Development of Fluorogenic Substrates of α-L-Fucosidase Useful for Inhibitor Screening and Gene-expression Profiling

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Supporting Information

Abbreviations

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Abbreviations: 2MeTG, 2-methyl TokyoGreen; ADDP, 1,1′-(azodicarbonyl)dipiperidine; AF, area of fluorescence; AV, average value; COSY, correlation spectroscopy; CV, coefficient of variation; DFJ, deoxyfuconojirimycin; DMSO, dimethyl sulfoxide; AcOEt, ethyl acetate; ESI, electrospray ionization; HMQC, heteronuclear multiple quantum correlation; MS, mass spectrometry; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline (not including Ca^{2+} and Mg^{2+}); RT, room temperature; SD, standard deviation; TBP, tributylphosphine; tFuc, tissue α-L-fucosidase; TFMU, 4-trifluoromethylumbelliferone; THF; tetrahydrofuran

General information for substrate synthesis: New compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, and HMQC spectrometry; mass spectrometry (MS); and elemental analysis. The nuclear magnetic resonance (NMR) spectra were recorded with a JEOL ECA500 spectrometer (JEOL, Tokyo, Japan; 500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts were expressed in ppm as downfield shifts from Me_4Si. Low-resolution mass spectra were obtained with a Waters Quattro Premier XE instrument (Waters, Milford, MA, USA) under positive and negative ion electrospray ionization (ESI) conditions. Column chromatography was performed using a Silica Gel 60N (Kanto Chemical, Tokyo, Japan; spherical neutral particle size: 100–210 μm). The progress of all reactions was monitored by thin-layer chromatography using a Silica Gel 60 F_{254} (0.25 mm; Merck Millipore, Billerica, MA, USA).

Synthesis of 1,2,3,4-tetra-O-acetyl-L-fucopyranose (7): L-Fucose (5.00 g; 30.5 mmol) was dissolved in 70 mL pyridine, followed by the addition of acetic anhydride (17.3 mL; 183 mmol). After stirring at room temperature (RT) for 24 h, the reaction mixture was poured into ethyl acetate (AcOEt). The organic layer was then washed with 1 M HCl, saturated $NaHCO_3$ (aq), and saturated NaCl (aq) and dried over Na_2SO_4. The obtained residue was purified by column chromatography on silica gel (2:1, hexane:AcOEt) to obtain 9.90 g (98% yield) of compound **7**. ESI-MS (positive mode): m/z = 355 $[M + Na]^+$.

1

2 *Synthesis of 2,3,4-tri-O-acetyl-L-fucopyranose (**8**):* Compound **7** (1.76 g; 5.29 mmol)
3 was dissolved in 10 mL of a mixed solution (100:1, 1,2-dimethoxyethane:H_2O), followed
4 by the addition of ammonium carbonate (4.80 g; 47.7 mmol). After stirring at RT for 7
5 days, the reaction mixture was filtered and concentrated *in vacuo*. The obtained residue
6 was purified by column chromatography on silica gel (1:1, hexane:AcOEt) to obtain 1.01
7 g (66% yield) of compound **8**. ESI-MS (positive mode): *m/z* = 313 $[M + Na]^+$.

8

9 *Synthesis of 2,3,4-tri-O-acetyl-L-fucopyranosyl 2,2,2-trichloroacetimidate (**9**)*:
10 Compound **8** (3.68 g; 12.7 mmol) was dissolved in dry 20 mL CH_2Cl_2, followed by the
11 addition of 1,8-diazabicyclo[5.4.0]-7-undecene (380 µL; 2.53 mmol) and
12 trichloroacetonitrile (3.80 mL; 38.0 mmol). After stirring at RT for 30 min under N_2
13 atmosphere, the mixture was poured into AcOEt. The organic layer was then washed
14 with H_2O and saturated NaCl (aq) and dried over Na_2SO_4. The obtained residue was
15 purified by column chromatography on silica gel (1:1, hexane:AcOEt) to obtain 4.92 g
16 (89% yield) of compound **9**, which was immediately used for the next step as it may
17 decompose when handled and stored.

18

19 *Synthesis of 4-(((tert-butyldimethylsilyl)oxy)methyl)phenyl*
20 *2,3,4-tri-O-acetyl-α-L-fucopyranoside (**10**)*: Compound **9** (1.39 g; 3.21 mmol) was
21 dissolved in dry 30 mL CH_2Cl_2, followed by the addition of
22 4-((*tert*-butyldimethylsilyloxy)methyl)phenol (1.15 g; 4.81 mmol; [1]) and boron
23 trifluoride ethyl ether complex (205 µL; 1.60 mmol). After stirring at −40 °C for 10 min
24 under N_2 atmosphere, the reaction mixture was poured into AcOEt. The organic layer
25 was then washed with saturated $NaHCO_3$ (aq) and saturated NaCl (aq) and then dried
26 over Na_2SO_4. The obtained residue was purified by flush column chromatography on
27 silica gel (2:1, hexane:AcOEt) to obtain 1.56 g (95% yield) of compound **10**, which was
28 immediately used for the next step as it may decompose when handled and stored.
29 ESI-MS (positive mode): *m/z* = 533 $[M + Na]^+$.

1

2 *Synthesis of 4-(hydroxymethyl)phenyl 2,3,4-tri-O-acetyl-α-L-fucopyranoside* ***(11)***:
3 Compound **10** (0.40 g; 0.79 mmol) was dissolved in 5 mL CH_3OH, followed by the
4 addition of 0.15 g DOWEX 50W-X8 (H^+ form). After stirring at RT for 4.5 h, the
5 reaction mixture was filtered and concentrated *in vacuo*. The obtained residue was
6 purified by column chromatography on silica gel (3:5, hexane:AcOEt) to obtain 0.29 g
7 (92% yield) of compound **11**. Characterization of compound **11** by ^{1}H-NMR, ^{13}C-NMR,
8 and MS agreed with previously reported data [2]. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.28 (d,
9 3H, $J_{5\text{-}6}$=6.3 Hz, H-6), 2.02 (s, 3H, -C$\underline{H}_3$, Ac), 2.06 (s, 3H, -C$\underline{H}_3$, Ac), 2.21 (s, 3H, -C$\underline{H}_3$,
10 Ac), 3.96 (qd, 1H, H-5), 4.65 (d, 2H, J=5.2 Hz, benzyl position), 5.04 (d, 1H, J=8.0 Hz),
11 5.11 (dd, 1H, J=3.5 Hz and 10.9 Hz), 5.31 (d, 1H, J=8.0 Hz), 5.47 (dd, 1H, J=8.0 Hz and
12 10.3 Hz), 7.00 (d, 1H, J=8.6 Hz), 7.31 (d, 1H, J=8.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$):
13 δ 16.17 (C6), 20.67 (-$\underline{C}H_3$, Ac), 20.73 (-$\underline{C}H_3$, Ac), 20.79 (-$\underline{C}H_3$, Ac), 64.87, 68.71, 69.52,
14 69.99, 71.20, 99.58 (C1), 116.95, 128.52, 135.60, 156.68, 169.53 (-$\underline{C}$=O, Ac), 170.27
15 (-$\underline{C}$=O, Ac), 170.72 (-$\underline{C}$=O, Ac). ESI-MS (positive mode): m/z = 419 $[M + Na]^+$.

16

17 *Synthesis of substrate* ***1***: Compound **11** (0.11 g; 0.28 mmol) was dissolved in 100 mL dry
18 tetrahydrofuran (THF), followed by the addition of 1,1′-(azodicarbonyl)dipiperidine
19 (ADDP; Tokyo Chemical Industry, Tokyo, Japan; A1051; 0.43 g; 1.66 mmol),
20 tributylphosphine (TBP; Tokyo Chemical Industry; T0361; 410 μL; 1.66 mmol), and
21 resorufin (Sigma-Aldrich, St. Louis, MO, USA; 424455; 73.0 mg; 0.33 mmol). After
22 stirring at 40 °C for 2.5 h under N_2 atmosphere, the reaction mixture was poured into
23 AcOEt. The organic layer was then washed with H_2O, and saturated NaCl (aq) and then
24 dried over Na_2SO_4. The obtained residue was purified twice by column chromatography
25 on silica gel (20:1, CH_2Cl_2:CH_3OH) to obtain 0.14 g (87% yield) of substrate **1**. ^{1}H-NMR
26 (500 MHz, $CDCl_3$): δ 1.28 (d, 3H, $J_{5\text{-}6}$=6.0 Hz, H-6), 2.02 (s, 3H, -C$\underline{H}_3$, Ac), 2.06 (s, 3H,
27 -C$\underline{H}_3$, Ac), 2.21 (s, 3H, -C$\underline{H}_3$, Ac), 3.97 (qd, 1H, H-5), 5.07 (d, 1H, $J_{1\text{-}2}$=8.0 Hz, H-1),
28 5.10-5.13 (m, 3H, H-3 and benzyl position), 5.31 (d, 1H, $J_{3\text{-}4}$=$J_{4\text{-}5}$=3.5 Hz, H-4), 5.48
29 (dd, 1H, $J_{1\text{-}2}$= 7.3 Hz, $J_{2\text{-}3}$=10.8 Hz, H-2), 6.32 (d, 1H, J=2.5 Hz), 6.84 (dd, 1H, J=2.5 Hz

and 9.8 Hz), 6.87 (d, 1H, *J*=2.0 Hz), 6.99 (dd, 1H, *J*=2.5 Hz and 9.0 Hz), 7.05 (d, 2H, *J*-=8.5 Hz), 7.38 (d, 2H, *J*=9.0 Hz), 7.42 (d, 1H, *J*=10.0 Hz), 7.71 (d, 1H, *J*=9.5 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 16.17 (C6), 20.63 (-CH_3, Ac), 20.69 (-CH_3, Ac), 20.77 (-CH_3, Ac), 68.71 (C2), 69.63 (C5), 69.98 (C4), 70.47 (benzyl position), 71.18 (C3), 99.37 (C1), 101.05, 106.79, 114.21, 117.11, 128.51, 129.24, 129.95, 131.62, 134.30, 134.71, 145.61, 145.77, 149.80, 157.27, 162.57, 169.46 (-C=O, Ac), 170.21 (-C=O, Ac), 170.64 (-C=O, Ac), 186.30 (-C=O, resorufin). ESI-MS (positive mode): *m/z* = 614 $[M + Na]^+$, (negative mode): *m/z* = 590 $[M - H]^-$. Elemental analysis: Calculated for $C_{31}H_{29}NO_{11}$: C, 62.94; H, 4.94; N, 2.37. Found: C, 62.96; H, 4.94; N, 2.33.

*Synthesis of substrate **4***: Substrate **1** (0.10 g; 0.18 mmol) was dissolved in 60 mL of mixed solution (5:1, CH_3OH:CH_2Cl_2), followed by the addition of 28% $NaOCH_3$ in CH_3OH (95 μL). After stirring at 0 °C for 17 h, the reaction mixture was poured into H_2O and extracted three times with AcOEt. The organic layer was then washed with saturated NaCl (aq) and dried over Na_2SO_4. The obtained residue was purified by column chromatography on silica gel (8:1, CH_2Cl_2:CH_3OH) to obtain 72.0 mg (88% yield) of substrate **4**. ^1H-NMR (500 MHz, DMSO-d6): δ 1.11 (d, 3H, $J_{5\text{-}6}$=6.5 Hz, H-6), 3.44-3.57 (m, 3H, H-2, H-3, and H-4), 3.74 (qd, 1H, H-5), 4.57 (d, 1H, -OH), 4.83-4.84 (m, 2H, $J_{1\text{-}2}$=8.0 Hz, H-1 and -OH), 5.16 (d, 1H, -OH), 5.19 (s, 1H benzyl position), 6.26 (d, 1H, *J*=2.0 Hz), 6.77 (dd, 1H, *J*=1.8 Hz and 9.8 Hz), 7.02 (d, 2H, *J*=9.0 Hz), 7.10 (dd, 1H, *J*=2.5 Hz and 9.0 Hz), 7.18 (d, 1H, *J*=3.0 Hz), 7.41 (d, 2H, *J*=8.5 Hz), 7.52 (d, 1H, *J*=9.5 Hz), 7.76 (d, 1H, *J*=9.0 Hz). ^{13}C-NMR (125 MHz, DMSO-d6): δ 16.68 (C6), 69.93, 70.12 (benzyl position), 70.32 (C5), 71.00, 73.45, 100.47 (C1), 101.21, 105.70, 114.50, 116.19, 128.02, 129.02, 129.79, 131.41, 133.79, 135.01, 145.24, 145.32, 149.84, 157.46, 162.46, 185.47 (-C=O, resorufin). ESI-MS (positive mode): *m/z* = 488 $[M + Na]^+$. Elemental analysis: Calculated for $C_{25}H_{23}NO_8{\bullet}1/2H_2O$: C, 63.29; H, 5.10; N, 2.95. Found: C, 62.96; H, 5.31; N, 2.69.

*Synthesis of substrate **2***: Compound **11** (0.11 g; 0.28 mmol) was dissolved in 20 mL dry

THF, followed by the addition of ADDP (0.42 g; 1.67 mmol), TBP (420 μL; 1.67 mmol), and 4-trifluoromethylumbelliferone (TFMU; Santa Cruz Biotechnology, Dallas, TX, USA; sc-210622A; 80.0 g; 0.33 mmol). After stirring at 40 °C for 20 min under N_2 atmosphere, the reaction mixture was poured into AcOEt. The organic layer was then washed with saturated $NaHCO_3$ (aq) and saturated NaCl (aq) and then dried over Na_2SO_4. The obtained residue was purified by column chromatography on silica gel (1:1, hexane:AcOEt) to obtain 0.16 g (94% yield) of substrate **2**. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.29 (d, 3H, $J_{5\text{-}6}$=6.5 Hz, H-6), 2.02 (s, 3H, $-C\underline{H}_3$, Ac), 2.06 (s, 3H, $-C\underline{H}_3$, Ac), 2.21 (s, 3H, $-C\underline{H}_3$, Ac), 3.98 (qd, 1H, H-5), 5.06 (d, 1H, $J_{1\text{-}2}$=8.0 Hz, H-1), 5.09-5.13 (m, 3H, H-3 and benzyl position), 5.31 (d, 1H, $J_{3\text{-}4}$=$J_{4\text{-}5}$=3.5 Hz, H-4), 5.48 (dd, 1H, $J_{1\text{-}2}$= 8.0 Hz, $J_{2\text{-}3}$=10.0 Hz, H-2), 6.63 (s, 1H), 6.93 (d, 1H, J=3.0 Hz), 6.98 (dd, 1H, J=2.8 Hz and 9.3 Hz), 7.04 (d, 2H, J=8.5 Hz), 7.37 (d, 2H, J=8.5 Hz), 7.63 (d, 1H, J=9.5 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 16.17 (C6), 20.67 ($-\underline{C}H_3$, Ac), 20.73 ($-\underline{C}H_3$, Ac), 20.80 ($-\underline{C}H_3$, Ac), 68.62 (C2), 69.57 (C5), 69.93 (C4), 70.21 (benzyl position), 71.15 (C3), 99.32 (C1), 102.32, 107.21, 112.33, 112.37, 114.03, 117.07, 126.40, 129.24, 129.86, 141.57 (q, $J_{C\text{-}F}$=33.4 Hz, $-\underline{C}F_3$), 156.22, 157.22, 159.40, 162.45 ($-\underline{C}$=O, TFMU), 169.52 ($-\underline{C}$=O, Ac), 170.25 ($-\underline{C}$=O, Ac), 170.68 ($-\underline{C}$=O, Ac). ESI-MS (positive mode): m/z = 631 $[M + Na]^+$, (negative mode): m/z = 607 $[M - H]^-$. Elemental analysis: Calculated for $C_{29}H_{27}F_3O_{11}$: C, 57.24; H, 4.47; F, 9.37; N, 0.00. Found: C, 57.53; H, 4.63; F, 9.33; N, 0.00.

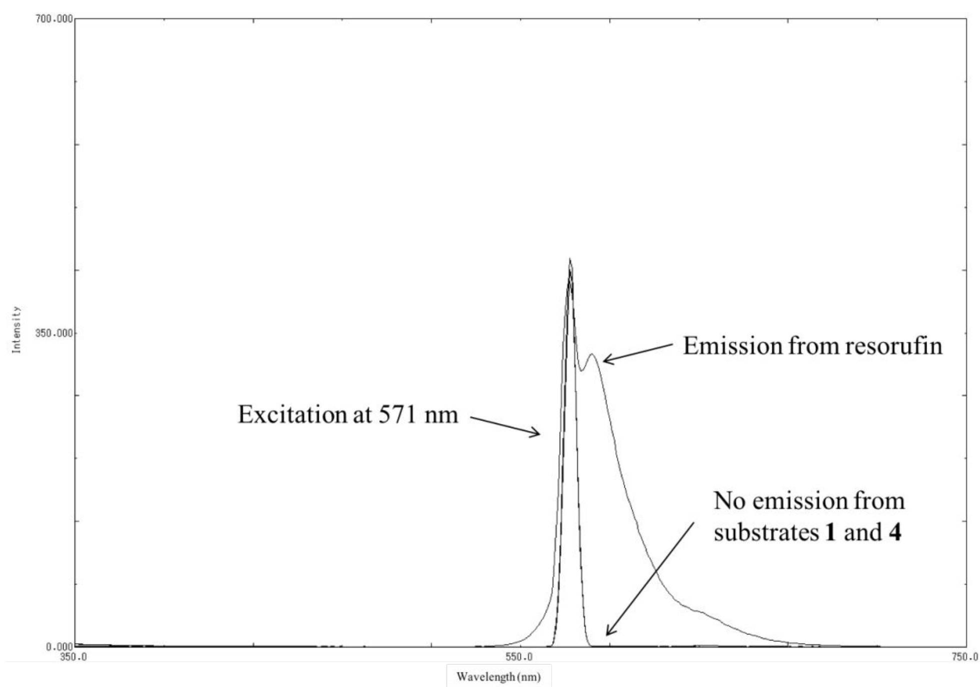
*Synthesis of substrate **5***: Substrate **2** (62.0 mg; 0.10 mmol) was dissolved in 3 mL CH_3OH, followed by the addition of 28% $NaOCH_3$ in CH_3OH (50 μL). After stirring at RT for 25 min, the reaction mixture was neutralized by DOWEX 50W-X8 (H^+ form), filtered, and concentrated *in vacuo*. The obtained residue was purified by column chromatography on silica gel (10:1, CH_2Cl_2:CH_3OH) to obtain 36.0 mg (73% yield) of substrate **5**. ^{1}H-NMR (500 MHz, DMSO-d6): δ 1.11 (d, 3H, $J_{5\text{-}6}$=6.0 Hz, H-6), 3.37-3.53 (m, 3H, H-2, H-3, and H-4), 3.74 (qd, 1H, H-5), 4.56 (d, 1H, $-O\underline{H}$), 4.83 (d, 2H, $J_{1\text{-}2}$=7.0 Hz, H-1 and $-O\underline{H}$), 5.16 (d, 1H, $-O\underline{H}$), 5.17 (s, 2H, benzyl position), 6.84 (s, 1H), 7.01 (d, 2H, J=8.5 Hz), 7.10 (dd, 1H, J=2.5 Hz and 9.0 Hz), 7.23 (d, 1H, J=2.0 Hz), 7.40 (d, 2H,

1 J=8.5 Hz), 7.61 (d, 1H, J=7.5 Hz). ^{13}C-NMR (125 MHz, DMSO-d6): δ 16.68 (C6),
2 69.88, 69.93 (benzyl position), 70.31 (C5), 71.02, 73.44, 100.48 (C1), 102.62, 106.57,
3 113.37, 114.10, 116.18, 121.81, 125.93, 129.02, 129.75, 139.48 (q, J_{C-F}=32.1 Hz, -$\underline{C}F_3$),
4 155.88, 157.44, 158.87, 162.18 (-$\underline{C}$=O, TFMU). ESI-MS (positive mode): m/z = 505 [M
5 + Na]$^+$, (negative mode): m/z = 481 [M - H]$^-$. Elemental analysis: Calculated for
6 $C_{23}H_{21}F_3O_8$: C, 57.26; H, 4.39; F, 11.81; N, 0.00. Found: C, 57.27; H, 4.36; F, 11.86; N,
7 0.00.
8
9 *Synthesis of substrate **3***: Compound **11** (0.14 g; 0.36 mmol) was dissolved in 30 mL dry
10 THF, followed by the addition of ADDP (0.56 g; 2.18 mmol), TBP (540 μL; 2.18 mmol),
11 and 2-methyl TokyoGreen (2MeTG; 0.14 g; 0.44 mmol; [3]). After stirring at RT for 60
12 min under N_2 atmosphere, the reaction mixture was concentrated *in vacuo*. The obtained
13 residue was purified by column chromatography on silica gel (1:5, hexane:AcOEt) to
14 obtain 0.22 g (87% yield) of substrate **3**. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.29 (d, 3H,
15 J_{5-6}=6.0 Hz, H-6), 2.02 (s, 3H, -C$\underline{H}_3$, Ac), 2.06 (s, 3H, -C$\underline{H}_3$, Ac), 2.07 (s, 3H, -C$\underline{H}_3$, Ac),
16 2.21 (s, 3H, -C$\underline{H}_3$, 2MeTG), 3.98 (qd, 1H, H-5), 5.08 (d, 1H, J_{1-2}=8.0 Hz, H-1), 5.10-5.13
17 (m, 3H, H-3 and benzyl position), 5.31 (d, 1H, J_{3-4}=3.3 Hz, J_{4-5}=1.3 Hz, H-4), 5.48 (dd,
18 1H, J_{1-2}= 8.0 Hz, J_{2-3}=10.0 Hz, H-2), 6.46 (d, 1H, J=2.0 Hz), 6.57 (dd, 1H, J=1.8 Hz, 9.8
19 Hz), 6.82 (dd, 1H, J=2.3 Hz and 8.8 Hz), 6.95 (d, 1H, J=10.0 Hz), 6.98 (d, 1H, J=9.5
20 Hz), 7.02 (d, 1H, J=2.5 Hz), 7.05 (d, 2H, J=9.0 Hz), 7.16 (d, 1H, J=7.5 Hz), 7.37-7.40
21 (m, 4H), 7.45 (dd, 1H, J=1.0 Hz and 7.5 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 16.16
22 (C6), 19.65 (-$\underline{C}H_3$, 2MeTG), 20.67 (-$\underline{C}H_3$, Ac), 20.73 (-$\underline{C}H_3$, Ac), 20.79 (-$\underline{C}H_3$, Ac),
23 68.61 (C2), 69.57 (C5), 69.90 (C4), 70.28 (benzyl position), 71.13 (C3), 99.27 (C1),
24 101.23, 105.81, 114.00, 114.50, 117.02, 118.33, 126.11, 129.05, 129.29, 129.45, 129.49,
25 129.92, 130.16, 130.57 (x2), 132.45, 136.15, 149.32, 154.52, 157.19, 158.88, 163.20,
26 169.50 (-$\underline{C}$=O, Ac), 170.25 (-$\underline{C}$=O, Ac), 170.67 (-$\underline{C}$=O, Ac), 185.82 (-$\underline{C}$=O, 2MeTG).
27 ESI-MS (positive mode): m/z = 703 [M + Na]$^+$. Elemental analysis: Calculated for
28 $C_{39}H_{36}O_{11}$: C, 68.81; H, 5.33; N, 0.00. Found: C, 68.83; H, 5.26; N, 0.00.
29

*Synthesis of substrate **6***: Substrate **3** (0.11 g; 0.16 mmol) was dissolved in 15 mL CH_3OH, followed by the addition of 28% $NaOCH_3$ in CH_3OH (15 µL). After stirring at 0 °C for 12 h, the reaction mixture was poured into H_2O and extracted three times with AcOEt. The organic layer was then washed with saturated NaCl (aq) and dried over Na_2SO_4. The obtained residue was purified by column chromatography on silica gel (8:1, CH_2Cl_2: CH_3OH) to obtain 63.0 mg (72% yield) of substrate **6**. ^{1}H-NMR (500 MHz, DMSO-d6): δ 1.12 (d, 3H, $J_{5\text{-}6}$=7.0 Hz, H-6), 2.00 (s, 3H, -C$\underline{H}_3$, 2MeTG), 3.36-3.53 (m, 3H, H-2, H-3, and H-4), 3.74 (qd, 1H, H-5), 4.52 (d, 1H, -O$\underline{H}$), 4.79 (d, 1H, -O$\underline{H}$), 4.83 (d, 1H, $J_{1\text{-}2}$=7.0 Hz, H-1), 5.12 (d, 1H, -O$\underline{H}$), 5.19 (s, 2H, benzyl position), 6.24 (d, 1H, *J*=2.0 Hz), 6.43 (dd, 1H, *J*=1.8 Hz and 9.8 Hz), 6.84 (d, 1H, *J*=10.0 Hz), 6.89 (d, 1H, *J*=8.5 Hz), 6.98 (dd, 1H, *J*=2.5 Hz and 8.5 Hz), 7.02 (d, 2H, *J*=8.5 Hz), 7.26 (d, 1H, *J*=7.0 Hz), 7.30 (d, 1H, *J*=2.5 Hz), 7.40 (d, 2H, *J*=8.5 Hz), 7.40-7.52 (m, 3H). ^{13}C-NMR (125 MHz, DMSO-d6): δ 16.61 (C6), 19.12 (-$\underline{C}H_3$, 2MeTG), 69.91, 70.04 (benzyl position), 70.28 (C5), 70.98, 73.43, 100.50 (C1), 101.57, 104.78, 113.78, 114.41 (x2), 116.19, 117.34, 126.23, 128.98, 129.07, 129.24, 129.56, 129.68, 130.51 (x2), 132.10, 135.64, 149.02, 153.98, 157.42, 158.42, 163.29, 184.05 (–$\underline{C}$=O, 2MeTG). ESI-MS (positive mode): *m/z* = 555 $[M + H]^+$. Elemental analysis: Calculated for $C_{33}H_{30}O_8$: C, 71.47; H, 5.45; N, 0.00. Found: C, 71.46; H, 5.64; N, 0.00.

Photochemical characterization of substrates 1 through 6: Solutions of resorufin and substrates **1** and **4** were prepared at 10 nM in phosphate-buffered saline (PBS) containing 2.0×10^{-4}% DMSO. Solutions of TFMU and substrates **2** and **5** were prepared at 100 nM in PBS containing 1.0×10^{-3}% DMSO. Solutions of 2MeTG and substrates **3** and **6** were prepared at 10 nM in PBS containing 1.0×10^{-4}% DMSO. The fluorescence emission spectra of these solutions were recorded at the following appropriate excitation wavelengths: resorufin and substrates **1** and **4**, 571 nm; TFMU and substrates **2** and **5**, 385 nm; and 2MeTG and substrates **3** and **6**, 491 nm. The fluorescence emission spectrum of each solution was recorded using a RF-5300PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). The results are summarized in Figures S1 to S3.

1

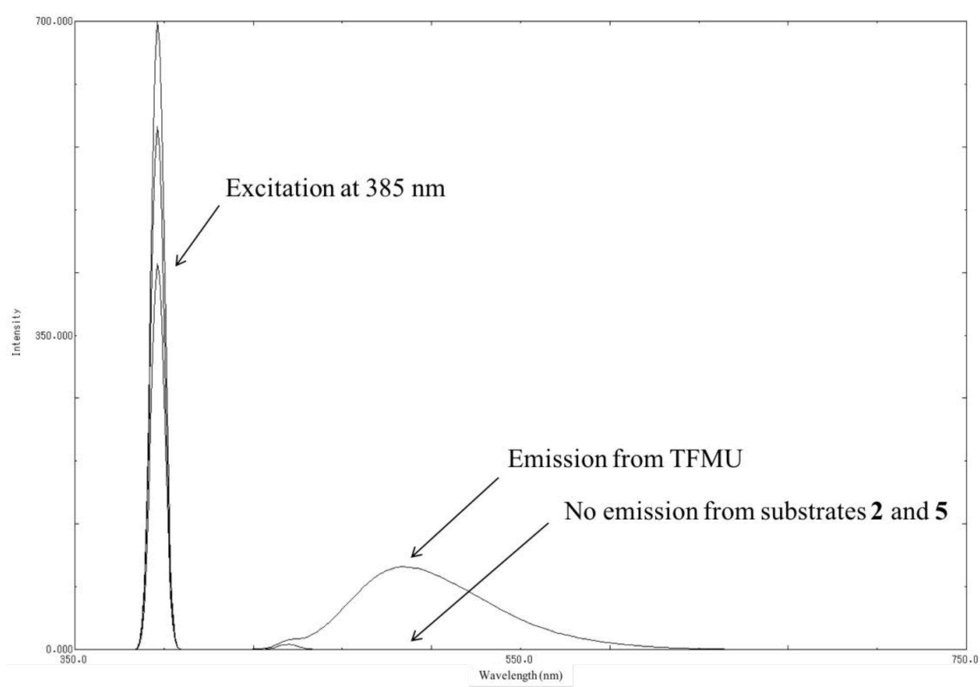


2

3 **Figure S1.** Overlay of the fluorescence spectra of resorufin and substrates **1** and **4**.

4

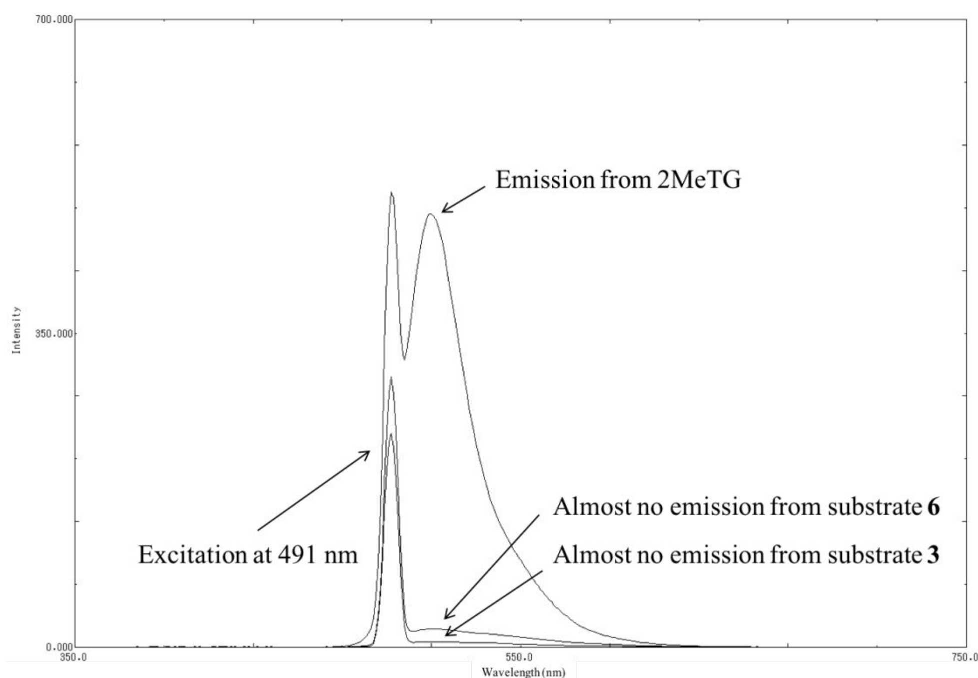
5



6

7 **Figure S2.** Overlay of the fluorescence spectra of TFMU and substrates **2** and **5**.

1



2

3 **Figure S3.** Overlay of the fluorescence spectra of 2MeTG and substrates **3** and **6**.

4

5 **Computational analysis**

6 *Calculated of distribution coefficient (clog D) values for substrates **1** through **6***: The
7 clog *D* values for substrates **1** through **6** were calculated using Structure Design Suite
8 version 12.01 from Advanced Chemistry Development Inc. (Toronto, Canada).

9

10 *Docking study of α-L-fucosidase versus substrates **4** through **6**, native substrate, and*
11 *2MeTG α-L-fucopyranoside:* Molecular docking simulations of *Thermotoga maritima*
12 α-L-fucosidase (PDB ID: 2ZXD) to substrates **4** through **6**, native substrate
13 (α-L-fucopyranoside α(1-6) *N*-acetylglucosamine), and 2MeTG α-L-fucopyranoside were
14 carried out using the Molegro Virtual Docker (version 7.0.0; Molexus, Odder, Denmark).
15 In these experiments, we used MolDock scoring function, which is based on a piecewise
16 linear potential, and a re-ranking procedure was applied to the highest ranked poses to
17 increase the docking accuracy. Affinity grid resolution was set to 0.3 Å. Ligand
18 evaluations were based on internal energy of binding, internal H-bond formation, and

Sp2-Sp2 (trigonal planar electron domain geometry) torsion angles. Candidate cavities for ligand docking were detected by inclusion of pockets with a volume 10–10000 Å^3. Among the candidate cavities, the cavity that was bound to a native ligand, 6-isopropyl-1-deoxyfuconojirimycin in X-ray structure of *T. maritima* α-L-fucosidase, was selected as an active site for docking. The cavity was measured for cavity volume (108.032 Å^3), surface (300.8 Å^2), and radius (15.0 Å). The customized search algorithm was set to MolDock Optimizer. Number of runs was 15. Parameter settings were set to 2000 iterations, 50 population sizes, 0.50 scaling factor, and 0.90 crossover rate. All dockings were performed at 0.70 Å RMSD threshold.

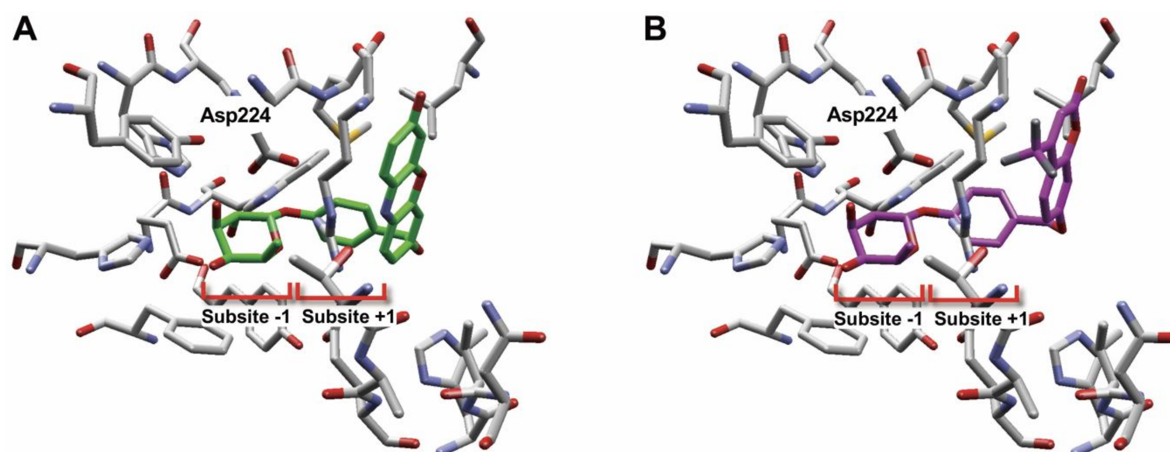


Figure S4. Evaluation of the suitability of the fluorogenic substrates for α-L-fucosidase by docking simulations. Docking models using (A) substrate **4** and (B) substrate **5**.

General information for cell-based assays: A human fibrosarcoma cell line (HT-1080; RCB1956), a human cervical cancer cell line (HeLa; RCB0007), and a human neuroblastoma cell line (SK-N-SH; RCB0426) were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. High-glucose Dulbecco's modified Eagle's medium (D6046) was purchased from Sigma-Aldrich. CELLECT fetal bovine serum (S1820) was purchased from MP Biomedical (Solon, OH, USA). The Lyso-ID Green assay kit (ENZ-51028-K100) was purchased from ENZO Life Sciences (Exeter, UK). DFJ•HCl (FC-007) was purchased from GlycoSyn (Lower Hutt, New Zealand).

Chloroquine diphosphate (038-17971), 0.5 w/v% trypsin–5.3 mmol/L EDTA ・4Na solution without phenol red (208-17251), and a 10% formalin solution (064-03843) for tissue fixation were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A 35-mm-high μ-Dish for cell imaging (ib81156) was purchased from Ibidi (Gräfelfing, Germany). Fluorescence intensities were recorded using a FluoView FV1000-D confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with the following filter sets: Alexa Fluor 488 (excitation, 473 nm; dichroic filter, 405/473; and emission, 490–590 nm) for 2MeTG measurements; fluorescein isothiocyanate (excitation, 473 nm; dichroic filter, 405/473; and emission, 490–590 nm) for Lyso-ID Green measurements; Alexa Fluor 568 (excitation, 559 nm; dichroic filter, 405/473/559; emission, 575–675 nm) for resorufin measurements; and 4′,6-diamidino-2-phenylindole (excitation, 405 nm; dichroic filter, 405/473; emission, 430–455 nm) for TFMU.

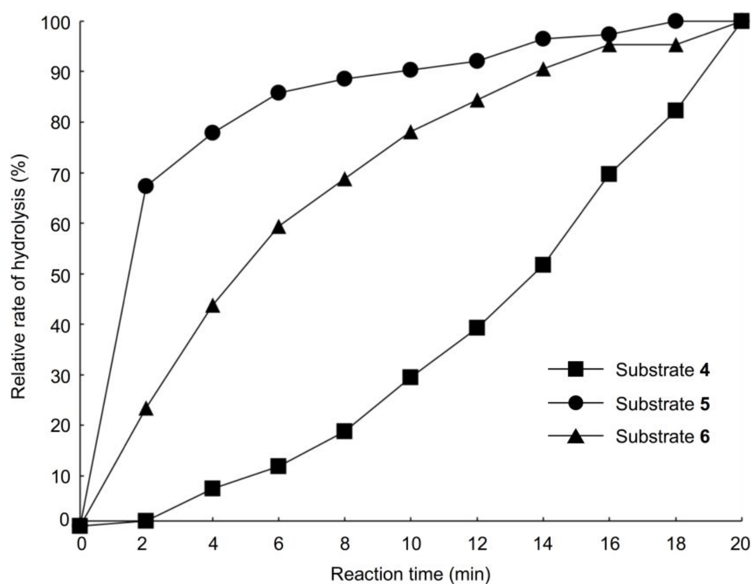
*Cell-based assays using substrates **1** through **6**:* HT-1080, HeLa, and SK-N-SH cells were seeded in μ-Dishes at 4.0×10^4 cells/dish. After incubation at 37 °C for 24 h in 5% CO_2, the medium was replaced with 1 mL of fresh medium. Subsequently, 5 μL of 2 mM DMSO solutions of substrates **1** through **6** were added to each dish (10 μM final concentration), followed by incubation at 37 °C for 1 h in 5% CO_2. The medium was removed, and cells were immediately fixed with 10% formalin. Fluorescence signals were recorded using a FluoView FV1000-D microscope (Olympus).

*Cell-based assays using Lyso-ID Green and substrates **1** and **4**:* HT1080 cells were seeded in μ-Dishes at 4.0×10^4 cells/dish. After incubation at 37 °C for 24 h in 5% CO_2, the medium was replaced with 1 mL of fresh medium. Subsequently, 5 μL of 2 mM DMSO solutions of substrates **1** and **4** was added to the dishes (10 μM final concentration), followed by incubation at 37 °C for 1 h in 5% CO_2. Next, 200 μL of dye solution from the Lyso-ID Green assay kit (ENZO Life Sciences) was added, and the cells were incubated at 37 °C in 5% CO_2 for 30 min in the dark. Cells were washed three times with 200 μL of assay buffer from the kit and immediately fixed with 10% formalin.

1 Fluorescence signals were recorded using a FluoView FV1000-D microscope (Olympus).
2
3 *Chloroquine treatment of cells assayed using Lyso-ID Green and substrate **1**:* HT1080
4 cells were seeded in a μ-Dish at 4.0×10^4 cells/dish. After incubation at 37 °C for 24 h in
5 5% CO_2, the medium was replaced with 1 mL of fresh medium. Subsequently, 5 μL of
6 15 mM chloroquine diphosphate water solution (75 μM final concentration) was added to
7 the dish, followed by incubation at 37 °C for 2 h in 5% CO_2. The medium was then
8 replaced with 1 mL of fresh medium, followed by addition of 5 μL of 2 mM DMSO
9 solution of substrate **1** (10 μM final concentration) to the dish. The cells were incubated
10 at 37 °C for 1 h in 5% CO_2, and the medium was replaced with 1 mL of fresh medium
11 along with 200 μL of dye solution from the Lyso-ID Green assay kit (ENZO Life
12 Sciences). The cells were then incubated at 37 °C for 30 min in 5% CO_2 in the dark. The
13 cells were washed three times with 200 μL of assay buffer from the kit and immediately
14 fixed with 10% formalin. Fluorescence signals were recorded using a FluoView
15 FV1000-D microscope (Olympus).
16
17 *Cell-based high-throughput screening for tissue α-L-fucosidase (tFuc) inhibitors:* HeLa
18 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After incubating the
19 cells at 37 °C for 24 h in 5% CO_2, the medium was replaced with 259 μL of fresh
20 medium. Subsequently, 1 μL of 3 mM DMSO solution of substrate **3** (11.5 μM final
21 concentration) was added to each well, followed by incubation at 37 °C for 6 h to 18 h in
22 5% CO_2. The medium was then removed, and the cells were washed three times with
23 PBS. After washing, 260 μL of PBS was added to each well, and fluorescence signals
24 from each well were recorded using a microplate reader (Synergy 2; excitation, 485/20
25 nm; and emission, 528/20 nm; BioTek Instruments, Winooski, VT, USA). We defined
26 the optimal assay parameters based on Z'-factor = $1-(3 \times SD_{\text{substrate } \mathbf{3}} + 3 \times SD_{DMSO})$ /
27 $(AV_{\text{substrate } \mathbf{3}} - AV_{DMSO})$, CV (%) = $(SD_{\text{substrate } \mathbf{3} \text{ or DMSO}} / AV_{\text{substrate } \mathbf{3} \text{ or DMSO}}) \times 100$, and
28 Signal / Background ratio = $AV_{\text{substrate } \mathbf{3}} / AV_{DMSO}$.
29

1 *Cell-based tFuc-inhibition assay:* HeLa cells were seeded in 6-wells plate at 4 × 10^4
2 cells/dish. After incubating the cells at 37 °C for 24 h in 5% CO_2, the medium was
3 replaced with 2 mL of fresh medium. Subsequently, 10 µL of 50 mM H_2O solution of
4 DFJ•HCl was added to each well (250 µM final concentration), followed incubation at
5 37 °C for 18 h in 5% CO_2. Next, 5 µL of 20 mM DMSO solution of substrate **3** was
6 added to each well (5 µM final concentration), followed by incubation at 37 °C for 1 h in
7 5% CO_2. The medium was then removed and cells were immediately fixed with 10%
8 formalin. Fluorescence signals were recorded using a fluorescence microscope
9 (BIOREVO BZ-9000; Keyence, Osaka, Japan) equipped with filter sets for green
10 fluorescence protein-bandpass (excitation, 470/40 nm; dichroic filter, 495 nm; and
11 emission, 535/50 nm) for 2MeTG measurements. The area of fluorescence (AF) of the
12 observed cells was analyzed using BZ-analyzer (v.2.1; Keyence, Osaka, Japan) and
13 WinROOF 2013 (v.1.2.0; Mitani, Tokyo, Japan). A region including approximately 100–
14 120 cells in the fluorescence image was selected to measure the AF stained by substrate
15 **3**. The average value per cell for both variables was calculated by dividing the total value
16 by the number of semi-manually counted cells. The value of wells was calculated by
17 averaging the values after performing the rejection test with the standard deviation for
18 the five measurement values of each well. The inhibition rate was defined as follows:
19 inhibition rate (%) = (AF DFJ − AF background) / (AF DMSO, negative control − AF
20 background) × 100.
21
22 **General information for *in vitro* assays:** Recombinant human tFuc from *FUCA1* gene
23 (7039-GH) was purchased from R&D Systems (Minneapolis, MN, USA). A
24 flat-bottomed 96 well plate for fluorescent measurement (237105) was purchased from
25 Thermo Fisher Scientific (Waltham, MA, USA). The assays were followed by
26 monitoring the fluorescence intensity change for fluorophore, resorufin, TFMU, and
27 2MeTG, respectively, for 20 min at 120 s intervals using a multi-mode microplate reader
28 (Spark 10M; TECAN, Zürich, Switzerland) at the following conditions: for resorufin
29 measurements (excitation; 570 nm, emission; 590 nm, and gain; 100), for TFMU

1 measurements (excitation; 385 nm, emission; 585 nm, and gain; 125), and for 2MeTG
2 measurements (excitation; 480 nm, emission; 525 nm, and gain; 50).
3
4 *In vitro α-L-fucosidase assays of recombinant human tFuc.* Substrates **4** to **6** were
5 prepared as 50 mM DMSO solutions. These substrate solutions were diluted to 100 μM
6 in 50 mM sodium acetate buffer including 5 mM $MgCl_2$ (pH 4.5). Recombinant human
7 tFuc was prepared as 2 ng/μL solutions in 50 mM sodium acetate buffer including 5 mM
8 $MgCl_2$ (pH 4.5). The assays were conducted by adding recombinant human tFuc solution
9 (50 μL) to each substrate solution (50 μL), followed by maintenance at 37 °C using a 96
10 well plate and monitoring of the fluorescence intensity change. For substrate blanks, the
11 enzyme solutions were replaced by the same assay buffer.
12 The fluorescence intensity by enzyme hydrolytic action was defined as follows:
13 fluorescence intensity = fluorescence intensity of substrate − fluorescence intensity of
14 substrate blank. Relative rate of hydrolysis was expressed by comparison with the
15 amount of fluorescence intensity that was released from corresponding substrates at 20
16 min, which was taken as 100%.
17



18

19 **Figure S5.** Hydrolytic activity of recombinant human tFuc for substrates **4** to **6**.

Gene-expression profiling by DNA microarray: HeLa cells were seeded in a 25-cm^2 flask, and after incubation at 37 °C for 5 h in 5% CO_2, the medium was replaced with 4 mL of fresh medium. Subsequently, 10 µL of 100 mM H_2O solution of DFJ•HCl (250 µM final concentration) and 10 µL of H_2O (negative control) were added to each flask, followed by incubation at 37 °C for 18 h in 5% CO_2. The medium was then removed, and the cells were washed three times with PBS. The cells were then detached using 1 mL of trypsin/EDTA solution and collected in a 1.5-mL tube. Extraction and purification of total RNA from these samples were performed using an RNeasy Mini Kit (74104; Qiagen) according to manufacturer instructions. Concentrations of purified RNA samples were measured using a micro-volume spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Waltham, MA, USA). The quality of the RNA samples was evaluated using an RNA 6000 Nano kit (5067-151; Agilent Technologies, Santa Clara, CA, USA) according to manufacturer instructions. Purified, high-quality RNA (1 µg) was converted, amplified, and labeled as cDNA using an Amino Allyl MessageAmp II RNA amplification kit (AM1753; Ambion, Austin TX, USA) and a Cy5 mono-reactive dye pack (PA25001; GE Healthcare, Pittsburgh, PA, USA) according to manufacturer instructions. Obtained cDNAs were hybridized to a 3D-Gene human oligo chip 25k (v.2.1; TRT-XR126; Toray Industries, Tokyo, Japan) according to manufacturer instructions. The chips were scanned (3D-Gene scanner 3000; Toray Industries), and all obtained microarray data were processed using Microsoft Excel (Microsoft, Redmond, WA, USA) and GenMAPP (v.2.1; http://www.genmapp.org/).

To compare the effects of DFJ treatment relative to negative control cells and identify differentially expressed genes relative to a negative control, normalized data from each array were globally normalized, such that the median of the signal intensity was adjusted to 25. The number of detected genes present at significant levels (global normalization value >100) was determined. Among the detected genes in the DFJ-treated cells, the expression levels of six genes were upregulated (fold change >2.5) and those of other genes were downregulated (fold change <0.45) relative to negative control cells

1 (Tables S1 and S2). Pathway analysis of microarray data to investigate the key molecular
2 events and functions involved in tFuc inhibition was performed using GenMAPP and
3 subjected to the following filtering criteria: 1) number of changed (i.e., significantly
4 differentially expressed) genes in a pathway ≥ 3; 2) z-score ≥ 2.0; and 3) permutation
5 p-value ≤ 0.03. As a result, two identified pathways were upregulated, and two were
6 downregulated (Table S3).
7
8 **Table S1.** List of upregulated (fold change >2.5) genes following tFuc inhibition in
9 HeLa cells.

Gene name	Fold change	$\log_2$ fold change	Protein name
PGA3	6.51	2.70	Pepsinogen 3, Pepsinogen A, group 1
RAB15	4.71	2.24	Member of RAS oncogene family
ALPI	2.98	1.57	Intestinal alkaline phosphatase
RUNX1	2.71	1.44	Runt-related transcription factor 1
IFIT1	2.67	1.42	Interferon-induced protein with tetratricopeptide repeats 1
ARRB2	2.51	1.33	Arrestin-β2

10
11 **Table S2.** List of downregulated (fold change >0.45) genes following tFuc inhibition in
12 HeLa cells.

Gene name	Fold change	$\log_2$ fold change	Protein name
JAG1	0.50	−1.01	Replication protein A 70-kDa DNA-binding subunit
COX7B	0.49	−1.03	Cytochrome c oxidase subunit 7B, mitochondrial
CDH2	0.49	−1.04	Cadherin-2
HIF1A	0.49	−1.04	Hypoxia-inducible factor-1α
DACH2	0.47	−1.11	Dachshund homolog 2
BAT2D1	0.46	−1.11	Protein PRRC2C
CSNK2A1	0.46	−1.13	Casein kinase II subunit-α

13

Table S3. Upregulated and downregulated pathways following tFuc inhibition in HeLa cells.

Pathway name	No. genes showing changes	Z score	P
Small-ligand GPCRs (up)	3	6.13	0.00
Kit-receptor signaling (up)	3	2.60	0.03
Histidine metabolism (down)	3	3.90	0.01
AMP-activated protein kinase signaling (down)	3	2.87	0.03

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