Supplementary Data

3-*O*-Alkyl-2,3-Dehydrosilibinins: Two Synthetic Approaches and Antiproliferative Effects toward Prostate Cancer Cells

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General synthetic procedures: NMR spectra were obtained on a Bruker Fourier 300 spectrometer in $CDCl_3$, CD_3OD, or DMSO-d_6. The chemical shifts are given in ppm referenced to the respective solvent peak, and coupling constants are reported in Hz. HRMS were obtained on an Orbitrap mass spectrometer with electrospray ionization (ESI) (Thermo Scientific). Anhydrous THF and dichloromethane were purified by PureSolv MD 7 Solvent Purification System from Innovative Technologies (MB-SPS-800). All other reagents and solvents were purchased from commercial sources and were used without further purification. Silica gel column chromatography was performed using silica gel (32-63 μm). Preparative thin-layer chromatography (PTLC) separations were carried out on thin layer chromatography plates

loaded with silica gel 60 GF254 (EMD Millipore Corporation, MA, USA). Silibinin (>98.0%) was purchased from Fisher Scientific (TCI America, Cat # 50-014-46874).

Synthesis of silibinin derivatives.

General procedure for the one-pot synthesis of 3-O-alkyl-2,3-dehydrosilibinins: To a solution of silibinin (200 mg, 0.41 mmol) in DMF (0.4 mL) was added anhydrous potassium acetate (122 mg, 1.24 mmol), and the reaction was allowed to proceed under air at 60 ^{o}C for 20 h. The reaction mixture was then cooled to room temperature, to which was added potassium carbonate (57 mg, 0.41 mmol). The reaction mixture was stirred for 3 minutes before adding alkyl iodide or alkyl bromide (0.41 mmol), and the reaction was allowed to proceed with stirring at room temperature for additional 50 h prior to being quenched with 1M HCl. After diluting with diethyl ether and ethyl acetate (200 mL, 1:1 (v/v)), the organic layer was rinsed with brine (20 mL x 5), dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was subjected to PTLC purification eluting with 5% methanol in DCM to yield the desired 3-*O*-alkyl-2,3-dehydrosilibinin. The yields were summarized in Table 1.

General procedure for the three-step synthesis of 3-O-alkyl-2,3-dehydrosilibinins: The first step is to synthesize 7-*O*-benzylsilibinin (**11**), which was achieved in 81% yield employing the procedure as described in our previous study.[1]

General procedure for the Synthesis of 3-O-alkyl-7-O-benzyl-2,3-dehydrosilibinins (step 2): To a solution of 7-*O*-benzyl-2,3-dedhydrosilbinin (100 mg, 0.17 mmol) in DMF (0.5 mL) was added anhydrous potassium carbonate (69 mg, 0.50 mmol) followed by alkyl iodide or alkyl bromide (0.19 mmol). The reaction mixture was allowed to stir at room temperature for 24 hours prior to being quenched with 1M HCl. After diluting with diethyl ether and ethyl acetate (200 mL, 1:1 (v/v)), the organic layer was rinsed with brine (15 mL x 5), dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was subjected to PTLC purification eluting with 5% methanol in

DCM to yield the respective 3-*O*-alkyl-7-*O*-benzyl-2,3-dehydrosilibinin. The yields are 54% (for compound **12**), 63% (for compound **13**), 48% (for compound **14**), 67% (for compound **15**), 52% (for compound **16**), 46% (for compound **17**), and 46% (for compound **18**).

General procedure for the synthesis of 3-O-alkyl-2,3-dehydrosibilibins (step 3): To a reaction flask, 3-*O*-alkyl-7-*O*-benzyl-2,3-dehydrosibilibin (0.12 mmol), palladium carbon (29 mg, 50% wet, 20%), ammonium formate (74 mg, 1.2 mmol), and methanol (4 mL) were added. After fully removing the air in the reaction mixture by vacuum, the reaction mixture was refluxed under argon atmosphere for 24 hours. The reaction mixture was cooled to room temperature and filtered through a silica gel pad in gravity filtration and washed with ethyl acetate (200 mL). The solvent was removed *in vacuo* to furnish the desired 3-*O*-alkyl-2,3-dehydrosilibinins. The yields for this step reaction are 83% (**2**), 59% (**3**), 98% (**4**), 84% (**5**), 87% (**6**), 86% (**7**), and 83% (**8**), respectively. The overall yields for 3-*O*-alkyl-2,3-dehydrosilibinin from this three-step procedure were listed in Table 1 in the paper.

3-*O*-Methyl-2,3-dehydrosilibinin (3): yellow solid, m.p. 157-158 ^{o}C. IR (neat) ν_{max}: 3243, 2923, 1652, 1605, 1500, 1435 cm^{-1}. ^{1}H NMR (300 MHz, CD_3COCD_3) δ: 12.74 (s, 1H), 7.71 (dd, J = 12.0, 2.1 Hz, 1H), 7.68 (s, 1H), 7.16 (d, J = 1.5 Hz, 1H), 7.05 (d, J = 8.4 Hz, 1H), 7.00 (dd, J = 8.1, 1.5 Hz, 1H), 6.91 (d, J = 8.1 Hz, 1H), 6.53 (s, 1H), 6.25 (s, 1H), 5.04 (d, J = 8.1 Hz, 1H), 4.28-4.23 (m, 1H), 3.89 (s, 6H), 3.79 (dd, J = 12.3, 2.1 Hz, 1H), 3.55 (dd, J = 12.3, 3.9 Hz, 1H). ^{13}C NMR (75 MHz, CD_3COCD_3) δ: 179.4, 165.4, 157.9, 155.8, 148.5, 148.1, 147.2, 144.8, 139.6, 128.8, 124.3, 123.0, 121.7, 117.9, 117.8, 115.8, 112.0, 105.7, 99.5, 94.6, 80.0, 77.3, 69.6, 61.6, 60.3, 56.4, 55.4. HR-MS (ESI) *m/z*: calcd for $C_{26}H_{23}O_{10}$ [M+H]: 495.1291; found 495.1285.

3-*O*-Ethyl-2,3-dehydrosilibinin (4): yellow solid, m.p. 215-216 ^{o}C. IR (neat) ν_{max}: 3190, 2926, 1652, 1605, 1586, 1453 cm^{-1}. ^{1}H NMR (300 MHz, CD_3COCD_3) δ: 12.79 (s, 1H), 7.79 (dd, J = 8.7, 1.5 Hz, 1H), 7.73 (d, J = 1.8 Hz, 1H), 7.17 (s, 1H), 7.09 (d, J = 8.4 Hz, 1H), 7.01 (d, J = 7.8 Hz, 1H), 6.91 (d, J

= 8.1 Hz, 1H), 6.54 (s, 1H), 6.26 (s, 1H), 5.06 (d, *J* = 7.8 Hz, 1H), 4.30-4.23 (m, 1H), 4.02 (q, *J* = 7.2 Hz, 2H), 3.89 (s, 3H), 3.80 (br.d, *J* = 13.2 Hz, 1H), 3.55 (br.d, *J* = 13.8 Hz, 1H), 1.31 (t, *J* = 7.2 Hz, 3H). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.60 (s, 1H), 11.07 (br.s, 1H), 9.31 (s, 1H), 7.64 (d, *J* = 8.7 Hz, 1H), 7.61 (s, 1H), 7.10 (d, *J* = 8.7 Hz, 1H), 7.01 (s, 1H), 6.87 (d, *J* = 8.1 Hz, 1H), 6.80 (d, *J* = 7.8 Hz, 1H), 6.42 (s, 1H), 6.18 (s, 1H), 5.13 (br.s, 1H), 4.93 (d, *J* = 7.5 Hz, 1H), 4.32-4.22 (m, 1H), 4.01 (q, *J* = 6.9 Hz, 2H), 3.77 (s, 3H), 3.51 (overlapped, 1H), 3.35 (br.d, *J* = 10.5 Hz, 1H), 1.21 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.4, 164.5, 161.5, 156.7, 155.3, 148.0, 147.4, 146.2, 143.7, 137.4, 127.5, 123.2, 122.4, 120.9, 117.3, 117.0, 115.7, 111.9, 104.5, 98.9, 94.1, 78.8, 76.2, 68.3, 60.4, 56.0, 15.6. HR-MS (ESI) *m/z*: calcd for $C_{27}H_{25}O_{10}$ [M+H]: 509.1448; found 509.1439.

3-*O*-Propyl-2,3-dehydrosilibinin (5): brown solid, m.p. 176–177 ^{o}C. IR (neat) ν_{max}: 3279, 2925, 1652, 1605, 1587, 1499, 1454 cm^{-1}. ^{1}H NMR (300 MHz, CD_3COCD_3) and ^{13}C NMR (75 MHz, CD_3COCD_3) δ: see Table 2 in the paper. HR-MS (ESI) *m/z*: calcd for $C_{28}H_{27}O_{10}$ [M+H]: 523.1604; found 523.1597.

3-*O*-Butyl-2,3-dehydrosilibinin (6): yellow solid, m.p. 205–206 ^{o}C. IR (neat) ν_{max}: 3247, 2925, 2737, 1652, 1606, 1499 cm^{-1}. ^{1}H NMR (300 MHz, CD_3COCD_3) δ: 12.78 (s, 1H), 7.91 (br.s, 1H), 7.73 (br.d, *J* = 8.7 Hz, 1H), 7.70 (d, *J* = 1.5 Hz, 1H), 7.15 (d, *J* = 1.2 Hz, 1H), 7.06 (d, *J* = 8.4 Hz, 1H), 7.00 (dd, *J* = 8.1, 1.2 Hz, 1H), 6.90 (d, *J* = 8.1 Hz, 1H), 6.52 (s, 1H), 6.24 (s, 1H), 5.05 (d, *J* = 7.8 Hz, 1H), 4.25-4.23 (m, 1H), 4.05 (t, *J* = 6.6 Hz, 2H), 3.88 (s, 3H), 3.79 (br.d, *J* = 12.3 Hz, 1H), 3.55 (br.d, *J* = 12.0 Hz, 1H), 1.70 (quin, *J* = 6.6 Hz, 2H), 1.44 (Hex, *J* = 7.5 Hz, 2H), 0.90 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, CD_3COCD_3) δ: 179.6, 164.9, 163.2, 157.9, 156.3, 148.6, 148.2, 147.1, 144.8, 138.8, 128.9, 124.4, 123.2, 121.7, 118.1, 117.7, 115.8, 111.9, 106.0, 99.4, 94.5, 80.1, 77.2, 72.9, 61.7, 56.4, 32.8, 19.8, 14.1. HR-MS (ESI) *m/z*: calcd for $C_{29}H_{29}O_{10}$ [M+H]: 537.1761; found 537.1753.

3-*O*-Pentyl-2,3-dehydrosilibinin (7): brown solid, m.p. 90-92 ^{o}C. IR (neat) ν_{max}: 3355, 2930, 1652, 1606, 1587, 1500, 1453 cm^{-1}. ^{1}H NMR (300 MHz, CD_3COCD_3) δ: 12.78 (s, 1H), 9.87 (br.s, 1H), 7.88

(br.s, 1H), 7.71 (d, *J* = 8.7 Hz, 1H), 7.70 (s, 1H), 7.15 (s, 1H), 7.05 (d, *J* = 8.1 Hz, 1H), 6.99 (d, *J* = 8.1 Hz, 1H), 6.90 (d, *J* = 7.8 Hz, 1H), 6.51 (s, 1H), 6.24 (s, 1H), 5.04 (d, *J* = 7.8 Hz, 1H), 4.30-4.18 (m, 1H), 4.05 (t, *J* = 6.6 Hz, 2H), 3.88 (s, 3H), 3.79 (br.d, *J* = 13.2 Hz, 1H), 3.55 (br.d, *J* = 11.7 Hz, 1H), 1.71 (quin, *J* = 6.3 Hz, 2H), 1.43-1.27 (m, 4H), 0.86 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, CD_3COCD_3) δ: 179.6, 164.9, 163.2, 157.9, 156.3, 148.6, 148.2, 147.2, 144.8, 138.8, 128.9, 124.4, 123.2, 121.7, 118.1, 117.7, 115.8, 112.0, 106.0, 99.4, 94.5, 80.1, 77.3, 73.2, 69.7, 56.4, 30.5, 28.9, 23.1, 14.3. HR-MS (ESI) *m/z*: calcd for $C_{30}H_{31}O_{10}$ [M+H]: 551.1917; found 551.1910.

3-*O*-Hexyl-2,3-dehydrosilibinin (8): brown solid. m.p. 108-109 ^{o}C. IR (neat) ν_{max}: 3383, 2933, 2870, 1654, 1588, 1500, 1456 cm^{-1}. ^{1}H NMR (300 MHz, CD_3COCD_3) δ: 12.79 (br.s, 1H), 7.71 (s, 2H), 7.16 (s, 1H), 7.06 (d, *J* = 7.5 Hz, 1H), 7.00 (d, *J* = 6.0 Hz, 1H), 6.90 (d, *J* = 7.8 Hz, 1H), 6.52 (br.s, 1H), 6.25 (s, 1H), 5.05 (d, *J* = 7.5 Hz, 1H), 4.29-4.20 (m, 1H), 4.06 (t, *J* = 6.3 Hz, 2H), 3.89 (s, 3H), 3.80 (d, *J* = 12.3 Hz, 1H), 3.55 (br.d, *J* = 11.7 Hz, 1H), 1.70 (quin, *J* = 6.6 Hz, 2H), 1.41 (quin, *J* = 6.3 Hz, 2H), 1.31-1.23 (m, 4H), 0.85 (t, *J* = 6.0 Hz, 3H). ^{13}C NMR (75 MHz, CD_3COCD_3) δ: 179.6, 165.1, 163.0, 157.9, 156.2, 148.5, 148.1, 147.1, 144.8, 138.8, 128.9, 124.4, 123.1, 121.7, 118.2, 117.7, 115.8, 112.0, 105.8, 99.4, 94.6, 80.1, 77.3, 73.2, 61.7, 56.4, 32.3, 30.7, 26.4, 23.3, 14.3. HR-MS (ESI) *m/z*: calcd for $C_{31}H_{33}O_{10}$ [M+H]: 565.2074; found 565.2069.

3-*O*-Heptyl-2,3-dehydrosilibinin (9): brown solid. m.p. 106–107 ^{o}C. IR (neat) ν_{max}: 3339, 2922, 2852, 1652, 1605, 1498, 1465 cm^{-1}. ^{1}H NMR (300 MHz, CD_3COCD_3) δ: 12.79 (s, 1H), 7.70 (s, 2H), 7.15 (s, 1H), 7.05 (d, *J* = 8.1 Hz, 1H), 7.00 (d, *J* = 8.1 Hz, 1H), 6.90 (d, *J* = 7.8 Hz, 1H), 6.52 (s, 1H), 6.25 (s, 1H), 5.04 (d, *J* = 7.8 Hz, 1H), 4.24-4.22 (m, 1H), 4.06 (t, *J* = 6.6 Hz, 2H), 3.89 (s, 3H), 3.80 (br.d, *J* = 13.2 Hz, 1H), 3.55 (br.d, *J* = 10.8 Hz, 1H), 1.70 (quin, *J* = 6.3 Hz, 2H), 1.40 (quin, *J* = 7.2 Hz, 2H), 1.33-1.22 (m, 6H), 0.85 (t, *J* = 6.0 Hz, 3H). ^{13}C NMR (75 MHz, CD_3COCD_3) δ: 179.6, 164.9, 163.1, 157.8, 156.2, 148.5, 148.1, 147.1, 144.7, 138.8, 128.8, 124.4, 123.1, 121.6, 118.1, 117.6, 115.8, 111.9,

105.9, 99.4, 94.5, 80.1, 77.2, 73.2, 61.7, 56.4, 32.5, 30.8, 29.8, 26.7, 23.2, 14.4. HR-MS (ESI) *m/z*: calcd for $C_{32}H_{35}O_{10}$ [M+H]: 579.2230; found 579.2222.

3-*O*-Benzyl-2,3-dehydrosilibinin (10): brown solid, m.p. 154-155 ^{o}C. IR (neat) ν_{max}: 3431, 3177, 1654, 1609, 1506 cm^{-1}. ^{1}H NMR (300 MHz, CD_3COCD_3) δ: 12.78 (s, 1H), 9.85 (s, 1H), 7.86 (s, 1H), 7.69 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.65 (d, *J* = 2.1 Hz, 1H), 7.45-7.42 (overlapped, 2H), 7.33-7.27 (overlapped, 3H), 7.15 (d, *J* = 1.8 Hz, 1H), 7.02 (d, *J* = 8.4 Hz, 1H), 7.00 (dd, *J* = 8.1, 1.8 Hz, 1H), 6.90 (d, *J* = 8.1 Hz, 1H), 6.53 (d, *J* = 1.8 Hz, 1H), 6.27 (d, *J* = 1.8 Hz, 1H), 5.13 (s, 2H), 5.05 (d, *J* = 8.1 Hz, 1H), 4.27-4.22 (m, 1H), 3.88 (s, 3H), 3.79 (br.d, *J* = 12.6 Hz, 1H), 3.55 (br.d, *J* = 12.0 Hz, 1H). ^{13}C NMR (75 MHz, CD_3COCD_3) δ: 179.6, 165.0, 163.2, 157.9, 156.8, 148.6, 148.2, 147.2, 144.7, 138.1, 137.8, 129.6, 129.1, 129.0, 128.9, 124.2, 123.3, 121.7, 118.2, 117.6, 115.8, 111.9, 105.9, 99.5, 94.6, 80.1, 77.2, 74.7, 61.7, 56.4. HR-MS (ESI) *m/z*: calcd for $C_{32}H_{27}O_{10}$ [M+H]: 571.1604; found 571.1599.

Cell culture: All cell lines were initially purchased from American Type Culture Collection (ATCCTM). The PC-3 and LNCaP prostate cancer cell lines were routinely cultured in RPIM-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. The DU145 prostate cancer cells were routinely cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. Cultures were maintained in a high humidity environment supplemented with 5% carbon dioxide at a temperature of 37^{o}C.

WST-1 cell proliferation assay: PC-3, DU145, or LNCaP cells were plated in 96-well plates at a density of 3,200 each well in 200 µL of culture medium. The cells were then treated with silibinin, or synthesized silibinin derivatives separately at 5 different doses for 3 days, while equal treatment volumes of DMSO (0.25%) were used as vehicle control. The cells were cultured in a CO_2 incubator at 37 ^{o}C for three days. 10 µL of the premixed WST-1 cell proliferation reagent (Clontech) was added to

each well. After mixing gently for one minute on an orbital shaker, the cells were incubated for additional 3 hours at 37 ^{o}C. To ensure homogeneous distribution of color, it is important to mix gently on an orbital shaker for one minute. The absorbance of each well was measured using a microplate reader (Synergy HT, BioTek) at a wavelength of 430 nm. The IC_{50} value is the concentration of each compound that inhibits cell proliferation by 50% under the experimental conditions and is the average from at least triplicate determinations that reproducible and statistically significant. For calculating the IC_{50} values, a linear proliferative inhibition was made based on at least five dosages for each compound.

Cell cycle analysis: PC-3 cells were plated in 24-well plates at a density of 200,000 each well in 400 μL of culture medium. After 3 hours of cell attachment, the cells were then treated with derivative **2** or **5** at 50 μM for 16 hours and 24 hours, while equal treatment volumes of DMSO were used as vehicle control. The cells were cultured in CO_2 incubator at 37°C for 16 hours and 24 hours, respectively. Both attached and floating cells were collected in a centrifuge tube by centrifugation at rcf value 450 g for 5 minutes. After discarding the supernatant, the collected cells were re-suspended with 500 μL 80% cold ethanol to fix for 30 minutes in 4°C. The fixed cell could store in -20°C for one week. After fixation, the ethanol was removed after centrifuging and the cells were washed with PBS. The cells were then re-suspend with 100 μL of 100 mg/mL ribonuclease and were cultured at 37°C for 30 minutes to degrade all RNA. The cells were stained with 200 μL of 50μg/mL propidium iodide stock solution for 30 minutes at -20°C, and then the fluorescence intensity of PI was detected in individual PC-3 cells using an Attune flow cytometer (Life Technologies) within 0.5 to 1 hour after staining.

F2N12S and CYTOX AADvanced double staining assay: PC-3 cells were plated in 24-well plates at a density of 200,000 each well in 400 μl of culture medium. After 3 hours of cell attachment, the cells were then treated with each test compound at different concentration for 15 hours, while equal

treatment volumes of DMSO were used as vehicle control. The cells were cultured in CO_2 incubator at 37°C for 15 hours. Both attached and floating cells were collected in a centrifuge tube by centrifugation at rcf value 450 g for 5 to 6 minutes. The collected cells were re-suspended with 500 µl HBSS to remove proteins which may affect flow signal and centrifuged again. After discarding the supernatant, the collected cells were re-suspended with 300 µl HBSS and stained with 0.3 µl of F2N12S for 3-5 minutes followed by 0.3 µl SytoxAAdvanced for an additional 5 minutes. The fluorescence intensity of the two probes was further measured in individual PC-3 cells using an Attune flow cytometer (Life Technologies) 0.5 to 1 hour after staining.

Statistical analysis: All data are represented as the mean ± standard deviation (S.D.) for the number of experiments indicated. Other differences between treated and control groups were analyzed using the Student's t-test. A p-value < 0.05 was considered statistically significant.

References:

1. Vue, B.; Zhang, S.; Zhang, X.; Parisis, K.; Zhang, Q.; Zheng, S.; Wang, G.; Chen, Q.-H. *Eur. J. Med. Chem.* **2016**, *109*, 36.