition of the ligand by AhR. In chlorinated BA, *ortho* chlorine-substitution of BA increased the ligand activity similarly to the case of MY. On the other hand, *para* chlorine-substitution also increased the ligand activity, unlike the case of MY. Since BA is smaller than MY, the molecular size of BA may not become too large by chlorine-substitution at position-4′. In contrast, enhancement of the AhR ligand activity of BA by *para* chlorine-substitution might be due to the chlorinated molecule approaching a more suitable size for an AhR ligand. In alkyl group substitution of BA, there was no significant substitution effect on the AhR ligand activity with methyl and ethyl groups. However, the ligand activity of BA disappeared by *tert*-butyl substitution at position-2′. Likewise, stilbene was deprived of AhR ligand activity due to the *cis*-conformation. In general, the planarity of the molecule is a requirement for binding to the Ah receptor. It is thought that the conformation of 2′-*tert*-butylbenzanilide is nonplanar because of the bulkiness of the *tert*-butyl group. These data indicated that the planar structure of a molecule is important for its AhR ligand activity in BA derivatives.

The enhancing effect of 2′-halogen substitution on the AhR ligand activity of MY and BA was not explained by the size or planarity of a molecule. With regard to MYs, the planarity of 2′-mono and 2′,6′-di-halogenated derivatives may not be lost because their λ_{max} values of UV absorption are almost the same as that of MY (Table 1). The enhancement of AhR ligand activity by halogen-substitution at the *ortho* position of the –N=N– or –NH–CO– bond in MY and BA might be due to an electronic effect (*i.e.*, inductive effect) of the halogen substituent(s) on ligand recognition by AhR. However, further studies are needed to better understand the mechanism of potentiation of the AhR ligand activity by *ortho* halogen-substitution in MY and BA.

The present study confirmed that the suitable size and planar structure of a molecule are important for the AhR ligand activity in MY and related congeners. It is expected that determination of the characteristic structure of AhR ligands described in the present study will be useful for prediction of the toxicity of some structurally related compounds.

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