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Electronic Supplementary Information

Relative Impact of 3- and 5- Hydroxyl Groups of Cytosporone B on Cancer Cell Viability

Zebin Xia,[1] Xihua Cao,[1] Elizabeth Rico-Bautista, Jinghua Yu, Liqun Chen, Jiebo Chen, Andrey Bobkov, Dieter A. Wolf, Xiao-Kun Zhang, and Marcia I. Dawson*

Cancer Center, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Rd., La Jolla, CA 92037, USA

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General chemistry methods:

Chemicals and solvents from commercial sources were used without further purification. The DIM-arene **4** (DIM-Ph-4-CF_3) was prepared as reported by Dawson et al.[1] Experimental procedures were not optimized. Anhydrous and/or oxygen-sensitive reactions were carried out under argon gas. Reactions were monitored by thin-layer chromatography on silica gel (mesh size 60, F_{254}) with visualization under UV light. Unless specified, the standard workup involved washing the organic extract with water and brine and drying over anhydrous sodium sulfate followed by concentration at reduced pressure. Chromatography refers to flash column chromatography on silica gel (Merck 60, 230-400 mesh). Melting points of samples were determined in capillary tubes using a Mel-Temp II apparatus and were uncorrected. Infrared spectra of powdered or liquid samples were obtained using an FT-IR Mason satellite spectrophotometer. ^{1}H NMR spectra were recorded on a 300-MHz Varian Unity Inova or a 400-MHz ECS Jeol spectrometer. Chemical shifts are expressed in ppm (δ) relative to $CHCl_3$ as the internal standard.

Synthetic methods of cytosporone B (1), 5-Me-Csn-B (2) and 3,5-$(Me)_2$-Csn-B (3):

Ethyl 2-(3,5-dimethoxyphenyl)acetate (6). To a solution of (3,5-dimethoxyphenyl)-acetic acid (**5**) (1.90 g, 9.68 mmol) in benzene (14 mL) was added ethanol (3.5 mL, 60 mmol) and concentrated H_2SO_4 (0.23 mL, 4.3 mmol). The resulting mixture was heated for 21 h at reflux (109 ˚C oil-bath) in a round-bottomed flask attached to a Dean-Stark trap to remove the water produced and then cooled. Solvents were removed at reduced pressure, and the residue was dissolved in ethyl acetate (100 mL). This solution was washed with 1 N aqueous sodium hydroxide (40 mL) and brine and dried. After removal of solvent at reduced pressure, the residue was purified by chromatography on silica gel (16–20% ethyl acetate/hexane) to give 2.03 g (93%) of **6** as a colorless liquid. IR 2943, 2845, 1734, 1597, 1151 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.26 (t, J = 7.4 Hz, 3H, OCH_2CH_3), 3.54 (s, 2H, CH_2CO), 3.78 (s, 6H, 2 OCH_3), 4.15 (q, J = 7.4 Hz, 2H, OCH_2CH_3), 6.37 (t, J = 2.3 Hz, 1H, 4-ArH), 6.44 ppm (d, J = 2.3 Hz, 2H, 2,6-ArH). The ^{1}H NMR data are in agreement with those reported.[2]

Ethyl 2-(3,5-dihydroxyphenyl)acetate (7). To a stirred solution of **6** (0.40 g, 1.79 mmol) in dichloromethane (20 mL) was added 1.0 M boron tribromide (8.97 mmol) in dichloromethane (8.97 mL) at –78 °C under argon. After 30 min, the temperature was raised to 0 °C and stirring was continued for 4 h, at which time the reaction mixture was quenched by addition of water (35 mL) and extracted with ethyl acetate (100 mL). The organic extract

was washed (brine) and dried. After solvent removal at reduced pressure, the residue was purified on silica gel (40–46% ethyl acetate/hexane) to give 295 mg (84%) of **7** as a white solid, mp 73–74 °C. IR 3346, 2939, 2841, 1726, 1602, 1178 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.27 (t, *J* = 6.9 Hz, 3H, OCH_2CH_3), 3.50 (s, 2H, CH_2CO), 4.16 (q, *J* = 6.9 Hz, 2H, OCH_2CH_3), 5.64 (s, 2H, OH), 6.23 (t, *J* = 2.3 Hz, 1H, 4-ArH), 6.33 ppm (d, *J* = 2.3 Hz, 2H, 2,6-ArH). The ^1H NMR data were in agreement with those reported.[3]

Ethyl 2-(3,5-dibenzyloxyphenyl)acetate (8). To a solution of **7** (161 mg, 0.82 mmol) in acetone (5 mL) was added benzyl bromide (315 mg, 1.81 mmol) followed by powdered potassium carbonate (272 mg, 1.97 mmol). The mixture was stirred at reflux temperature for 22 h. Acetone was removed at reduced pressure, and 0.5N hydrochloric acid (25 mL) was added. The acidified mixture was extracted with ethyl acetate (80 mL), and the organic layer was washed and dried. After removal of solvent at reduced pressure, the residue was purified on silica gel (15–16% EtOAc/hexane) to give 303 mg (98%) of **8** as a colorless liquid. IR 2923, 2870, 1728, 1591, 1142, 1025 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.26 (t, *J* = 7.3 Hz, 3H, OCH_2CH_3), 3.54 (s, 2H, CH_2CO), 4.13 (q, *J* = 7.3 Hz, 2H, OCH_2CH_3), 5.02 (s, 4H, $PhCH_2O$), 6.55 (m, 3H, 2,4,6-ArH), 7.3–7.43 ppm (m, 10H, C_6H_5). Compound **8** has been reported without characterization data [3]

Ethyl 2-(3,5-dibenzyloxy-2-octanoylphenyl)acetate (9) and ethyl 2-(3-benzyloxy-5-hydroxy-2-octanoylphenyl)acetate (10). To a stirred mixture of **8** (301 mg, 0.80 mmol) and *n*-octanoic acid (127 mg, 0.88 mmol) in trifluoroacetic anhydride (489 μL) was added 85% aqueous phosphoric acid (60 μL). This mixture was stirred for 24 h, quenched with 5% aqueous sodium bicarbonate (30 mL) and extracted with ethyl acetate (90 mL). The organic layer was washed and dried. After removal of solvent at reduced pressure, the residue was purified by chromatography on a silica gel (20–33% ethyl acetate/hexane) to give 15 mg (4%) of **9** as a colorless liquid and 140 mg (42%) of **10** as a light-yellow liquid.

9: IR 2927, 2855, 1735, 1680, 1602, 1316, 1157 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.86 (t, *J* = 6.9 Hz, 3H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.16–1.32 (m, 8H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.25 (t, *J* = 7.3 Hz, 3H, OCH_2CH_3), 1.55 (m, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 2.82 (t, *J* = 7.6 Hz, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 3.62 (s, 2H, CH_2CO), 4.12 (q, *J* = 7.3 Hz, 2H, OCH_2CH_3), 5.02 (s, 2H, 3-$PhCH_2O$), 5.04 (s, 2H, 5-$PhCH_2O$), 6.49 (d, *J* = 2.3 Hz, 1H, 4-ArH), 6.54 (d, *J* = 2.3 Hz, 1H, 6-ArH), 7.32–7.41 ppm (m, 10H, C_6H_5). Compound **9** has been reported but without characterization data.[3]

10: IR 3352, 2927, 2854, 1734, 1712, 1604, 1318, 1161 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.86 (t, *J* = 6.9 Hz, 3H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.14–1.29 (m, 8H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.27 (t, *J* = 7.3 Hz, 3H, OCH_2CH_3), 1.55 (m, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 2.80 (t, *J* = 7.8 Hz, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 3.56 (s, 2H, CH_2CO), 4.15 (q, *J* = 7.3 Hz, 2H, OCH_2CH_3), 4.99 (s, 2H, 3-$PhCH_2O$), 5.61 (s, 1H, 5-OH), 6.31 (d, *J* = 2.3 Hz, 1H, 4-ArH), 6.36 (d, *J* = 2.3 Hz, 1H, 6-ArH), 7.31–7.37 ppm (m, 5H, C_6H_5).

Cytosporone B (1). A solution of **10** (138 mg, 0.33 mmol), 1.0 M boron tribromide (1.7 mmol) in dichloromethane (1.7 mL) and dichloromethane (4 mL) was stirred for 2 h at –78 °C, then quenched with water (30 mL) and extracted with ethyl acetate (80 mL). The extract was washed (brine) and dried. After solvent removal at reduced pressure, the residue was purified on silica gel (25 to 33% ethyl acetate/hexane) to give 105 mg (98%) of **1** as a light-yellow solid, mp 80–82 °C. IR 3339, 2927, 2856, 1710, 1610, 1268, 1162 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.86 (t, *J* = 6.9 Hz, 3H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.14–1.29 (m, 8H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.27 (t, *J* = 7.3 Hz, 3H, OCH_2CH_3), 1.70 (m, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 2.83 (t, *J* = 7.6 Hz, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 3.84 (s, 2H, CH_2CO), 4.20 (q, *J* = 7.3 Hz, 2H, OCH_2CH_3), 5.70 (s, 1H, 5-OH), 6.27 (d, *J* = 2.3 Hz, 1H, 4-ArH), 6.31 (d, *J* = 2.3 Hz, 1H, 6-ArH), 12.22 ppm (s, 1H, 3-OH). All characterization data are in agreement with those reported. [3–5]

3,5-Dimethylcytosporone B (3). To a stirred solution of *n*-octanoic acid (0.57 g, 3.9 mmol) in trifluoroacetic anhydride (2.18 mL, 15.7 mmol) was added 85% aqueous phosphoric acid (453 mg, 3.93 mmol). After 5 min, **6** (800 mg, 3.57 mmol) was added and stirrring was continued for 23 h. The reaction mixture was quenched by adding 5% aqueous sodium bicarbonate (40 mL) and extracted with ethyl acetate (100 mL). The organic layer was washed and dried. After removal of solvent at reduced pressure, the residue was purified on silica gel (15% ethyl acetate/hexane) to give 1.2 g (96%) of **3** as a light-yellow liquid. IR 2929, 2855, 1736, 1681, 1603, 1317, 1155 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.9 Hz, 3H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.22–1.34 (m, 8H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 1.25 (t, *J* = 7.3 Hz, 3H, OCH_2CH_3), 1.64 (m, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 2.82 (t, *J* = 7.6 Hz, 2H, $C(O)CH_2CH_2(CH_2)_4CH_3$), 3.61 (s, 2H, CH_2CO), 3.81 (s, 6H, 2 × OCH_3), 4.14 (q, *J* = 7.3 Hz, 2H, OCH_2CH_3), 6.37 (d, *J* = 2.3 Hz, 1H, 4-ArH), 6.40 ppm (d, *J* = 2.3 Hz, 1H, 6-ArH). The ^1H NMR data are in agreement with those reported.[5]

5-Methylcytosporone B (2). A solution of **3** (380 mg, 1.08 mmol), 1.0 M boron tribromide (4.4 mmol) in dichloromethane (4.4 mL) and dichloromethane (10 mL) was stirred at –78 °C under argon for 2 h, quenched with water (30 mL) and extracted with ethyl acetate (100 mL). The extract was washed (brine) and dried. After solvent

removal at reduced pressure, the residue was purified on silica gel (15–16% ethyl acetate/hexane) to give 334 mg (98%) of **2** as a white solid, mp 70–72 °C. IR 3344, 2928, 2855, 1737, 1709, 1611, 1314, 1158 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.9 Hz, 3H, C(O)$CH_2CH_2(CH_2)_4CH_3$), 1.23–1.34 (m, 8H, C(O)$CH_2CH_2(CH_2)_4CH_3$), 1.26 (t, *J* = 7.3 Hz, 3H, OCH_2CH_3), 1.70 (m, 2H, C(O)$CH_2CH_2(CH_2)_4CH_3$), 2.83 (t, *J* = 7.6 Hz, 2H, C(O)$CH_2CH_2(CH_2)_4CH_3$), 3.81 (s, 3H, 5-OCH_3), 3.86 (s, 2H, CH_2CO), 4.18 (q, *J* = 7.3 Hz, 2H, OCH_2CH_3), 6.33 (d, *J* = 2.3 Hz, 1H, 4-ArH), 6.39 ppm (d, *J* = 2.3 Hz, 1H, 6-ArH). The ^1H NMR data are in agreement with those reported.[5]

Computational studies:

Docking to the crystal structure of the human NR4A1 ligand-binding domain (LBD) (PDB code 2QW4) was achieved using BioMed Cache 6.2 software (Fujitsu Limited, Beaverton, OR). The ligand-binding pocket (LBP) was derived by selecting all neighboring residues within 10 Å (radius) of that reported for cytoB.[1, 3] As the objective was to compare the conformation of cytosporone B as reported by Liu et al.[3] with those of the two methyl ether derivatives, the octanoyl side chains were kept fully extended and the ethyl acetate side chain was drawn as shown in its reported docking pose.[3] Potential energies of the various conformers obtained by rotating either the octanoyl or the acetate side chain about the bond connecting it to the benzene ring were then calculated to produce potential energy maps. The lowest energy conformations within 3 kcal in each low-energy well were then used for docking. During the docking process the energy-minimized ligands were kept rigid and side chains of pocket residues were allowed to be flexible. On the basis of their docking scores, we chose those poses having the lowest energy as the final binding modes to be illustrated in Fig. 3. The final docking poses of Csn-B, 5-Me-Csn-B and 3,5-$(Me)_2$-Csn-B (**1**–**3**) were analyzed by measuring interatom distances after superposing the helical backbones of the NR4A1 LBD poses.

The docking of compounds Csn-B and 5-Me-Csn-B, having conformations in which an intra-molecular H-bond existed between the oxygen of the 2-octanoyl side chain and the 3-hydroxyl group of the benzene ring was also performed by keeping ligand conformations rigid and the LBP residue side chains flexible (**A** and **B**) or keeping both flexible (**C** and **D**). The docking results are shown in Figure S1. All docked poses exhibited an H-bond between Y122 O and the OH at 3-position of the benzene ring with the distances between Y122 O and 3-OH O from 2.50 to 2.66 Å. The 3-OH and the 2-octanoyl O of Csn-B and 5-Me-Csn-B still forms an intra-molecular H-bond, but compared to 2.78 Å (A and B), the distance of O···O was changed during the full flexible docking, e.g. 2.89 Å (C) and 2.71 Å (D).

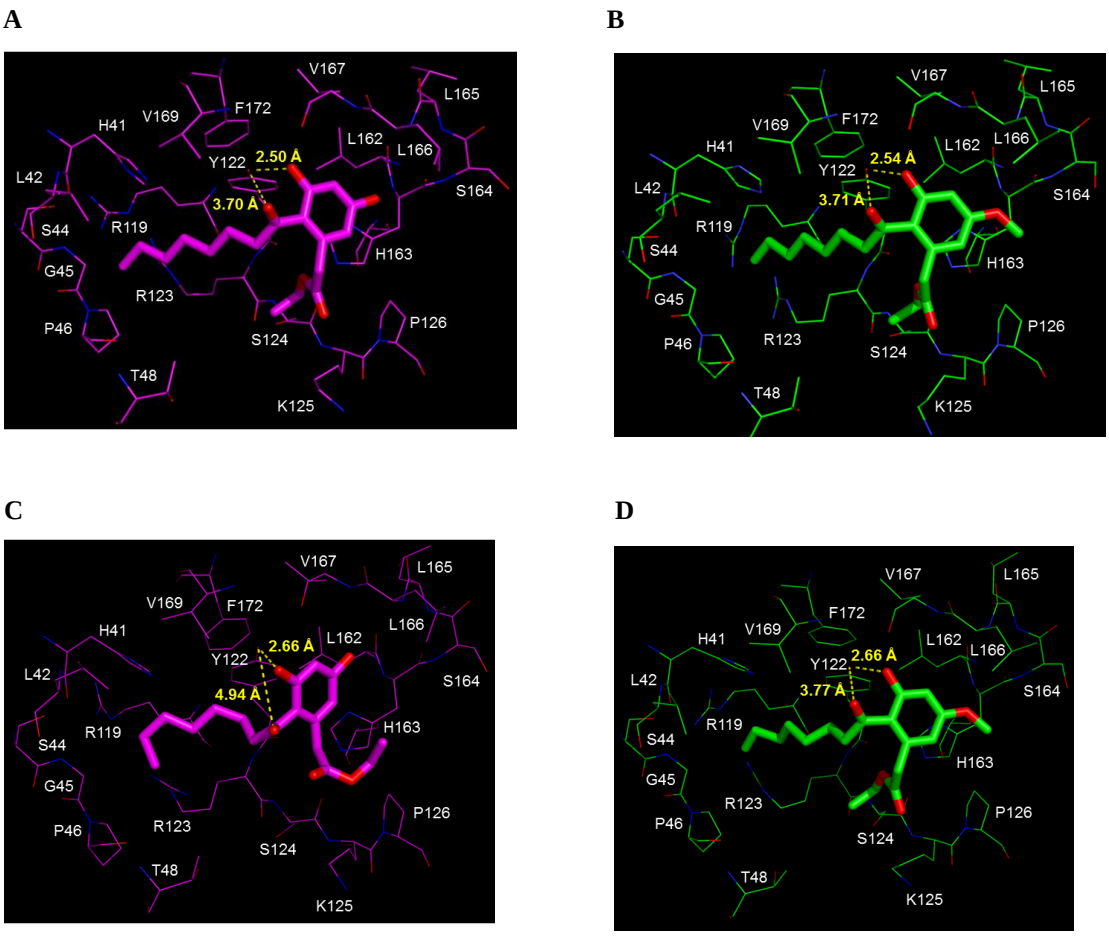

Fig. S1 Cytosporone B (Csn-B, **1**) and 5-Me-Csn-B (**2**) having an intra-molecular H-bond between the O atom of the octanoyl side chain and the 3-OH group of the benzene ring were docked to the human NR4A1 ligand-binding domain (PDB crystal structure code 2QW4) as described in the methods. The docked poses in **A** and **B**, respectfully, were obtained by keeping the ligands rigid and the LBP residue side chain flexible, whereas the docked poses in **C** and **D** were obtained by keeping both ligand and side chains flexible. Ligand poses are shown in stick format and residue side chains in line format. Carbon atoms in the pose for Csn-B and the accompanying NR4A1 binding-pocket side chains are shown in magenta (**A** and **C**), those for 5-Me-Csn-B and accompanying side chains are in green (**B** and **D**). Nitrogen atoms are colored blue; oxygens, red; and sulfurs, yellow. Residue numbering is that used for the human NR4A1 LBD.

Bioevaluation methods:

Inhibition of cancer cell viability:

Cancer cell lines were obtained from ATTC® (Manassas, VA). NCI-H460 NSCLC cells were cultured for 72 h in RPMI-1640 medium (Mediatech, Manassas, VA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT) in the presence of compound at 0.1, 1.0 and 10 μM or vehicle alone (DMSO, 0.1% final concentration). Cell viability was measured by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St. Louis, MO). Briefly, 2×10^{-3} exponentially growing cells/well were seeded into 96-well plates and incubated overnight before each compound was added at the indicated concentrations. Incubation was continued for

72 h, after which time MTT was added (0.5 mg/mL) and cells were incubated for 4 h more. Formazan products were solubilized with DMSO and their absorption at 490 nm was measured using a multi-well scanning microplate reader (SpectraMAX 190, Molecular Devices, Sunnyvale, CA).

LNCaP cells (5×10^3/well) in RPMI-1640 medium containing 10% FBS were plated in 96-well plates, allowed to attach for 24 h, and then treated for 72 h with increasing concentrations (1–20 μM) of compound or with DMSO alone (0.2% final concentration). Cell viability was assessed using the MTT cell proliferation assay (ATCC®) according to the manufacturer's protocol.

Points shown in Fig. 4A and Fig. 4B are the means of six replicates ± SD. IC_{50} values were calculated by intrapolation of the dose–response curves.

NR4A1 nuclear export:

H460 cells were incubated overnight in RPMI-1640 medium without serum in cover-slipped wells of 24-well plates and then treated with 10 μM compound or vehicle alone for 8 h. Cells were fixed (3.7% paraformaldehyde), permeabilized (0.3% Triton X-100 in PBS) for 10 min, incubated for 10 min in PBS containing 3% bovine serum albumin (BSA) and then stained with anti-rabbit NR4A1 XP® monoclonal antibody (D63C5, Cell Signalling, Beverly, MA), followed by goat anti-rabbit IgG conjugated with Alexa Fluor® 594 (1:1000 dilution, Life Technologies, Grand Island, NY). Nuclei were stained with 4´,6-diamidino-2-phenylindole (DAPI, Sigma). Fluorescent images were collected and analyzed using a confocal microscope (ConfocalFluoview 1000, Olympus America, Center Valley, PA). Images (Fig. 5) were overlaid to demonstrate localization of NR4A1.

Induction of apoptosis:

H460 cells in RPMI-1640 medium without serum were seeded into 6-well plates for Western analysis or into 24-well plates having coverslips for nuclear staining, allowed to attach for 12 h and then treated with 10 μM compound for 8 h. For Western analysis, cells were then collected and lysed in 50 mM Tris·HCl, pH 7.9, containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and a protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Lysates were loaded on 12% SDS-PAGE gels and separated proteins transferred to nitrocellulose membranes, which were then blocked in 5% nonfat milk in 20 mM Tris·HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20 (TBST) for 30 min, washed twice (TBST) and probed with rabbit polyclonal anti-PARP antibody (Santa Cruz Biotech, Santa Cruz, CA) overnight at 4 °C followed by horseradish peroxidase-conjugated secondary anti-rabbit IgG for 2 h at room temperature. Blots were developed using enhanced chemoluminescence (GE Healthcare, Waukesha, WI) and then reprobed with anti-β-actin antibody (Sigma) to confirm equivalent protein loading per lane.

For nuclear staining, cells were fixed with 3.7% paraformaldehyde and permeablized with 0.3% Triton X-100 in PBS for 120 min. Fixed cells were incubated in PBS containing 3% BSA for 10 min and then treated with DAPI to visualize nuclear morphology using fluorescence microscopy. The percentage of apoptotic cells showing nuclear fragmentation and/or chromatin condensation was determined by scoring 300-cell fields. Results shown in Fig. 6B represent the average of triplicates ± SD.

Ligand binding studies using nuclear magnetic resonance spectroscopy:

Human NR4A1 LBD protein (NR4A1residues 358−598) was expressed in *Escherichia coli* Bl21 as an N-His_6 construct and purified using a nickel-tethered resin (His60 Ni Superflow, Clontech Laboratories, Mountain View, CA) followed by dialysis. One-dimensional (1D) ^1H nuclear magnetic resonance (NMR) spectra were recorded using 128 transients, a 12,376-Hz sweep width, and a 3-sec repetition time on a 600-MHz NMR spectrometer (Avance, Bruker, Billerica, MA) equipped with a triple resonance cryoprobe (TCI, Bruker) to maintain the sample temperature at 11 °C. Spectra (Fig. 6) were obtained on solutions of each compound alone using Csn-B (**1**) at 100 µM and 5-Me-Csn-B (**2**) or 3,5-$(Me)_2$-Csn-B (**3**) at 50 µM in dialysis buffer containing 92.5% D_2O and 2% DMSO-d_6 or combined in the same buffer with the LBD protein at 10 µM for Csn-B and at 5 µM for 5-Me-Csn-B or 3,5-$(Me)_2$-Csn-B.

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