Supplementary Information

TokyoGreen Derivatives as Specific and Practical Fluorescent Probes for UDP-Glucuronosyltransferase (UGT) 1A1

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Materials and Methods

Materials. General chemicals were of the best grade available, supplied by Tokyo Chemical Industries (Japan), Wako Pure Chemical (Japan), and Aldrich Chemical Co., and were used without further purification. DMSO used as a cosolvent was of fluorometric grade (Dojindo, Japan). Reagents for UGT assays (UGT Supersomes containing respective human recombinant isozymes and UGT Reaction Mix Solutions) were purchased from BD Biosciences. Human liver microsomes (Pool of Human Liver Microsomes Mixed Gender, Pool of 50, 20 mg/mL) were purchased from Xeno Tech.

Instruments. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA300 instrument (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR) or a JEOL JNM-LA400 instrument (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR); δ values are in ppm relative to tetramethylsilane. Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuToF for ESI. UV-visible spectra were obtained on a Shimadzu UV-1650. Fluorescence spectroscopic studies were performed on a Hitachi F4500 or JASCO FP-6500. For assays using microplates, a PerkinElmer EnVision multifunctional plate reader was used.

Fluorometric analysis. The slit width was 2.5 nm for both excitation and emission. The photon multiplier voltage was 700 V for the F4500 spectrofluorimeter, and 400 V for the FP-6500 spectrofluorimeter. Fluorescence quantum efficiencies of TokyoGreen (TG) derivatives were obtained by comparing the area under the emission spectrum of the test sample excited at 490 nm with that of a solution of fluorescein in 0.1 M NaOH

aq., which has a quantum efficiency of 0.85.[S1] Unless otherwise stated, measurements were performed at 25 °C.

HPLC analysis (Fig. S11 and S12). HPLC analysis was performed on an Inertsil ODS-3 column (4.6 x 250 mm, GL Sciences Inc., Japan) using an HPLC system composed of a pump (PU-980, JASCO, Japan) and a detector (MD-2015 or FP-2025, JASCO). The linear gradient (A/B = 80/20 to 20/80 over 30 min) was formed by mixing solvent A (0.1% TFA, H_2O) with solvent B (0.1% TFA, 80% acetonitrile, 20% H_2O). Flow rate was 1.0 mL/min.

LC/MS analysis (Fig. 1d and S1). A mixture of 2-Me TG (100 μM), UDP-glucuronic acid (2 mM), alamethicin (25 μg/mL), $MgCl_2$ (8 mM), and UGT1A1 Supersomes (0.5 mg/mL) in Tris-HCl buffer (0.1 M, pH 7.4) containing 10% DMSO (total volume 100 μL) was incubated at 37 °C for 4 h. An aliquot (25 μL) was taken and diluted with 0.1% formic acid (100 μL), and then protein was removed with an Amicon Ultracentrifuge kit (Millipore, cutoff 30 kDa). The filtrate (10 μL) was subjected to LC/MS analysis. LC/MS analysis was performed on a reverse-phase ODS column (Inertsil ODS-3, 3.0 x 250 mm, GL Sciences) fitted on a Agilent 1200 HPLC system equipped with a G6130 single quadrupole mass spectrometer, with a flow rate of 0.5 mL/min. Absorbance at 440 nm and MS signals (ESI− scan mode: *m/z* 200-1000) were monitored. A linear gradient of eluents A/B = 95/5 to 5/95 in 30 min was used (eluent A: 0.1% formic acid, eluent B: 80% acetonitrile, 20% water, 0.1% formic acid). Control samples were prepared by replacing UGT1A1 Supersomes with Control Supersomes.

UGT assay with separation (Fig. 1b, 1c, 5b, 5c, S2, S11, S12). A mixture of fluorescein or TokyoGreen derivative (150 μM), UDP-glucuronic acid (2 mM), alamethicin (25 μg/mL), $MgCl_2$ (8 mM), and UGT1A1 Supersomes (1.0 mg/mL) in Tris-HCl buffer (0.1 M, pH 7.5) containing 20% DMSO (total volume 200 μL) was incubated at 37 °C for the period described in each legend. The reaction was stopped by adding 6% acetic acid/acetonitrile (50 μL), and the resulting mixture was centrifuged for 5 min at 3000 rpm. The supernatant (100 μL) was taken, diluted with 0.3 M sodium phosphate buffer (pH 9.0) to 3.0 mL, and subjected to spectroscopic measurements.

Homogeneous UGT assay (Fig. 2, Table S1, Fig. S3, S4, S5). A mixture of a fluorescein or TokyoGreen derivative (1 μM for a fluorescent compound, and 10 μM for a non-fluorescent compound), UDP-glucuronic acid (2 mM), alamethicin (25 μg/mL), $MgCl_2$ (8 mM), and UGT1A1 Supersomes (0.1 mg/mL) in Tris-HCl buffer (0.1 M, pH 7.5) containing up to 1% DMSO (total volume 3.0 mL) was incubated in a quartz cuvette at 37 °C with stirring. Spectroscopic measurements were performed at every 1 or 10 seconds. Excitation and emission wavelengths were set to the maxima of each compound. The changes of fluorescence intensity or absorbance were normalized using the value at $t = 0$, and the reaction rates (v) were determined from the initial slope.

QSAR (Fig. S6). The lipophilicity parameters, π values,[S2] for benzene moieties were obtained from Bio-Loom.[S3] When the measured log P value of a relevant benzene derivative was not available, the log P value calculated with Bio-Loom was used. For example, the log P values of toluene and benzene are reported as 2.73 and 2.13,

respectively, in Bio-Loom. Therefore, the π value of a methyl moiety was calculated as being 0.60, as shown below.

$$\begin{aligned}\pi_{CH_3} &= \log P_{\text{toluene}} - \log P_{\text{benzene}} \\ &= 2.73 - 2.13 \\ &= 0.60\end{aligned}$$

The linear regression equation of the correlation between π_R and log v (Fig. S6) was as follows (calculated R^2 value is 0.61).

$$\log v = 0.81(\pm 0.17) \times \pi_R - 1.14(\pm 0.40)$$

Michaelis-Menten plot (Fig. S7). A mixture of 2-Me-4-OMe TG (0.1 ~ 4 μM), UDP-glucuronic acid (2 mM), alamethicin (25 μg/mL), $MgCl_2$ (8 mM), and UGT1A1 Supersomes (0.1 mg/mL) in Tris-HCl buffer (0.1 M, pH 7.5) containing less than 0.5% DMSO (total volume 0.6 mL) was incubated in a quartz cuvette at 37 °C. Fluorescence measurements (Ex. 490 nm / Em. 510 nm) were performed every second. The initial reaction rate (v) for each substrate concentration ([S]) was calculated by fitting the data to a linear equation. The relationship between [S] and v was plotted, and fitted to the classical Michaelis-Menten equation.

$$v = \frac{V_{\max} \times [S]}{[S] + K_m}$$

Detection limit of UGT1A1 (Fig. S8). The assay was performed on a plastic 96-well microplate (Costar, half area black plate). A mixture of 2-Me-4-OMe TG (0.2 μM), UDP-glucuronic acid (2 mM), alamethicin (25 μg/mL), $MgCl_2$ (8 mM), and UGT1A1 Supersomes (20 ng/mL to 2 μg/mL) in Tris-HCl buffer (0.1 M, pH 7.4) containing less than 0.1% DMSO (total volume 50 μL) was incubated for 4 h at 37 °C. Fluorescence measurement was then performed using an EnVision plate reader (PerkinElmer) with a

FITC filter set, and the average fluorescence intensity of each group was compared with that of the control group without UGT1A1.

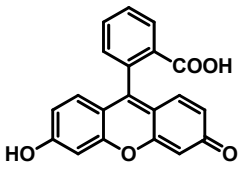
Isozyme specificity assay (Fig. 3a and S13). A mixture of 2-Me-4-OMe TG or 2-Me TG (1 μM), UDP-glucuronic acid (2 mM), alamethicin (25 μg/mL), $MgCl_2$ (8 mM), and different UGT Supersomes (0.05 mg/mL) in Tris-HCl buffer (0.1 M, pH 7.5) containing 10% DMSO (total volume 1.5 mL) was incubated in a quartz cuvette at 37 °C. Fluorescence measurements (Ex. 490 nm / Em. 510 nm) were performed every second, and the changes of fluorescence intensity over 100 s were plotted as relative reactivity.

Inhibition assay (Fig. 3b and S9). The assay was performed on a plastic 96-well microplate. A mixture of 2-Me-4-OMe TG (0.2 μM), UDP-glucuronic acid (2 mM), alamethicin (25 μg/mL), $MgCl_2$ (8 mM), inhibitor (0 ~ 100 μM), and UGT1A1 Supersomes (0.1 mg/mL) in Tris-HCl buffer (0.1 M, pH 7.5) containing less than 5% DMSO (total volume 0.2 mL) was incubated for 230 s at room temperature. Fluorescence measurements were performed using an EnVision plate reader (PerkinElmer) with a FITC filter set. Inhibition percentage was calculated using the data of positive and negative control samples (without inhibitors and without UGT1A1, respectively).

Assay of human liver microsomes (Fig. 4, and S10). A mixture of 2-Me-4-OMe TG (1 μM), UDP-glucuronic acid (2 mM), alamethicin (25 μg/mL), $MgCl_2$ (8 mM), a drug (atazanavir or propofol), and human liver microsomes (0.05 mg/mL) in Tris-HCl buffer (0.1 M, pH 7.5) containing 10% DMSO (total volume 0.5 mL) was incubated for 10

min in a quartz cuvette at 37 °C. Fluorescence intensity (Ex. 490 nm / Em. 510 nm) was measured every second, and the change over 100 s was divided by that of the control sample without a drug. Experiments were performed in triplicate. For comparison, assays using UGT1A1 (0.05 mg/mL) in place of microsomes were performed.

Chemical structures of the probes



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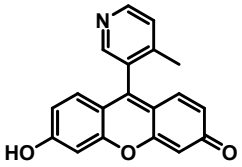

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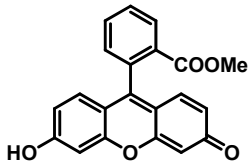

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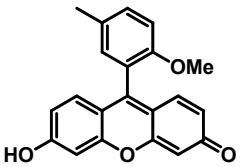

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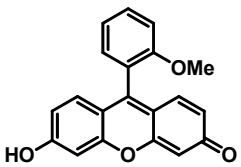

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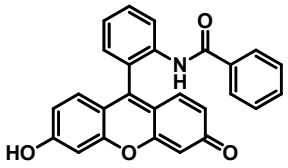

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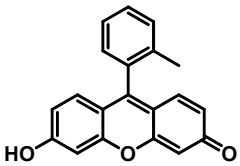

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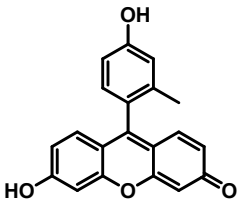

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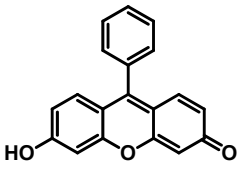

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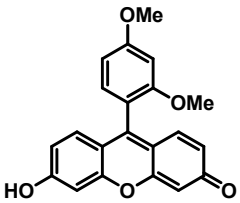

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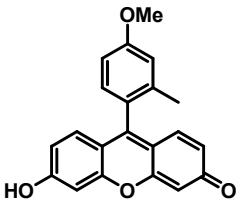

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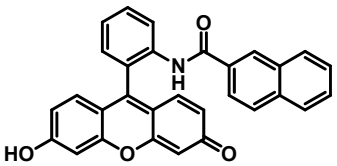

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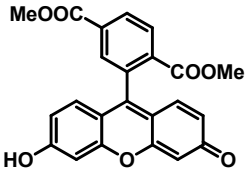

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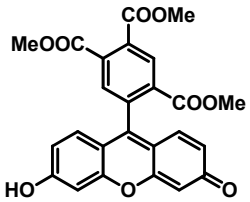

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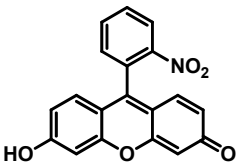

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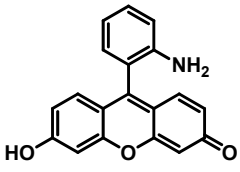

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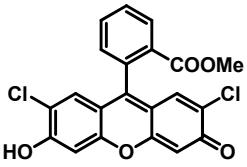

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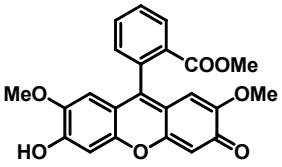

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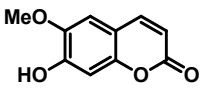
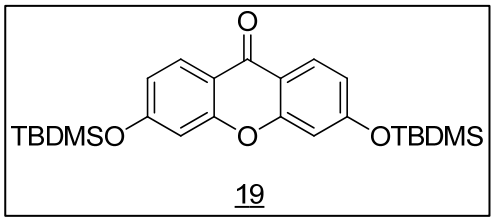
c.f.)



scopoletin

Synthesis of the compounds

Compounds 4, 5, 7, 9, 10, 11 and xanthone-diTBDMS (19) were synthesized as described in reference S4. Compounds 13 and 14 were synthesized as described in reference S5. Compounds 3, 17, 18 were synthesized as described in references S6, S7, and S8, respectively. Compound 8 was synthesized as described in reference S9. Synthetic procedures for compounds 2, 6, 12, 15, and 16 were as follows.



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Synthesis of 6-hydroxy-9-(4-methylpyridin-3-yl)-3*H*-xanthen-3-one (2)

To a solution of 3-bromo-γ-picoline (10 mmol) in distilled THF (15 mL) kept at -78 °C was added a 1.5 M *t*-BuLi solution in *n*-pentane (10 mL). The mixture was stirred at -78 °C for 15 min under an Ar atmosphere, then a solution of 19 (0.50 mmol) in distilled THF (10 mL) was added and stirring was continued for 30 min. The reaction was quenched by adding 2 N HCl aq. (10 mL), and the organic solvent was evaporated. The resulting mixture was washed 3 times with $CHCl_3$. Then, an excess of 2 N NaOH aq. was added and the mixture was washed 3 times with $CHCl_3$. Saturated NaH_2PO_4 solution was added to adjust the pH to ca. 5, and the resulting yellow precipitate was collected by filtration. The solid was washed with a small amount of sodium phosphate buffer (pH 5), and dried in vacuo to obtain 2 as a yellow powder (yield 17%).

^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.19 (s, 3H), 6.70 (m, 4H), 7.01 (d, 2H, J = 10.5 Hz), 7.57 (d, 1H, J = 5.4 Hz), 8.40 (s, 1H), 8.64 (d, 1H, J = 5.4 Hz). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 18.6, 103.6, 125.3, 128.9, 129.8, 145.4, 150.3. HRMS (ESI$^+$): m/z calcd for $[M+H]^+$; 304.0973, found; 304.0955.

Synthesis of 6-hydroxy-9-(2-nitrophenyl)-3*H*-xanthen-3-one (15)

To a solution of 2-nitrobromobenzene (6.0 mmol) in distilled THF (15 mL) kept at -78 °C was added a solution of 1.5 M phenyllithium solution in *n*-pentane (10 mL). The mixture was stirred at -78 °C for 30 min under an Ar atmosphere, then a solution of 19 (1.0 mmol) in distilled THF (10mL) was added and stirring was continued for 90 min. The reaction was quenched by adding 2 N HCl aq. (10 mL), and the organic solvent was evaporated. Then, an excess of 2 N NaOH aq. was added and the mixture was washed 3 times with CH_2Cl_2. Saturated NaH_2PO_4 solution was added to adjust the pH to ca. 5, and the resulting yellow precipitate was collected by filtration. The crude solid was purified with silica gel column chromatography to obtain pure 15 as a red solid (yield 61%).

^{1}H NMR (CD_3OD, 300 MHz) δ 6.49-6.54 (m, 4H), 6.84 (dd, 2H, J = 1.2, 9.7 Hz), 7.53 (d, 1H, J = 7.3 Hz), 7.87 (dd, 1H, J = 7.5, 8.0 Hz), 7. 95 (dd, 1H, J = 7.3, 7.5 Hz), 8.39 (d, 1H, J = 8.0 Hz). ^{13}C NMR (CD_3OD, 75 MHz) δ 104.9, 112.1, 124.8, 126.2, 130.3, 130.8, 132.1, 133.0, 135.1, 149.8, 160.1, 180.0. HRMS (ESI^+): m/z calcd for $[M+H]^+$; 334.0716, found; 334.0676.

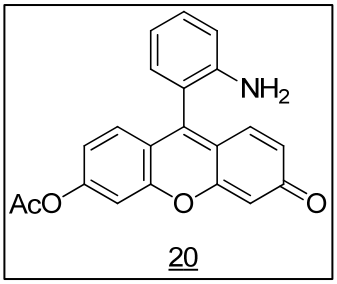
Synthesis of 6-hydroxy-9-(2-aminophenyl)-3*H*-xanthen-3-one (16)

Triethylamine (42 μL) and 10% Pd/C catalyst (1 portion) were added to a solution of 15 (0.30 mmol) in MeOH/CH_2Cl_2 (4 mL/16 mL). The reaction mixture was stirred under a H_2 atmosphere for 40 min at room temperature. The catalyst was removed by filtration and the solvent was evaporated to obtain a crude solid, which was dissolved in 2 N HCl aq. The mixture was washed 3 times with AcOEt. The aqueous layer was basified with 2 N NaOH aq., and saturated NaH_2PO_4 solution was added to adjust the pH to ca. 5. The

resulting precipitate was collected by filtration and dried in vacuo to obtain pure 16 as a red solid (yield 53%).

^{1}H NMR (CD_3OD, 300 MHz) δ 6.50 (d, 2H, J = 2.1 Hz), 6.54 (dd, 2H, J = 2.1, 9.2 Hz), 6.82-6.92 (m, 2H), 7.00 (dd, 1H, J = 1.6, 7.6 Hz), 7.12 (d, 2H, J = 9.2 Hz), 7.27-7.33 (m, 1H). ^{13}C NMR (CD_3OD, 75 MHz) δ 104.7, 112.8, 117.0, 118.3, 119.3, 124.4, 131.0, 131.4, 132.1, 146.4, 155.2, 160.6, 183.1. HRMS (ESI^+): m/z calcd for $[M+H]^+$; 304.0973, found; 334.0925.

Synthesis of 9-(2-aminophenyl)-3-oxo-3*H*-xanthen-6-yl acetate (20)



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To a solution of 16 (3.7 mmol) and $Na_2HPO_4 \cdot 12H_2O$ (3.7 mmol) in DMF (20 mL) was added acetic anhydride (4.0 mmol). The mixture was stirred at room temperature for 4 hours. The solid was removed by filtration and CH_2Cl_2 (100 mL) was added to the filtrate. The organic layer was washed 3 times with water and dried over anhydrous Na_2SO_4. The solid was removed by filtration and the solvent was evaporated to give crude title compound, which was purified by silica gel column chromatography (eluent: AcOEt/*n*-hexane = 50/50 to100/0) to afford pure 20 as an orange solid (yield 79%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.35 (s, 3H), 6.42 (d, 1H, J = 1.5 Hz), 6.60 (dd, 1H, J = 1.5, 9.7 Hz), 6.82-7.06 (m, 4H), 7.17 (d, 1H, J = 9.7 Hz), 7.27-7.38 (m, 2H). MS (ESI^+): m/z 346 $[M+H]^+$.

Synthesis of *N*-[2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)phenyl]benzamide (6)

To a solution of 20 (0.10 mmol) and triethylamine (140 μL) in CH_2Cl_2 (2 mL) was added benzoyl chloride (0.16 mmol) in CH_2Cl_2 (1 mL) on ice. The reaction mixture was stirred for overnight at ambient temperature. The solvent was removed by evaporation and the residue was dissolved in MeOH (9.5 mL), then 2 N NaOH aq. (0.5 mL) was added and the mixture was stirred for 1 hour at room temperature. The solvent was removed by evaporation, and the residue was partitioned between water and AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and filtered. The solvent was evaporated and the residue was purified by silica gel column chromatography (eluent: AcOEt/MeOH = 100/0 to 95/5) to give pure 6 as an orange solid (yield 12%).

^{1}H NMR (acetone-d_6, 300 MHz) δ 6.51 (d, 2H, J = 1.8 Hz), 6.50 (dd, 2H, J = 1.8, 9.3 Hz), 7.15 (d, 2H, J = 9.3 Hz), 7.26-7.31 (m, 2H), 7.40-7.53 (m, 5H), 7.69 (m, 1H), 8.09-8.12 (m, 1H). HRMS (ESI$^+$): m/z calcd for [M-H]$^-$; 406.1079, found; 406.1124.

Synthesis of Naphthalene-2-carboxylic acid [2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)phenyl]amide (12)

This compound was synthesized by the same procedure as described for 6 using 2-naphthoyl chloride as a reactant (yield 9.9%).

^{1}H NMR (acetone-d_6, 300 MHz) δ 6.53 (s, 2H), 6.62 (d, 2H, J = 9.3 Hz), 7.19 (d, 2H, J = 9.3 Hz), 7.50-7.89 (m, 11H), 8.21-8.25 (m, 1H). HRMS (ESI$^+$): m/z calcd for [M-H]$^-$; 456.1236, found; 456.1228.

Table S1. Reactivity of the compounds with UGT1A1. See page S8 for the chemical structures. See Fig. S3, S4, and S5 for raw experimental data.

Compound	π_R of the benzene moiety[a]	Rate (nM / s)
1	0.760	< 0.01
2	0.602	0.42
3	1.869	0.65
4	1.758	0.83
5	1.259	1.01
6	1.378	1.46
7	2.099	1.77
8	1.432	1.87
9	1.880	1.93
10	1.348	3.44
11	2.018	6.84
12	2.552	8.57
15	1.643	1.33
16	0.710	0.07
17	1.869	0.23
18	1.869	3.07
scopoletin[b]	N.A.[c]	0.37

[a] The values were calculated with Bio-Loom software.[S3]

[b] Presented for comparison.

[c] Not available due to the overall structure difference.

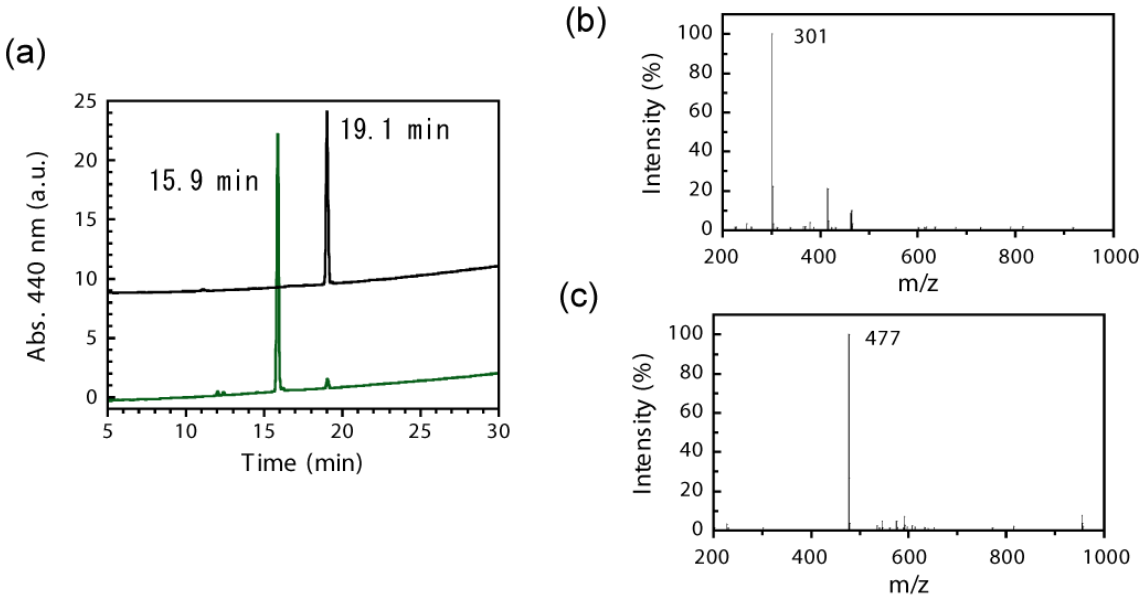

Fig. S1 LC/MS analysis of the reaction mixtures of 2-Me TG. (a) HPLC chromatogram of the samples (black: UGT −, green: UGT +) monitored by measuring absorbance at 440 nm. (b) Negative ions detected at 19.1 min, and (c) negative ions detected at 15.9 min. See also Fig. 1d.

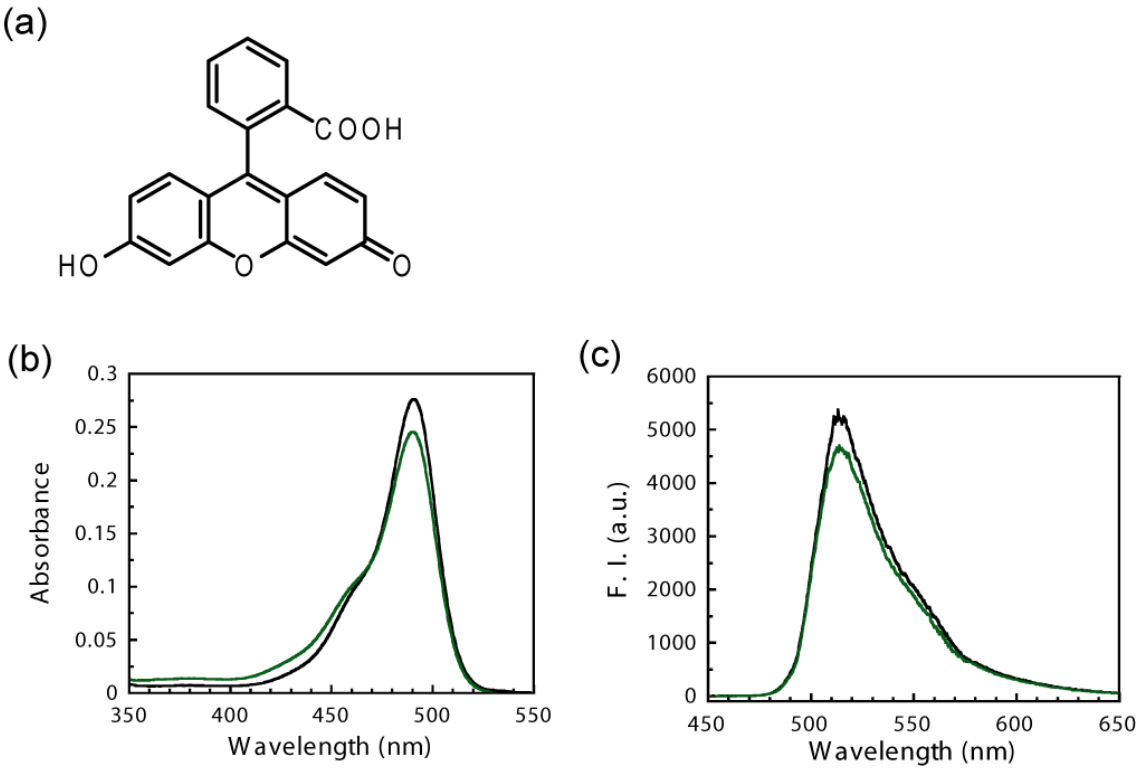

Fig. S2 Glucuronidation of fluorescein (1). (a) Chemical structure of fluorescein. (b, c) Absorption (b) and emission (c) spectra of fluorescein after 0 h (black) and 4.5 h (green) incubation with UGT1A1. Excitation wavelength was 490 nm.

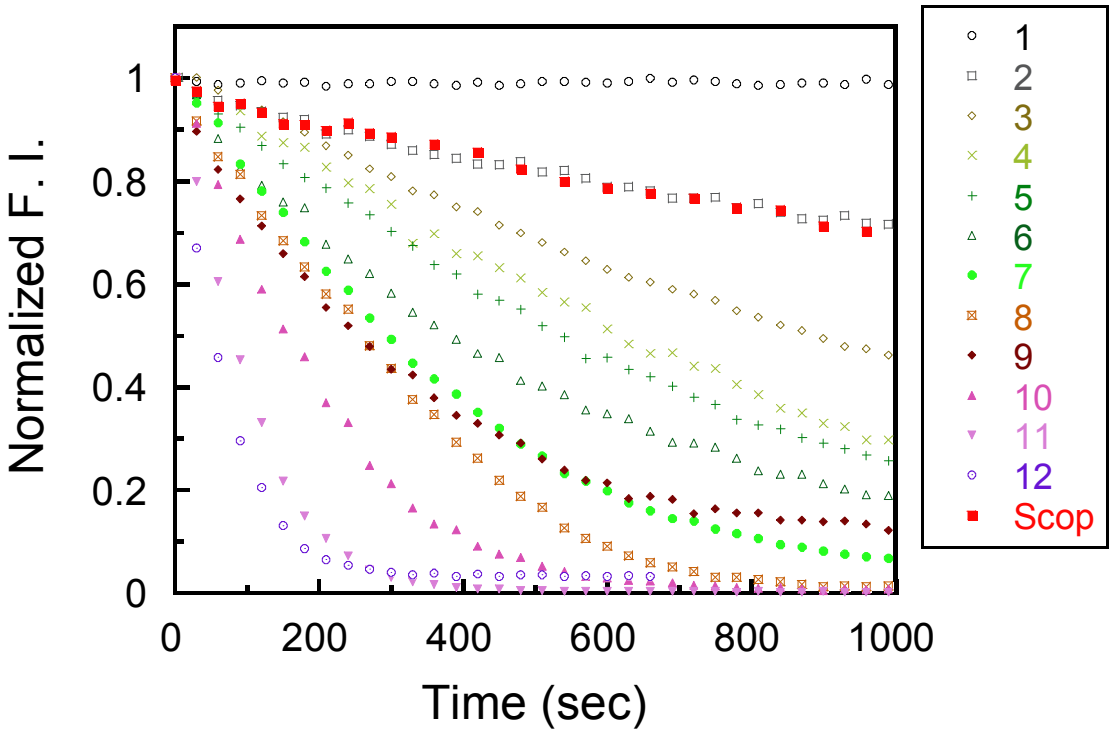

Fig. S3 UGT1A1-catalyzed reaction of fluorescent TGs (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) monitored by measuring the change of fluorescence intensity. Initial concentration of each TG was 1 μM. For comparison, results for scopoletin (Scop) are also shown.

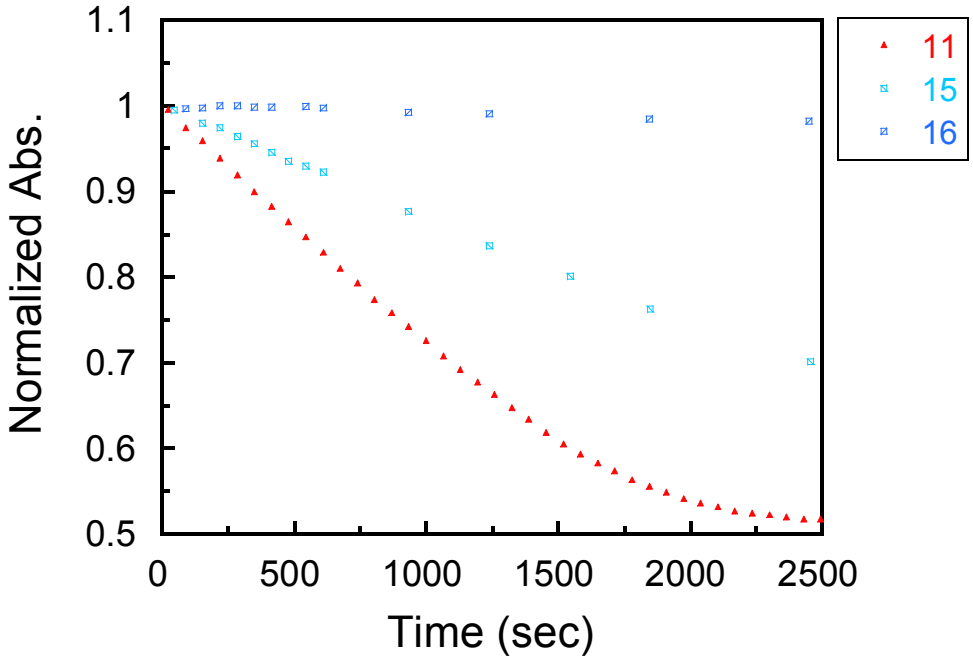

Fig. S4 UGT1A1-catalyzed reaction of non-fluorescent TGs (15, 16) monitored by measuring the change of absorbance. Initial concentration of each TG was 10 μM. For comparison, results for 2-Me-4-OMe TG (11) are also shown.

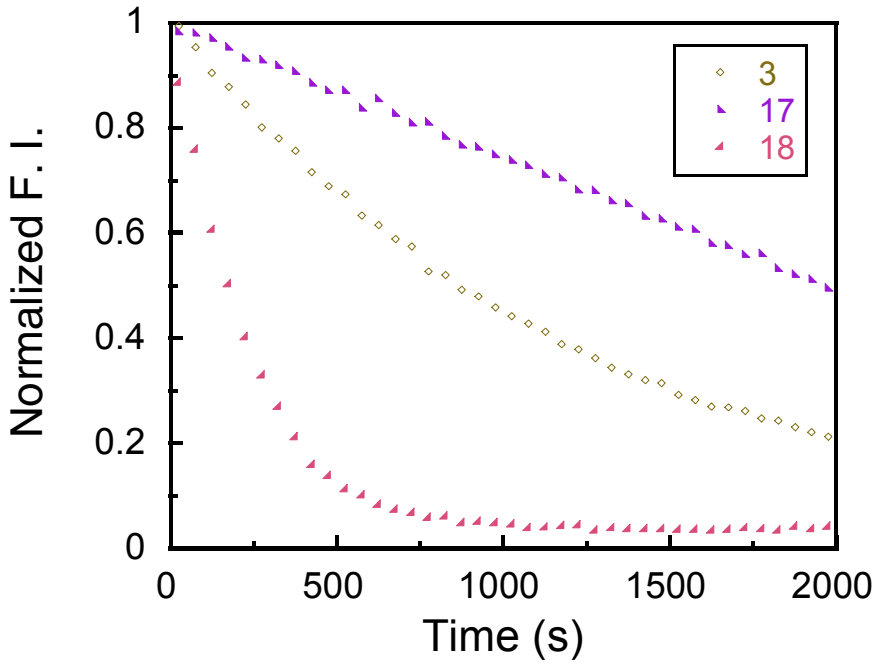

Fig. S5 Effect of substituents at the 2 and 7 positions of the xanthene ring. Change of fluorescence intensity (Ex. 490 nm, Em. 510 nm) of compounds 17 and 18 was plotted as a function of incubation time. Initial concentration of each fluorescein derivative was 1 μM. For comparison, results for 2-COOMe fluorescein (3) are also shown.

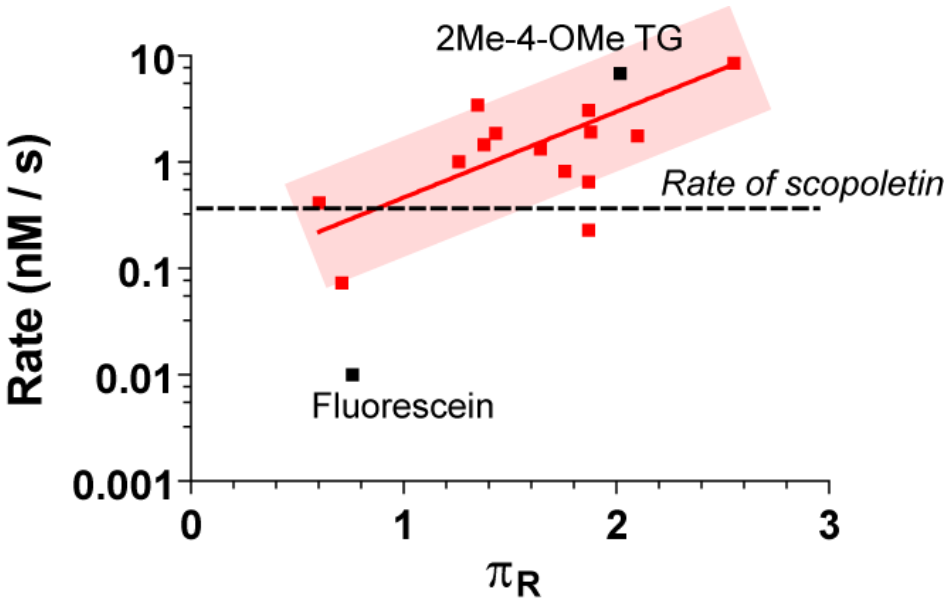

Fig. S6 Correlation between the substituent constants of benzene moieties (π_R)[S2] and the reaction rates of TG derivatives. For complete data, see Table S1. The rate of the standard fluorescent substrate, scopoletin, is also shown for comparison (dotted line).

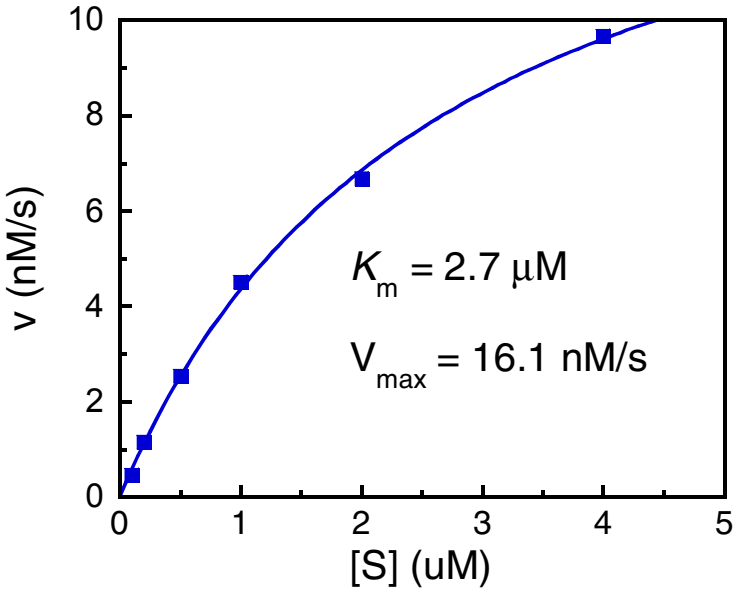

Fig. S7 Michaelis-Menten plot of 2-Me-4-OMe TG (11) for UGT1A1. From the plot, K_m and V_{max} values were calculated to be 2.7 μM and 16.1 nM/s, respectively. However, monotonic increase was not observed at higher concentrations, presumably due to substrate and/or product inhibition. Similar "atypical" behavior has been observed for several UGT isozymes.[S10]

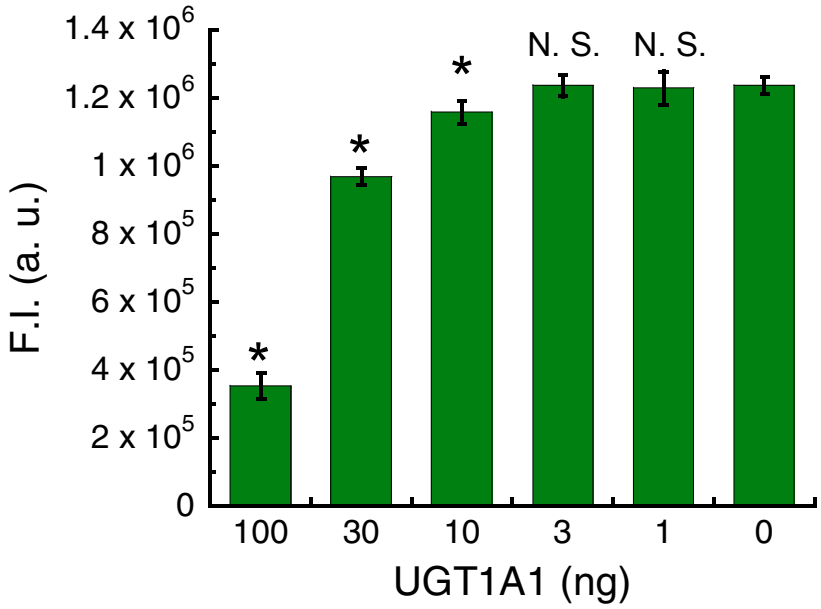

Fig. S8 Detection limit of UGT1A1 with 2-Me-4-OMe TG (11) as the substrate on a 96-well plate. Data are shown as mean ± S. E. (n = 5). * indicates $p < 0.05$, and N. S. stands for not significant, versus the control group without enzyme (one-sided Student's *t*-test).

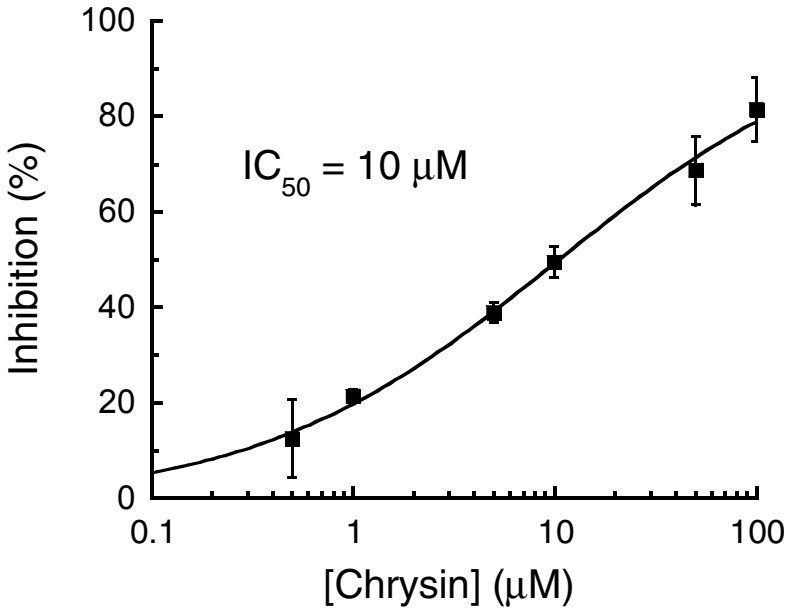

Fig. S9 UGT1A1 inhibition by chrysin detected with 2-Me-4-OMe TG (11) as the substrate on a 96-well plate. Data are shown as mean ± S. D. (n = 3).

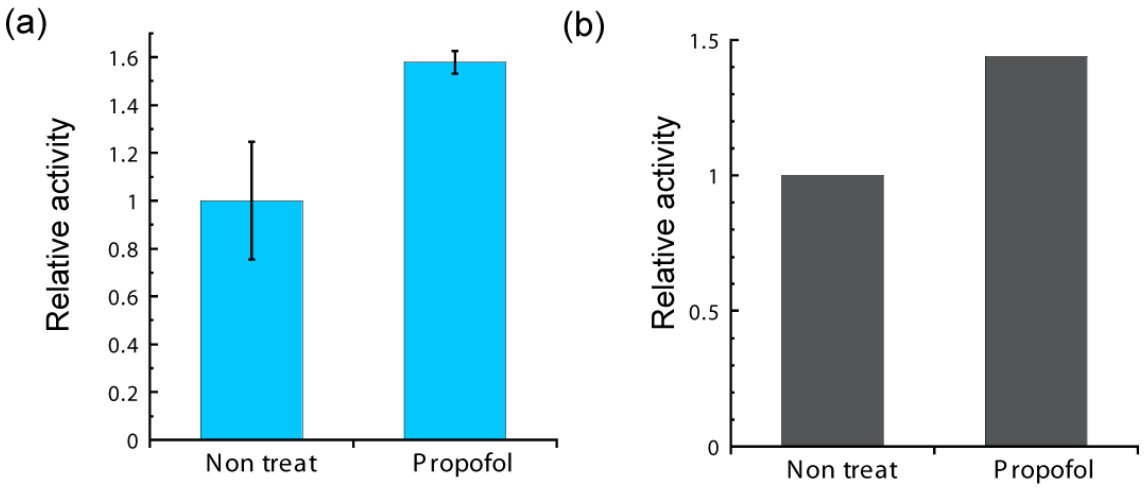

Fig. S10 Activation of UGT1A1 by propofol (200 μM) in fluorescence assays using (a) human liver microsomes, and (b) recombinant UGT1A1 enzyme. 2-Me-4-OMe TG (11) was used as the substrate. Data are shown as (a) mean ± S. D. (n = 3) or (b) average of two independent experiments.

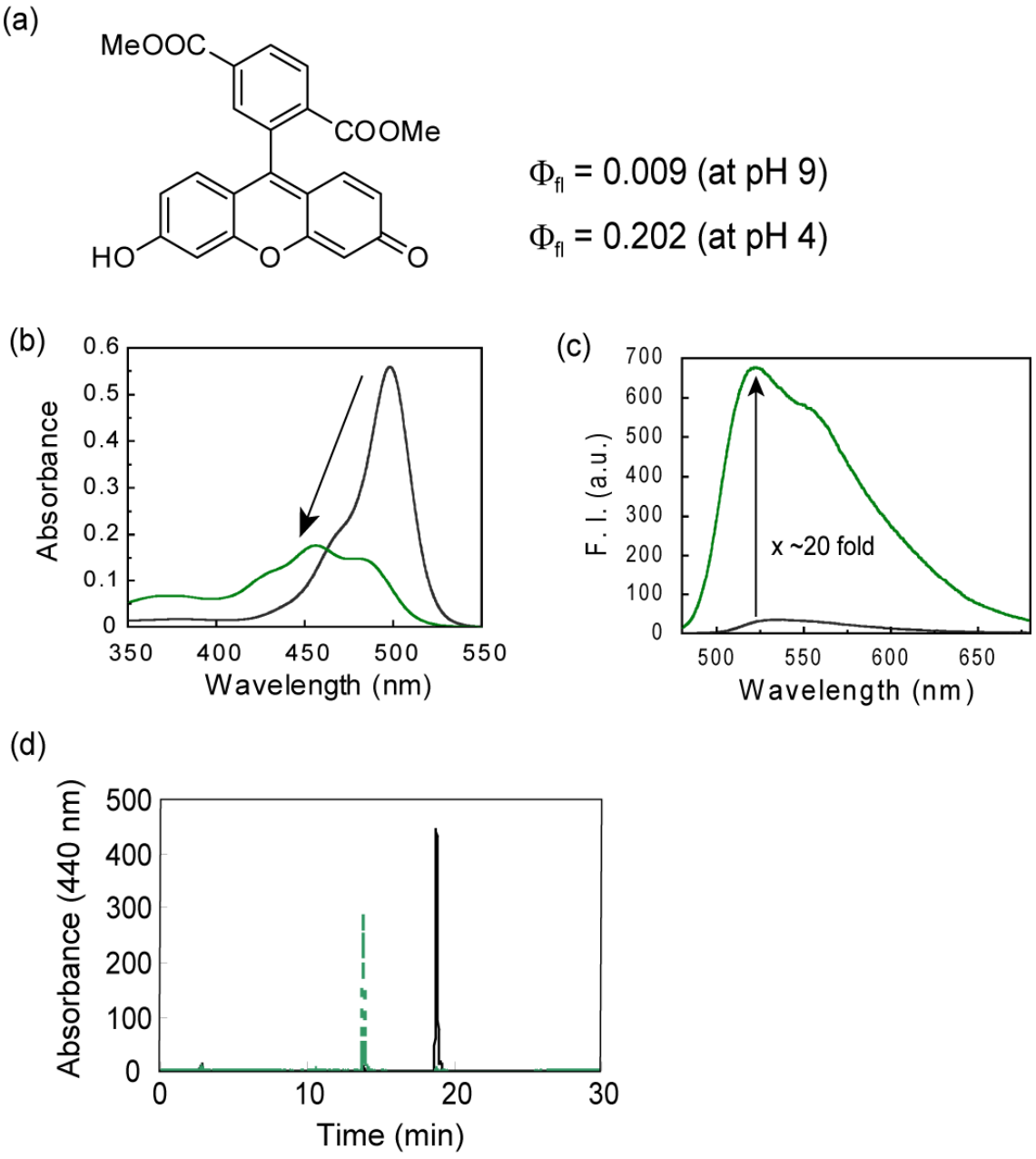

Fig. S11 Another turn-on probe for UGT1A1: 6-carboxyfluorescein dimethylester (13). (a) Chemical structure and fluorescence quantum yields at different pH values. (b, c) Absorption (b) and emission (c) spectra of the compound after 0 h (black) and 7 h (green) incubation with UGT1A1. Excitation wavelength was 455 nm. (d) HPLC chromatogram before (black solid line) and after (green dashed line) the reaction. Absorbance was measured at 440 nm.

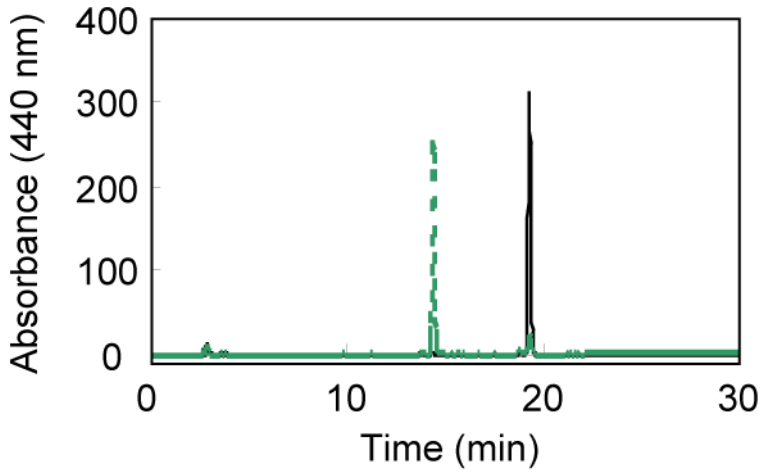

Fig. S12 HPLC chromatogram of 5,6-dicarboxyfluorescein trimethylester (14) before (black solid line) and after 5 h incubation (green dashed line) with UGT1A1. Absorbance was measured at 440 nm.

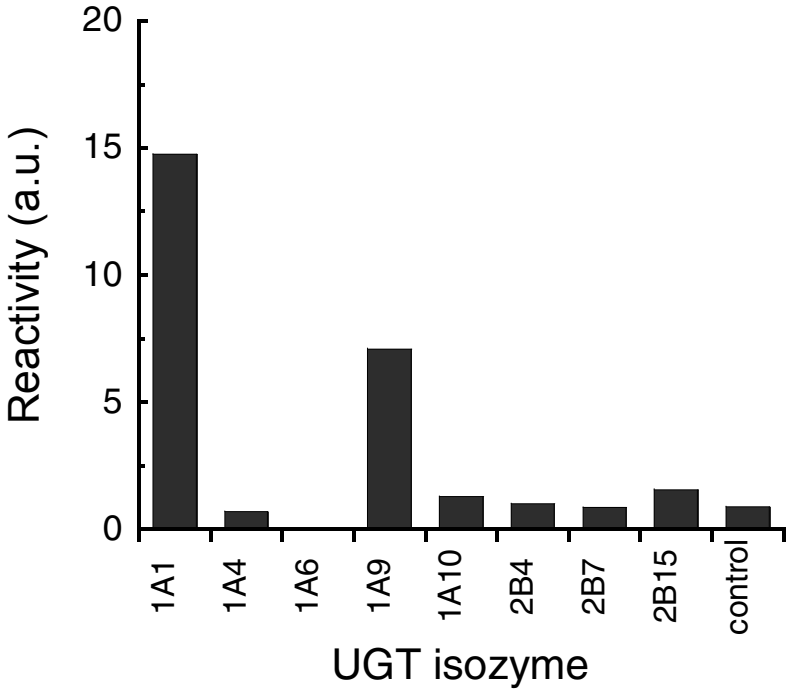

Fig. S13 Isozyme specificity of 2-Me TG (7). Relative reactivity was calculated using the same procedure as described for Fig. 3a.

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