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SYNTHETIC MODELS RELATED TO METHOXALEN – CYP2A6 INTERACTIONS. DIMETHOXYBENZOFURAN DERIVATIVES AS POTENT AND SELECTIVE INHIBITORS OF CYP2A6

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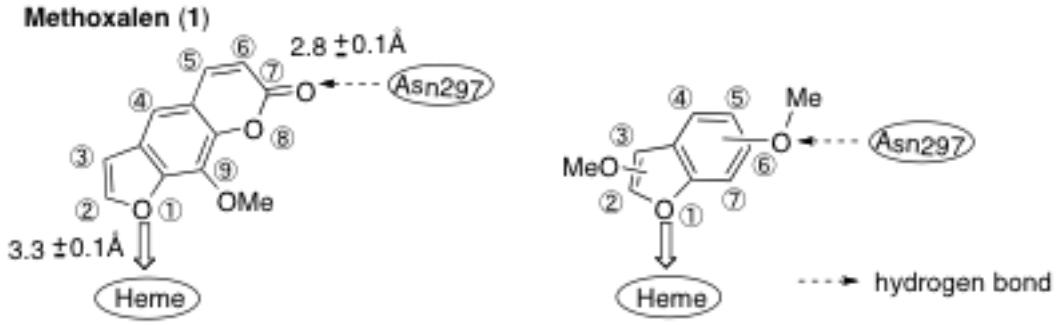
Abstract – The human CYP2A6 enzyme metabolizes several xenobiotics including nicotine, the addictive component in tobacco. Reduced activity of CYP2A6, either for genetic reasons or by administering inhibitors of CYP2A6, reduces tobacco smoking. The reported compound methoxalen had a potent inhibitory effect on activity of CYP2A6 with an IC_{50} value of 1.27 μM. We selected methoxalen as a lead compound and prepared various dimethoxybenzofuran derivatives that have inhibitory effects on activity of human cytochrome P450 (CYP) 2A6. Synthetic benzofuran derivatives (3,6-dimethoxybenzofuran: IC_{50}=1.92 μM and 3,7-dimethoxybenzofuran: IC_{50}=2.00 μM) also exhibited comparable activities against CYP2A6 and were selective inhibitors of CYP2A6. These compounds can be used as lead compounds in the development of drugs for smoking reduction therapy.

Nicotine, the primary component present in tobacco, plays a significant role in establishing and maintaining tobacco dependence. CYP2A6 is known to be responsible for the metabolism of nicotine to cotinine in humans, approximately 70-80% of nicotine being metabolized by CYP2A6 to cotinine.[1] CYP2A6 does not seem to have an extensive role in human drug metabolism, but it has been shown to be involved in the mutagenic activation of promutagens such as the tobacco-specific nitrosoamine, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK).[2] It has also been shown that methoxalen (9-methoxyfuranocoumarin), a potent human CYP2A6 inhibitor, is a strong chemopreventive agent against NNK induction of lung tumorigenesis.[3] Pharmacogenomic studies have suggested that male smokers completely lacking CYP2A6 were more resistant to lung cancer.[4] In addition, inhibition of

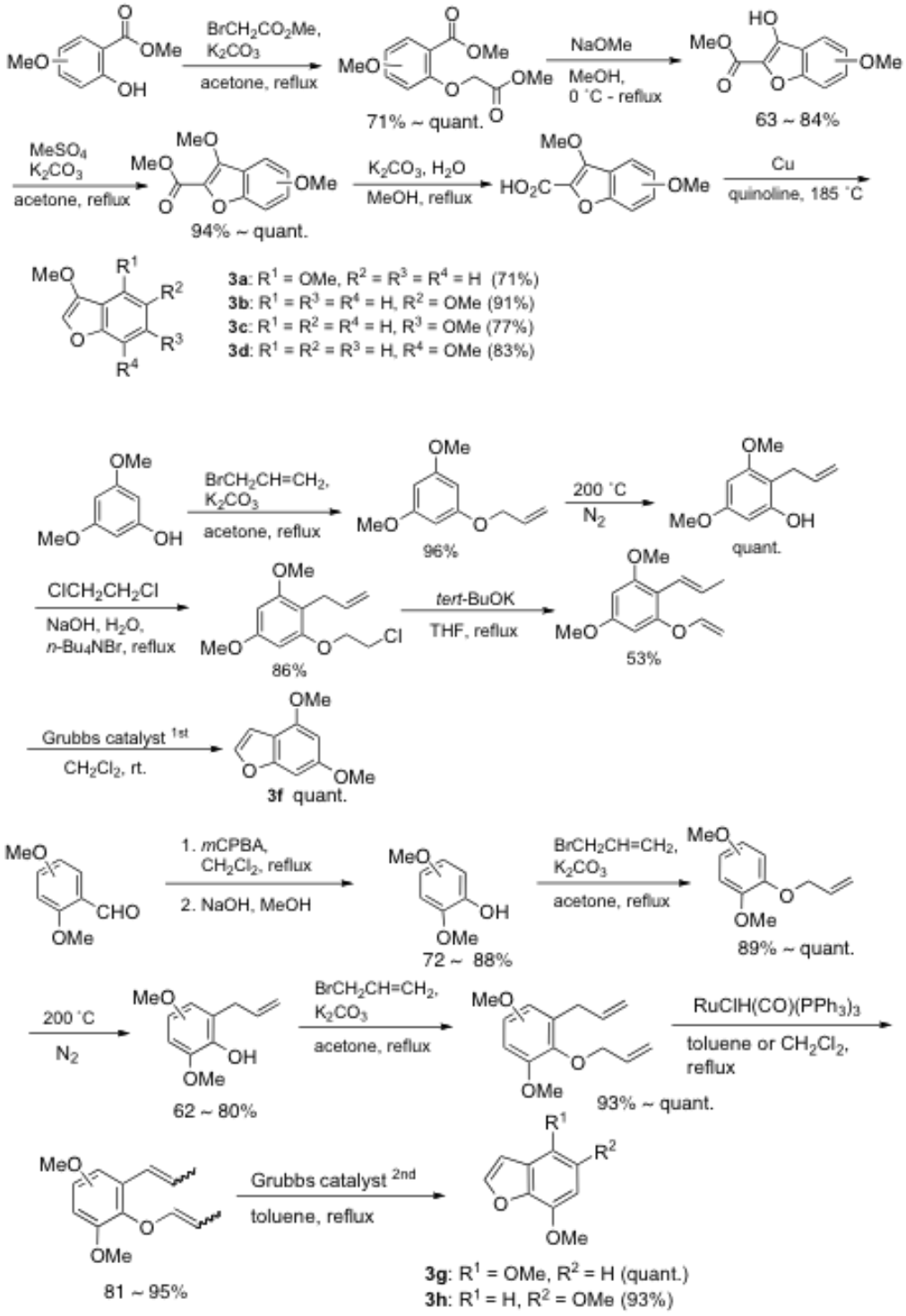
CYP-mediated metabolism would lead to slower elimination of nicotine from the bodies of smokers and, consequently, to a decrease in the number of cigarettes needed to maintain a constant level of nicotine in the body. This decrease in smoking would decrease the exposure to carcinogenic nitrosoamine.[5] For this reason, CYP2A6 inhibitors have been proposed as novel targets for reducing tobacco-related cancer risk. An ideal drug candidate for use as a CYP2A6 inhibitor and a smoking cessation agent must be highly potent and selective to avoid undesirable effects.[5]

Although a number of compounds, including methoxalen, have been used as inhibitors of CYP2A6, these compounds generally lack selectivity for targeting CYP2A6 and many of these compounds also inhibit activities of other drug-metabolizing enzymes (such as CYP3A4) and therefore can result in untoward drug-drug interactions.[6] For example, methoxalen (**1**) showed an IC_{50} value for CYP3A4 of 5.46 μm.[6] Furthermore, menthofuran ((*R*)-4,5,6,7-tetrahydro-3,6-dimethylbenzofuran), an excellent selective inhibitor of CYP2A6, has disadvantage in toxicity.[7] We selected a benzofuran ring system that has the partial structure common to two CYP2A6 inhibitors (methoxalen and menthofuran). Herein we report a synthesis of dimethoxybenzofuran derivatives that have a simple ring system than that of furanocoumarin derivatives and present results of assays of their inhibitory effects on CYP2A6 activity with a view to defining the relationship between structures and inhibitory effects on CYP2A6 activity.

To better understand the binding methoxalen to CYP2A6, Johnson *et al.* determined the structure of the enzyme by X-ray crystallography.[8] The CYP2A6 structure shows a compact, hydrophobic active site with one hydrogen bond donor, Asn297. In the crystal structure, Asn297 acts as a hydrogen donor to the carbonyl oxygen of methoxalen, and the oxygen of the furan ring is closest to the heme iron in the active site of CYP2A6 for reigioselective oxidation.[8] We also found that bergapten **2** (methoxy group at the 4-position on the furanocoumarin ring) showed a much weaker inhibitory effect on CYP2A6 than that of methoxalen **1**, indicating that the potency of inhibition is also very sensitive to location of the methoxy group on the furanocoumarin ring.[9] We selected methoxalen (**1**) as a lead compound and prepared various dimethoxybenzofuran derivatives. We expected that the methoxy group on the benzofuran ring would act as a hydrogen acceptor of Asn297 instead of carbonyl oxygen of methoxalen (**1**) (Scheme 1). A series of benzofuran derivatives (**3a** ~ **3h**) derivatives were prepared as shown in Scheme 2.



Scheme 1

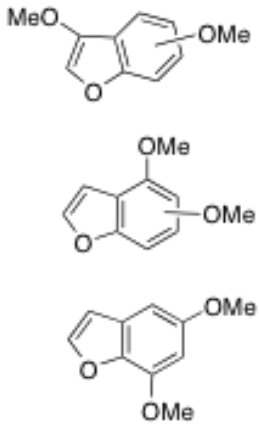


Scheme 2

3,4-Dimethoxybenzofuran (**3a**) was synthesized from methyl 2-hydroxy-6-methoxybenzoate.[10] Methyl 2-hydroxy-6-methoxybenzoate was converted into ether using methyl bromoacetate and K_2CO_3. This compound treated with sodium methoxide in methanol to give 3-hydroxybenzofuran derivatives in 83% yield. Reaction of 3-hydroxybrnzofuran with dimethyl sulfate followed by hydrolysis and copper-catalyzed decarboxylation gave 3,4-dimethoxybenzofuran (**3a**). Dimethoxybenzofuran derivatives

(**3b**~**d**) were similarly prepared. 4,5-Dimethoxybenzofuran (**3e**) was prepared from 3-hydroxy-4-methoxybenzaldehyde by the reported procedure.[11] 4,6-Dimethoxybenzofuran (**3f**) was synthesized from 3,5-dimethoxyphenol by an isomerization-ring-closing metathesis strategy.[12] 3,5-Dimethoxyphenol was converted into allyl ether using allyl bromide and K_2CO_3. Subsequent Claisen rearrangements gave the corresponding phenol quantitatively. This phenol was alkylated with 1,2-dichloroethane followed by treatment with potassium *tert*-butoxide to give an aryl enol ether. The aryl enol ether was cyclized to give 4,7-dimethoxybenzofuran by using first-generation Grubbs metathesis catalyst. 4,7-Dimethoxybenzofuran (**3g**) was synthesized from 2,5-dimethoxyphenol (prepared from 2,5-dimethoxybenzaldehyde by Baeyer-Villiger oxidation) by an isomerization-ring-closing metathesis strategy.[11] 2,5-Dimethoxyphenol was converted into allyl ether using allyl bromide and K_2CO_3. Subsequent Claisen rearrangements gave the corresponding phenol in good yield. This phenol was re-allylated to afford 1-allyl-2-allyloybenzene. The two allyl groups were isomerized with ruthenium-based catalyst [RuClH(CO)(PPh_3)$_3$] to give an aryl enol ether.[13] This compound was not characterized further and was used directly in the next reaction. The aryl enol ether was cyclized to give 4,7-dimethoxybenzofuran quantitatively by using the second-generation Grubbs metathesis catalyst. 5,7-Dimethoxybenzofuran (**3h**) was similarly prepared from 2,4-dimethoxyphenol.

Assays of inhibition of CYP2A6 activity by benzofuran derivatives were based on microsomal coumarin 7-hydroxylation.[14] The results are shown in Scheme 3 and Table 1.



3a: 3,4-dimethoxy
3b: 3,5-dimethoxy
3c: 3,6-dimethoxy
3d: 3,7-dimethoxy
3e: 4,5-dimethoxy
3f: 4,6-dimethoxy
3g: 4,7-dimethoxy
3h: 5,7-dimethoxy

Scheme 3

Table 1. IC_{50} Values of Benzofuran Derivatives for Coumarin 7-Hydroxylation

Compounds	CYP2A6 IC_{50} (μM)
Methoxalen	1.27
3a	> 10
3b	> 10
3c	1.92
3d	2.00
3e	7.51
3f	> 10
3g	9.29
3h	5.16

At first, the inhibitory activities of 3-methoxybenzofuran derivatives (**3a**, **3b**, **3c**, and **3d**) were compared. Introduction of a methoxy group at the 6 (**3c**) or 7 (**3d**) position on the benzofuran ring resulted in significantly increased inhibitory potency (1.92 μM and 2.00 μM, respectively), whereas the same substituent in the 4 (**3a**) or 5 (**3b**) position gave rise to low potency (> 10 μM) of CYP2A6 inhibition. Next, the inhibitory activities of 4-methoxybenzofuran derivatives (**3e**, **3f**, and **3g**) were examined.

Though **3e** and **3f** showed moderate potency of CYP2A6 inhibition (7.51 μM and 9.29 μM, respectively), none of the derivatives exhibited activities comparable to those of **3c** and **3d** against CYP2A6, indicating that the existence of a methoxy group at the 3-position of benzofuran is important for potency of CYP2A6 inhibition. 4,6-Dimethoxybenzofuran (**3f**) showed poor inhibitory activity against CYP2A6. Finally, CYP2A6 inhibition by 5,7-dimethoxybenzofuran (**3h**) was studied. 5,7-Dimethoxybenzofuran (**3h**) also showed moderate inhibitory activity against CYP2A6. It was found that the potency of inhibition was very sensitive to location of the methoxy group on the benzofuran ring.

In this experiment, 8 candidate molecules were selected and tested for inhibition potency. 3,6-Dimethoxyethoxybenzofuran **3c** and 3,7-dimethoxybenzofuran **3d** were most potent as CYP2A6 inhibitors with IC_{50} values of 1.92 μM and 2.00 μM, respectively. These results and our findings provide a clue for elucidating the relationship between structures and inhibitory effects on CYP2A6 activity. As previously described, in the crystal structure, Asn297 acts as a hydrogen donor to the carbonyl oxygen of methoxalen, and the oxygen of the furan ring is closest to the heme iron in the active site of CYP2A6.[7] A hydrogen bond with Asn297 and van der Waals interaction between the hydrophobic amino acids and inhibitor are important determinants of inhibition potency. We speculated that the presence of a hydrogen bond between a 6- or 7-methoxy group on the benzofuran ring and Asn297 in the active site of CYP2A6 has an effect on inhibition potency. Furthermore, Rahnasto *et al.* reported that the crystal structure shows that electron-rich regions (furan ring of methoxalen) in suicide inhibitors coordinate to the heme iron.[15] Thus, the 3-methoxy group, an electron-donating substituent, on the furan ring in **3c** and **3d** is also a determinant of inhibition potency.

In order to further explore the selectivity of CYP 2A6 inhibition, compounds **3c** and **3d** – a selection of the most potent inhibitors of CYP2A6 among compounds examined in this study – were tested for their inhibitory potency for the most prominent drug-metabolizing enzyme CYP3A4. Both of them (**3c** and **3d**) showed IC_{50} values on CYP3A4 of > 10 μm.[16]

A number of compounds, including methoxalen, have been reported to be inhibitors of CYP2A6. However, these compounds generally lack selectivity for targeting CYP2A6, and many also inhibit other drug-metabolizing enzymes such as CYP3A4A.[6] Fortunately, of the compounds examined, compounds **3c** and **3d** were found to have highly selective inhibitory potency for CYP2A6. These compounds may serve as lead structures to develop even more potent and selective inhibitors of CYP2A6 that are more stable and accessible than methoxalen and have comparable activity against CYP2A6.

There is considerable interest in development of clinically useful inhibitors of the CYP2A6 enzyme. None of the currently known CYP2A6 inhibitors are suitable for routine clinical use, and thus there is a need to develop novel inhibitors with suitable potency, selectivity and toxicity profiles. The dose-response relationship between the number of cigarettes smoked and development of related cancer was established many years ago by epidemiological studies.[17] Reducing the number of cigarettes smoked

and decreasing the toxicity and carcinogenicity of tobacco will result in less tobacco-related cancer risk.

EXPERIMENTAL

All melting points were determined using a Yamato melting point apparatus (MP-J3) and are uncorrected. NMR spectra were obtained using JEOL JNM-ECA 500 spectrometers. The chemical shifts are reported in ppm (δ) relative to TMS (0.0 ppm) as the internal standard. MS spectra (MS, HRMS) were obtained using a Shimadzu GC MS 9100-MK gas chromatograph-mass spectrometer. Column chromatography was conducted using silica gel (Merck, Kieselgel 60, 70-230 mesh).

Preparation of inhibitors

General procedure for preparation of 3-methoxybenzofuran derivatives (**3a**, **3b**, **3c**, and **3d**)

Appropriate phenols and methyl bromoacetate were refluxed for 4 h in the presence of K_2CO_3 to give diesters.

Methyl 2-methoxy-6-(2-methoxy-2-oxoethoxy)benzoate. mp 63-64 ºC. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.77 (3H, s), 3.82 (3H, s), 3.92 (3H, s), 4.64 (2H, s), 6.43 (1H, d, J = 8.0 Hz), 6.60 (1H, d, J = 8.0 Hz), 7.26 (1H, t, J = 8.0 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 52.3, 52.6, 56.2, 66.3, 105.1x2, 114.0, 131.2, 155.8, 157.6, 166.6, 169.1. ESI-LRMS *m/z*: 277 (MNa^+). ESI-HRMS *m/z*: 277.0689 (Calcd for $C_{12}H_{14}NaO_6$: 277.0688).

Methyl 5-methoxy-2-(2-methoxy-2-oxoethoxy)benzoate. mp 81-83 ºC (lit.[17] 82-85 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.79 (6H, s), 3.90 (3H, s), 4.66 (2H, s), 6.91 (1H, d, J = 9.2 Hz), 6.99 (1H, dd, J = 2.9, 9.2 Hz), 7.35 (1H, d, J = 2.9 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 52.3, 52.4, 55.9, 68.4, 116.0, 117.8, 119.7, 122.4, 151.8, 154.5, 166.2, 169.5. ESI-LRMS *m/z*: 277 (MNa^+). Its spectral data are in accordance with previously reported data.[18]

Methyl 4-methoxy-2-(2-methoxy-2-oxoethoxy)benzoate. Yellow oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.80 (3H, s), 3.82 (3H, s), 3.86 (3H, s), 4.70 (2H, s), 6.39 (1H, d, J = 2.3 Hz), 6.56 (1H, dd, J = 2.3, 9.2 Hz), 7.88 (1H, d, J = 9.2 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 51.9, 52.4, 55.6, 66.7, 101.4, 106.3, 113.4, 134.2, 159.6, 164.1, 165.9, 169.0. Its spectral data are in accordance with previously reported data.[19]

Methyl 3-methoxy-2-(2-methoxy-2-oxoethoxy)benzoate. mp 71-73 ºC (lit.,[18] 75.5 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.80 (3H, s), 3.85 (3H, s), 3.88 (3H, s), 4.68 (2H, s), 7.05 (1H, dd, J = 1.8, 8.0 Hz), 7.11 (1H, t, J = 8.0 Hz), 7.31 (1H, dd, J = 1.8, 8.0 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 52.1, 52.4, 56.2, 69.9, 115.9, 122.3, 124.6, 126.5, 146.7, 153.1, 166.6, 169.8. EI-LRMS *m/z*: 254 (M^+). Its spectral data are in accordance with previously reported data.[20]

Diester and NaOMe were refluxed in MeOH to give 3-hydroxybenzofuran derivatives. These compounds were not characterized further and were used directly in the next reaction. 3-Hydroxybenzofuran derivatives and Me_2SO_4 were refluxed in the presence of K_2CO_3 to give 3-methoxybenzofuran

derivatives.

Methyl 3,4-dimethoxybenzofuran-2-carboxylate. mp 77-78 ºC (lit.,[9] 70 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.95 (3H, s), 3.96 (3H, s), 4.10 (3H, s), 6.65 (1H, d, J = 8.0 Hz), 7.09 (1H, d, J = 8.6 Hz), 7.35 (1H, dd, J = 8.0, 8.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 52.1, 56.0, 63.2, 103.6, 105.6, 113.0, 129.5, 131.7, 150.0, 154.8, 154.9, 159.8. ESI-LRMS *m/z*: 259 (MNa^+). Its physical and spectral data are in accordance with previously reported data.[1]

Methyl 3,5-dimethoxybenzofuran-2-carboxylate. mp 106-109 ºC (lit.,[10] 109 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.85 (3H, s), 3.96 (3H, s), 4.23 (3H, s), 7.07 (1H, dd, J = 2.3, 9.2 Hz), 7.12 (1H, d, J = 2.3 Hz), 7.38 (1H, d, J = 9.2 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 52.1, 56.0, 61.9, 101.8, 113.7, 118.7, 122.6, 131.8, 148.4, 149.9, 156.1, 159.8. ESI-LRMS *m/z*: 259 (MNa^+). Its physical and spectral data are in accordance with previously reported data.[10]

Methyl 3,6-dimethoxybenzofuran-2-carboxylate. mp 69-70 ºC (lit.,[10] 70 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.85 (3H, s), 3.95 (3H, s), 4.26 (3H, s), 6.89 (1H, dd, J = 2.3, 8.6 Hz), 6.95 (1H, d, J = 2.3 Hz), 7.65 (1H, d, J = 8.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 51.9, 55.8, 61.4, 96.0, 113.7, 115.1, 121.9, 129.7, 150.2, 155.0, 159.9, 161.2. ESI-LRMS *m/z*: 259 (MNa^+). Its physical and spectral data are in accordance with previously reported data.[10]

Methyl 3,7-dimethoxybenzofuran-2-carboxylate. mp 68-70 ºC (lit.,[10] 68 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.95 (3H, s), 3.99 (3H, s), 4.26 (3H, s), 6.91 (1H, d, J = 8.0 Hz), 7.18 (1H, t, J = 8.0 Hz), 7.34 (1H, d, J = 8.0 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 52.0, 56.0, 61.6, 109.3, 113.0, 123.6, 123.9, 130.9, 143.1, 146.2, 150.0, 159.8. ESI-LRMS *m/z*: 259 (MNa^+). Its physical and spectral data are in accordance with previously reported data.[10]

3-Methoxybenzofuran derivatives and K_2CO_3 were refluxed in aqueous MeOH to give benzofuran-2-carboxylic acid derivatives.

3,4-Dimethoxybenzofuran-2-carboxylic acid. mp 214-216 ºC (lit.,[10] 218 ºC). ^{1}H-NMR (500 MHz, acetone-d_6) δ: 3.97 (3H, s), 4.03 (3H, s), 6.81 (1H, d, J = 8.0 Hz), 7.08 (1H, d, J = 8.6 Hz), 7.42 (1H, dd, J = 8.0, 8.6 Hz). ^{13}C-NMR (125 MHz, acetone-d_6) δ: 55.4, 62.3, 103.9, 105.0, 112.9, 129.6, 132.3, 149.5, 154.7, 154.9, 159.1. ESI-LRMS *m/z*: 245 (MNa^+). ESI-HRMS *m/z*: 245.0440 (Calcd for $C_{11}H_{10}NaO_5$: 245.0426). Its physical and spectral data are in accordance with previously reported data.[10]

3,5-Dimethoxybenzofuran-2-carboxylic acid. mp 181-184 ºC (lit.,[10] 184 ºC). ^{1}H-NMR (500 MHz, acetone-d_6) δ: 2.99 (1H, br s), 3.85 (3H, s), 4.18 (3H, s), 7.10 (1H, dd, J = 2.3, 9.2 Hz), 7.22 (1H, d, J = 2.3 Hz), 7.42 (1H, d, J = 9.2 Hz). ^{13}C-NMR (125 MHz, acetone-d_6) δ: 55.4, 61.3, 101.7, 113.3, 118.5, 122.8, 132.3, 148.1, 149.5, 156.4, 159.2. ESI-LRMS *m/z*: 245 (MNa^+). Its physical and spectral data are in accordance with previously reported data.[10]

3,6-Dimethoxybenzofuran-2-carboxylic acid. mp 174-177 ºC (lit.,[10] 177ºC). ^{1}H-NMR (500 MHz, acetone-d_6) δ: 2.97 (1H, br s), 3.87 (3H, s), 4.21 (3H, s), 6.92 (1H, dd, J = 2.3, 8.6 Hz), 7.05 (1H, d, J =

2.3 Hz), 7.72 (1H, d, J = 8.6 Hz). ^{13}C-NMR (125 MHz, acetone-d_6) δ: 55.3, 61.0, 95.7, 113.6, 115.2, 121.8, 130.2, 149.9, 154.8, 159.2, 161.4. ESI-LRMS *m/z*: 245 (MNa^+). Its physical and spectral data are in accordance with previously reported data.[10]

3,7-Dimethoxybenzofuran-2-carboxylic acid. mp 216-218 ºC (lit.,[10] 216 ºC). ^{1}H-NMR (500 MHz, acetone-d_6) δ: 3.09 (1H, br s), 3.97 (3H, s), 4.20 (3H, s), 7.07 (1H, d, J = 8.0 Hz), 7.22 (1H, t, J = 8.0 Hz), 7.37 (1H, d, J = 8.0 Hz). ^{13}C-NMR (125 MHz, acetone-d_6) δ: 55.5, 61.2, 109.7, 112.7, 123.9, 124.0, 131.4, 142.9, 146.3, 149.6, 159.2. ESI-LRMS *m/z*: 245 (MNa^+). Its physical and spectral data are in accordance with previously reported data.[10]

Dimethoxybenzofuran-2-carboxylic acid derivatives and Cu powder were heated at 185 °C in quinoline to convert appropriate dimethoxybanzofurans (**3a** ~ **3d**).

3,4-Dimethoxybenzofuran (**3a**). mp 95-97 ºC (lit.,[10] 99 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.88 (3H, s), 3.96 (3H, s), 6.63 (1H, d, J = 8.0 Hz), 7.01 (1H, d, J = 8.0 Hz), 7.16 (1H, s), 7.20 (1H, t, J = 8.0 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 56.0, 58.8, 103.0, 105.2, 111.2, 123.3, 125.8, 145.6, 154.1, 155.4. EI-LRMS *m/z*: 178 (M^+). Its physical and spectral data are in accordance with previously reported data.[10]

3,5-Dimethoxybenzofuran (3b). Color less oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.81 (3H, s), 3.86 (3H, s), 6.89 (1H, dd, J = 2.9, 9.2 Hz), 6.99 (1H, d, J = 2.9 Hz), 7.28 (1H, d, J = 9.2 Hz), 7.49 (1H, s). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 56.0, 58.2, 100.5, 112.4, 114.4, 122.0, 125.3, 145.6, 148.8, 155.6. EI-LRMS *m/z*: 178 (M^+). Its spectral data are in accordance with previously reported data.[10]

3,6-Dimethoxybenzofuran (3c). mp 73-76 ºC (lit.,[9] 76 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.83 (3H, s), 3.85 (3H, s), 6.85 (1H, dd, J = 2.3, 8.6 Hz), 6.90 (1H, d, J = 2.3 Hz), 7.16 (1H, s), 7.43 (1H, d, J = 8.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 55.8, 58.2, 96.1, 111.5, 115.3, 119.0, 123.5, 145.6, 154.9, 158.7. EI-LRMS *m/z*: 178 (M^+). Its physical and spectral data are in accordance with previously reported data.[10]

3,7-Dimethoxybenzofuran (3d). mp 42-45 ºC (lit.,[10] 45 ºC). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.86 (3H, s), 3.93 (3H, s), 6.88 (1H, dd, J = 1.7, 6.9 Hz), 7.09-7.13 (2H, m), 7.51 (1H, s). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 56.1, 58.3, 106.9, 111.1, 123.0, 123.4, 124.7, 143.2, 145.5, 145.8. EI-LRMS *m/z*: 178 (M^+). Its physical and spectral data are in accordance with previously reported data.[10]

Procedure for preparation of 4,6-dimethoxybenzofuran (**3f**)

3,5-Dimethoxyphenol and allyl bromide were refluxed in the presence of K_2CO_3 for 2 h to give the allyloxybenzene derivative.

1-(Allyloxy)-3,5-dimethoxybenzene. Yellow oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.76 (6H, s), 4.49 (2H, d, J = 5.7 Hz), 5.28 (1H, m), 5.41 (1H, m), 6.01-6.10 (4H, m). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 55.4, 69.0, 93.2, 93.7, 117.9, 133.2, 160.6, 161.6. EI-LRMS *m/z*: 194 (M^+). EI-HRMS *m/z*: 194.0940 (Calcd for $C_{11}H_{14}O_3$: 194.0943).

Allyloxybenzene was heated at 200 °C for 45 min to give the 2-allylphenol derivative.

2-Allyl-3,5-dimethoxyphenol. Yellow oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.38 (2H, dt, J = 1.7, 6.3 Hz),

3.76 (3H, s), 3.77 (3H, s), 5.06-5.14 (3H, m), 5.99 (1H, m), 6.08 (1H, d, J = 2.3 Hz), 6.10 (1H, d, J = 2.3 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 27.1, 55.4, 55.8, 91.7, 93.8, 105.7, 115.4, 136.9, 156.0, 158.8, 159.8. EI-LRMS *m/z*: 194 (M^+). EI-HRMS *m/z*: 194.0945 (Calcd for $C_{11}H_{14}O_3$: 194.0943).

This phenol and 1,2-dichloroethane were refluxed in the presence of TBAB and NaOH for 1.5 h to give 2-allyl-1-(2-chloroethoxy)-3,5-dimethoxybenzene.

2-Allyl-1-(2-chloroethoxy)-3,5-dimethoxybenzene. Colorless oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.34 (2H, dt, J = 1.7, 6.3 Hz), 3.78-3.80 (8H, m), 4.19 (2H, t, J = 6.0 Hz), 4.89-4.98 (2H, m), 5.92 (1H, m), 6.09 (1H, d, J = 2.3 Hz), 6.16 (1H, d, J = 2.3 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 27.1, 42.1, 55.5, 55.9, 68.7, 91.6, 91.9, 109.9, 113.9, 137.3, 157.3, 159.0, 159.5. EI-LRMS *m/z*: 256 (M^+). EI-HRMS *m/z*: 256.0865 (Calcd for $C_{13}H_{17}ClO_3$: 256.0866).

2-Allyl-1-(2-chloroethoxy)-3,5-dimethoxybenzene and potassium *tert*-butoxide were refluxed in THF for 30 min under N_2 to give the propenyl vinylbenzene derivative.

(*E*)-1,5-Dimethoxy-2-(prop-1-en-1-yl)-3-(vinyloxy)benzene. Colorless oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.89 (3H, d, J = 4.6 Hz), 3.78 (3H, s), 3.82 (3H, s), 4.39 (1H, dd, J = 1.8, 6.0 Hz), 4.68 (1H, dd, J = 1.8, 13.8 Hz), 6.17 (1H, d, J = 2.3 Hz), 6.24 (1H, d, J = 2.3 Hz), 6.45-6.47 (2H, m), 6.54 (1H, m). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 20.1, 55.5, 55.8, 94.2, 94.6, 95.8, 110.5, 120.5, 129.1, 149.0, 155.5, 159.0, 159.1. EI-LRMS *m/z*: 220 (M^+). EI-HRMS *m/z*: 220.1100 (Calcd for $C_{13}H_{16}O_3$: 220.1099).

The aryl enol ether and the first-generation Grubbs catalyst (5% mol) were refluxed for 2 h to give 4,7-dimethoxybenzofuran.

4,6-Dimethoxybenzofuran (3f). Colorless oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.84 (3H, s), 3.90 (3H, s), 6.33 (1H, d, J = 2.3 Hz), 6.67 (1H, m), 6.78 (1H, m), 7.46 (1H, d, J = 2.3 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 55.6, 55.8, 88.3, 94.3, 103.9, 111.4, 142.6, 153.7, 156.8, 159.2. EI-LRMS *m/z*: 178 (M^+). EI-HRMS *m/z*: 178.0633 (Calcd for $C_{10}H_{10}O_3$: 178.0630).

General procedure for preparation of 7-methoxybenzofuran derivatives (**3g** and **3h**)

Appropriate dimethoxybenzaldehydes and *m*-chloroperbenzoic acid were refluxed in CH_2Cl_2 for 14 h to give dimethoxyphenols.

2,5-Dimethoxyphenol. Colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.74 (3H, s), 3.84 (3H, s), 5.68 (1H, s), 6.37 (1H, dd, J = 3.4, 9.2 Hz), 6.56 (1H, d, J = 3.4 Hz), 6.77 (1H, d, J = 9.2 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 55.7, 56.7, 101.8, 104.3, 111.5, 141.0, 146.5, 154.6. Its spectral data are in accordance with previously reported data.[21]

2,4-Dimethoxyphenol. Colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.76 (3H, s), 3.86 (3H, s), 5.23 (1H, s), 6.38 (1H, dd, J = 2.8, 8.6 Hz), 6.49 (1H, d, J = 2.8 Hz), 6.82 (1H, d, J = 8.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 55.9, 56.0, 99.5, 104.3, 114.2, 139.9, 147.1, 153.6. Its spectral data are in accordance with previously reported data.[22]

The corresponding phenols and allyl bromide were refluxed in the presence of K_2CO_3 for 20 h to give the

allyloxybenzene derivative.

2-(Allyloxy)-1,4-dimethoxybenzene. Colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.75 (3H, s), 3.83 (3H, s), 4.59 (2H, dt, J = 1.7, 5.2 Hz), 5.27-5.42 (2H, m), 6.07 (1H, m), 6.40 (1H, dd, J = 2.9, 8.6 Hz), 6.52 (1H, d, J = 2.9 Hz), 6.79 (1H, d, J = 8.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 55.7, 56.7, 69.9, 102.2, 103.6, 112.5, 118.1, 133.3, 143.9, 148.9, 154.2. Its spectral data are in accordance with previously reported data.[12]

1-(Allyloxy)-2,4-dimethoxybenzene. Yellow oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.76 (3H, s), 3.84 (3H, s), 4.54 (2H, dd, J = 1.7, 4.6 Hz), 5.26-5.39 (2H, m), 6.07 (1H, m), 6.36 (1H, dd, J = 2.3, 9.2 Hz), 6.50 (1H, d, J = 2.3 Hz), 6.81 (1H, d, J = 9.2 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 55.7, 55.9, 70.9, 100.5, 103.0, 114.8, 117.9, 133.8, 142.3, 150.6, 154.7. Its spectral data are in accordance with previously reported data.[23]

The corresponding allyloxybenzenes were heated at 200 °C for 1 h under N_2 to give 2-allylphenol derivatives.

2-Allyl-3,6-dimethoxyphenol. Yellow oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.34 (2H, d, J = 6.3 Hz), 3.77 (3H, s), 3.84 (3H, s), 4.95-5.04 (2H, m), 5.71 (1H, s), 5.99 (1H, m), 6.35 (1H, d, J = 8.6 Hz), 6.67 (1H, d, J = 8.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 27.6, 56.1, 56.5, 101.1, 108.2, 114.4, 115.0, 136.5, 141.2, 144.4, 152.6. Its spectral data are in accordance with previously reported data.[12]

2-Allyl-4,6-dimethoxyphenol. Yellow oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.37 (2H, d, J = 6.9 Hz), 3.75 (3H, s), 3.85 (3H, s), 5.04-5.11 (2H, m), 5.30 (1H, s), 5.99 (1H, m), 6.28 (1H, d, J = 2.9 Hz), 6.37 (1H, d, J = 2.9 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 34.2, 55.8, 56.1, 97.3, 105.5, 115.7, 125.7, 136.6, 137.5, 147.0, 153.0. Its spectral data are in accordance with previously reported data.[24]

The corresponding phenols and allyl bromide were refluxed in the presence of K_2CO_3 for 20 h to give 1-allyl-2-allyloybenzene derivatives.

2-Allyl-3-(allyloxy)-1,4-dimethoxybenzene. Yellow oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.42-3.44 (2H, m), 3.77 (3H, s), 3.80 (3H, s), 4.47-4.48 (2H, m), 4.92-4.96 (2H, m), 5.21 (1H, m), 5.38 (1H, m), 6.00 (1H, m), 6.10 (1H, m), 6.56 (1H, d, J = 9.2 Hz), 6.73 (1H, d, J = 9.2 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 28.5, 56.1, 56.3, 74.0, 105.7, 110.2, 114.5, 117.1, 123.3, 134.5, 137.1, 146.9, 147.4, 152.3. Its spectral data are in accordance with previously reported data.[12]

1-Allyl-2-(allyloxy)-3,5-dimethoxybenzene. Yellow oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.39 (2H, dt, J = 1.7, 6.9 Hz), 3.76 (3H, s), 3.81 (3H, s), 4.40 (2H, dt, J = 1.7, 6.9 Hz), 5.03-5.09 (2H, m), 5.19-5.37 (2H, m), 5.94 (1H, m), 6.08 (1H, m), 6.28 (1H, d, J = 2.9 Hz), 6.36 (1H, d, J = 2.9 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 34.6, 55.6, 55.8, 74.1, 98.3, 105.0, 115.8, 117.2, 134.3, 134.6, 137.2, 139.9, 153.5, 156.1. Its spectral data are in accordance with previously reported data.[12]

1-Allyl-2-allyloybenzenederivatives and $RuClH(CO)(PPh_3)_3$ (5% mol) were heated in degassed toluene for 22 h to give aryl enol ethers. These compounds were not characterized further and were used directly

in the next reaction. The aryl enol ethers and the second-generation Grubbs catalyst (5% mol) were refluxed for 20h under N_2 to afford dimethoxybenzofurans.

4,7-Dimethoxybenzofuran (**3g**). Yellow oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.89 (3H, s), 3.97 (3H, s), 6.53 (1H, d, J = 8.6 Hz), 6.70 (1H, d, J = 8.6 Hz), 6.86 (1H, d, J = 2.3 Hz), 7.55 (1H, d, J = 2.3 Hz). ^{13}C-NMR (500 MHz, $CDCl_3$) δ: 55.9, 56.6, 102.8, 104.5, 106.5, 119.5, 140.5, 144.0, 145.3, 147.7. EI-LRMS *m/z*: 178, EI-HRMS *m/z*: 178.0627 (Calcd for $C_{10}H_{10}O_3$: 178.0630). Its physical and spectral data are in accordance with previously reported data.[12]

5,7-Dimethoxybenzofuran (**3h**). Colorless oil, ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.83 (3H, s), 3.97 (3H, s), 6.45 (1H, d, J = 2.3 Hz), 6.63 (1H, d, J = 2.3 Hz), 6.69 (1H, d, J = 2.3 Hz), 7.58 (1H, d, J = 2.3 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 55.9, 56.1, 94.6, 97.1, 107.2, 128.8, 139.7, 145.5, 145.8, 156.9. EI-LRMS *m/z*: 178 (M^+). EI-HRMS *m/z*: 178.0630 (Calcd for $C_{10}H_{10}O_3$: 178.0630). (M^+). Its spectral data are in accordance with previously reported data.[12]

Assay of inhibition

Assays of inhibition of CYP2A6 activity (GENTEST Co. Human CYP2A6 + Cytochrome b5 + P450 reductase (Baculovirus)) by benzofuran and coumarin derivatives were based on microsomal coumarin 7-hydroxylation. The 7-hydroxylation of coumarin was used as the index of CYP2A6 activity. The rate of coumarin 7-hydroxylation was determined by the method of Yamazaki *et al*.[14] Expressed human CYP2A6 (0.5 pmol) was incubated with coumarin (final concentration of 1 μM) and an inhibitor (0.01-10 μM) at 37 °C in the presence of phosphate buffer (10 0 mM, pH 7.4), $NADP^+$ (0.8 mM), glucose-6-phosphate (7.9 mM), and glucose-6-phosphate dehydrogenase (1 unit/mL) in a total volume of 400 μL. The reaction mixture was preincubated for 2 min at 37 °C, and the reaction was started by the addition of an NADPH-generating system of the microsomal solution. The reaction was stopped after 6-min incubation with 50 μL of 60% $HClO_4$. After further mixing, the reaction mixture was centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was analyzed by the HPLC-fluorescence method (Ex 338 nm, Em 458 nm). The HPLC system consisted of a Hitachi model L7100 pump, Hitachi D-7500 detector, and Hitachi F-1050 fluorescence spectrophotometer.

Assays of inhibition of CYP3A4 activity (GENTEST Co. Human CYP3A4 + cytochrome b5 + P450 reductase (Baculovirus)) by benzofuran and coumarin derivatives were based on microsomal testosterone 6β-hydroxylation. The rate of testosterone 6β-hydroxylation was determined by the method of Guo *et al*.[16] Expressed human CYP3A4 (2.5 pmol) was incubated with testosterone (final concentration of 0.2 mM) and an inhibitor (0.01-10 μM) at 37 °C in the presence of phosphate buffer (100 mM, pH 7.4), $NADP^+$ (0.8 mM), glucose-6-phosphate (7.9 mM), and glucose-6-phosphate dehydrogenase (1 unit/mL) in a total volume of 500 μL. The reaction mixture was preincubated for 2 min at 37 °C, and the reaction was started by the addition of an NADPH-generating system of the microsomal solution. The reaction

was stopped after 15-min incubation with 3 mL of AcOEt. After 11α-hydroxy progesterone (internal standard) had been added, the reaction mixture was centrifuged for 5 min at 2,800 rpm. The supernatant was analyzed by the HPLC-UV method (240 nm). The HPLC system consisted of a Shimadzu model LC-10ATvp pump, Shimadzu C-R8A detector, and Shimadzu SPD-10Avp UV spectrophotometer.

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