Chemical Analysis of Norrisolide-Induced Golgi Vesiculation

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Supporting Information

Table of Contents

1. Materials and methods ........................................ S2
2. Golgi membranes localization assay and competition studies ........................................ S3
3. Localization and competition studies with 6 ........................................ S3
4. Experimental procedures/data ........................................ S4-S10
5. NMR Spectra ........................................ S11-S26
Materials and methods

Reagents and cells: NRK cells were plated on 12 mm glass coverslips coated with Pronectin F (Sigma) and grown in complete medium (500 µL per coverslip), consisting of alpha MEM medium (GIBCO) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 25 mM Hepes pH 7.4, at 37 °C in a 5% CO₂ cell incubator. Stock solution (25 mg/mL) of norrisolide and analogues were made in DMSO and stored at -20 °C. The working concentration of the compound 4 was 80 µM per coverslip for living cells (Golgi fragmentation assay) and 25 µM for fixed cells (Golgi localization assay). The working concentration of norrisolide and other analogues was 25 µM per coverslip.

Golgi membranes fragmentation assay: To half of the coverslips (70% confluent) were added norrisolide or analogues (0.5 µL of the stock solutions). To the other half were added 0.5 µL of DMSO as negative control. Both groups of cells were incubated at 37 °C for 60 min. Part of the treated cells (Fig. 3b, 4b, 4e, 4h) and part of the control cells (Fig. 3a, 4a, 4d, 4g) were then fixed with 4% formaldehyde for 10 min and processed for immunofluorescence microscopy. The remaining cells were washed four times with phosphate-buffered saline (PBS) (150 mM NaCl, 1.8 mM NaH₂PO₄, 8.4 mM Na₂HPO₄) and then incubated in fresh complete medium at 37 °C for 90 min, then fixed with 4% formaldehyde for 10 min and processed for immunofluorescence microscopy (Fig. 3c, 4c, 4f, 4i).

Immunofluorescence microscopy: For fluorescent labeling, cells were incubated in blocking buffer (PBS containing 2.5% fetal bovine serum and 0.1% Tween 20) for 30 min at room temperature. The cells were then incubated for 1h at room temperature in primary antibody diluted in blocking buffer. Rabbit Mannosidase II antibody (1:2000) (a gift from Dr. Kelly Moreman, Vanderbilt University, TN) was used to visualize Golgi apparatus. The cells were then washed three times with PBS and incubated with secondary antibody, diluted in blocking buffer, for 1h at room temperature. Alexa Fluor 594 goat anti rabbit (1:500) from Molecular Probes was used. Cells were washed three times with PBS containing Hoescht (1:100,000) (H33342, Molecular Probes) to stain DNA. Coverslips were then mounted onto glass slides and visualized using a Nikon micophot-FXA fluorescence microscope at 60x magnification.
**Golgi membrane localization assay:** The cells were first fixed with 2% formaldehyde for 90 sec. This mild fixation induces a minimal perturbation of cellular proteins, allowing the binding between compound 4 and its cellular receptor. Cells were processed for immunofluorescence microscopy as described above. Before the coverslips were mounted onto glass slides, compound 4 was added at 25 µM for 30 min (Fig. 3d, 3e, 3f).

**Competition experiments:** Norrisolide was added after compound 4 at 100µM for 30 min. Coverslips were then mounted onto glass slides and visualized using a Nikon micophot-FXA fluorescence microscope at 60x magnification. The intracellular visualization of compound 4 was accomplished using excitation at 370 nm and emission at 460 nm.

**Golgi localization and competition experiments with 6 (Figure S1).**

**Figure S1.** Intracellular localization of compound 6 (a-c) and competition experiments between 6 and norrisolide (d-f). Golgi localization studies (a-c): Fixed cells stained with Golgi specific antibody (a); and then (b) incubated with 6. In (c) is shown the co-localization between the Golgi antibody and 6 (yellow color). The red coloring of Figure S1a is due to antibody staining, while the green coloring of Figure S1b is due to coloring by 6. Figure S1c shows in yellow/orange color the co-localization between the Golgi antibody and 6.

Competition experiment (d-f): Fixed cells preincubated with 6 (d) were treated with DMSO (e) and norrisolide (100 µM) (f). The yellow/orange color of Figure S1d is due to co-localization of antibody and 6. This color persists after treatment with DMSO as shown in Figure S1e. However, treatment of these cells with norrisolide displaces compound 6 from the Golgi membranes as evidenced by the loss of green color. The Golgi are now stained red due to the presence of antibody.
**General Chemical Techniques.** All reagents were commercially obtained (Aldrich, Acros) at highest commercial quality and used without further purification except where noted. The coumarin probe was prepared as described by Fritz, M. G.; Seebach D. in *Helv. Chim. Acta* 1998, 81, 2414-2429. The norrisolide fragments were prepared as described in reference 3. Yields refer to chromatographically and spectroscopically ($^1$H NMR, $^{13}$C NMR) homogeneous materials. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as the visualizing agent and 10% ethanolic phosphomolybdic acid (PMA) or $p$-anisaldehyde solution and heat as developing agents. E. Merck silica gel (60, particle size 0.040-0.063 mm) was used for flash chromatography. Preparative thin-layer chromatography separations were carried out on 0.25 or 0.50 mm E. Merck silica gel plates (60F-254). NMR spectra were recorded on Varian Mercury 400 and/or Unity 500 MHz instruments and calibrated using the residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. High resolution mass spectra (HRMS) were recorded on a VG 7070 HS mass spectrometer under chemical ionization (CI) conditions or on a VG ZAB-ZSE mass spectrometer under fast atom bombardment (FAB) conditions.

**Experimental procedures and data**

**Fluorescent probe 4:** Alcohol $9^3$ (15 mg, 0.08 mmol), carboxylic acid $S1^{10}$ (25 mg, 0.1 mmol), EDC (39 mg, 0.2 mmol), and DMAP (12 mg, 0.1 mmol) were dissolved in CH$_2$Cl$_2$ (1 mL) and stirred for 6 hours at 25 °C. The solvent was removed on the rotary evaporator and the residue
was applied to silica gel chromatography (25% ether in hexanes) to afford 29 mg of compound 4 (87%). 4: Rf = 0.4 (100% ether); ¹H NMR (400 MHz, CDCl₃) δ 7.39 (d, J= 9.2 Hz, 1H), 6.60 (d, J= 8.8 Hz, 1H), 6.52 (s, 1H), 6.05 (s, 1H), 4.54 (t, J= 8.4 Hz, 1H), 3.67 (s, 2H), 3.05 (s, 6H), 2.09 (m, 1H) 1.60 - 1.09 (m, 6 H), 1.11 - 0.99 (m, 4H), 0.87 (s, 3H), 0.84 (s, 3H), 0.74 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 168.9, 161.7, 155.9, 152.9, 148.7, 125.4, 110.6, 108.9, 108.5, 98.4, 84.3, 52.6, 42.6, 41.3, 40.1, 38.6, 37.5, 33.0, 32.8, 26.2, 20.7, 20.2, 19.3, 12.6; HRMS FAB, calcd for C₂₅H₃₃NO₄ [M⁺]: 411.2410, found 411.2413.

Aldehyde S3: Lactol S2³ (0.3g, 0.7 mmol), PDC (1.3 g, 3.5 mmol), and Mol Sieves (4 Å, 4g) were dissolved in CH₂Cl₂ (15 mL) and stirred for 8 hours at 25 °C. The reaction was filtered through a plug of celite and the solvent removed on the rotary evaporator. The residue was applied to silica gel chromatography (100% ether) to afford 180 mg of compound S3 (57%). S3: Rf = 0.6 (100% ether); [α]₂₅D = +11 (c= 0.3, CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ 9.69 (s, 1H), 7.66-7.61 (m, 4H), 7.43-7.33 (m, 6H), 6.08 (d, J= 5.2 Hz, 1H), 3.89-3.79 (dd, J= 10.8, 3.2 Hz, 1H), 3.81-3.73 (m, 2H), 3.48 (m, 1H), 2.75 (m, 1H), 2.61-2.30 (m, 4H), 1.07 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 199.0, 175.0, 135.5, 132.7, 129.8, 127.7, 107.2, 82.1, 63.0, 41.5, 41.3, 35.9, 28.6, 26.8, 19.2; HRMS, calcd for C₂₅H₃₀O₅Si [M+Na⁺] 461.1761, found: 461.1757.

Lactone S4: To aldehyde S3 (50 mg, 0.1 mmol), in MeOH (5 ml) cooled to 0 ° was added sodium borohydride (4 mg, 0.1 mmol) and the reaction was stirred for 10 minutes. The reaction
was quenched with 20 ml of saturated ammonium chloride (aq) and extracted with CH₂Cl₂ (3 x 20 ml). The solvent was dried (MgSO₄) and the solvent removed on the rotary evaporator. The crude alcohol (Rf= 0.3, 75% ether) was taken to the next step directly. The alcohol (50 mg, 0.1 mmol), carboxylic acid S₁ (25 mg, 0.1 mmol), DMAP (12 mg, 0.1 mmol), and EDC (20 mg, 0.1 mmol) were stirred together in CH₂Cl₂ (2mL) for 6 hours at 25 °C. The reaction was concentrated and applied to silica gel chromatography (100% ether) to afford 30 mg of compound S₄ (40%, 2 steps). S₄: Rf= 0.6 (100% ether); [α]₂₅D= +7.4 (c= 0.21, CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ 7.69-7.63 (m, 4H), 7.41-7.31 (m, 7H), 6.64 (dd, J= 8.8, 2.4 Hz, 1H), 6.53 (d, J= 2.4 Hz, 1H), 6.05 (s, 1H), 6.01 (d, J= 6 Hz, 1H), 4.19-4.08 (m, 2H), 3.95-3.66 (m, 3H), 3.70 (s, 2H), 3.07 (s, 6H), 2.63-2.21 (m, 4H), 2.83-2.67 (m, 2H), 1.05 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 175.0, 167.2, 164.7, 135.5, 132.7, 129.8, 127.7, 125.3, 125.0, 110.7, 109.3, 106.2, 99.1, 82.2, 63.1, 60.9, 41.3, 40.5, 37.9, 37.3, 30.1, 29.5, 27.7, 25.5, 20.0; HRMS, calcd for C₃₈H₄₃NOSi [M+Na⁺] 692.2656, found: 692.2647.

Fluorescent probe 5: To a solution of silyl ether S₄ (12 mg, 0.01 mmol) in THF (2 ml) was added TBAF (1M in THF, 0.05 mL, 0.05mmol) and the reaction was stirred for 2 hours at 25 °C. The solvent was removed on the rotary evaporator and the residue was subjected to silica gel chromatography (100% hexanes) to afford 3 mg of 5 (85%). 5: Rf= 0.3 (100% ether); [α]₂₅D= +9.2, c=0.21, CH₂Cl₂; ¹H NMR (400 MHz, CDCl₃) δ 7.38 (d, J= 1.2 Hz, 1H), 6.63 (dd, J= 8.8, 2.4 Hz, 1H), 6.53 (d, J= 2.4 Hz, 1H), 6.05 (s, 1H), 5.99 (d, J= 5.6 Hz, 1H), 4.19-4.08 (m, 2H), 3.91-3.63 (m, 3H), 3.70 (s, 2H), 3.07 (s, 6H), 2.59-2.23 (m, 4H), 2.81-2.65 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 174.9, 166.8, 164.9, 125.5, 125.1, 110.7, 109.0, 106.7, 99.4, 82.4, 63.3, 61.0, 41.5, 40.1, 38.1, 37.5, 30.3, 29.7, 28.0, 25.7; HRMS, calcd for C₂₂H₂₅NO₈ [M+H⁺] 432.1653, found: 432.1658.
Fluorescent probe 6: Alcohol \( \text{S5} \) (5 mg, 0.015 mmol), carboxylic acid \( \text{S1} \) (10 mg, 0.04 mmol), EDC (10 mg, 0.05 mmol), and DMAP (10 mg, 0.08 mmol) were dissolved in \( \text{CH}_2\text{Cl}_2 \) (1 mL) and stirred for 6 hours at 25 °C. The solvent was removed on the rotary evaporator and the residue was applied to silica gel chromatography (25% ether in hexanes) to afford 8.1 mg of compound 6 (91%). 6: \( R_f = 0.3 \) (100% ether); \( ^1\text{H} \) NMR (400 MHz, \( \text{CDCl}_3 \)) \( \delta \) 7.40 (d, J= 9.2 Hz, 1H), 6.63 (dd, J= 8.8, 2.8 Hz, 1H), 6.52 (d, J= 2.8 Hz, 1H), 6.05 (s, 1H), 6.00 (d, J= 5.6 Hz, 1H), 5.18 (s, 1H), 4.86 (s, 1H), 4.58 (dd, J= 12, 3 Hz, 1H), 4.18 (m, 1H), 4.05 (dd, J= 12.4, 5.2 Hz, 1H), 3.72 (s, 2H), 3.13 (m, 1H), 3.06 (s, 6H), 2.64-2.57 (m, 2H), 2.49-2.42 (m, 1H), 1.92 (m, 1H), 1.71-0.99 (m, 1H), 0.83 (s, 3H), 0.83 (s, 3H), 0.52 (s, 3H); HRMS FAB, calcd for \( \text{C}_{34}\text{H}_{43}\text{NO}_7 \) [M+] 577.3040, found: 577.3041.

Benzyl ether 7a: Methyl-2,4-dihydroxy benzoate (7) (2.0 g, 12 mmol), benzyl bromide (5.1 g, 30 mmol) and \( \text{K}_2\text{CO}_3 \) (7 g, 50 mmol) were refluxed in acetone (30 mL) for 6 hours. The reaction was cooled to room temperature and the solids were removed by filtration. The solvent was removed on the rotary evaporator and the residue was purified by silica gel chromatography (25% ether in hexanes) to afford 3.9 g of 7a (95%). 7a: \( R_f = 0.7 \) (50% ether in hexanes); \( ^1\text{H} \) NMR (400 MHz, \( \text{CDCl}_3 \)) \( \delta \) 7.88 (d, J= 8.4 Hz, 1H), 7.49 (m, 2H), 7.40-7.36 (m, 8H), 6.49 (m, 2H), 5.14 (s, 2H), 5.06 (s, 2H), 3.87 (s, 3H); \( ^{13}\text{C} \) NMR (100 MHz, \( \text{CDCl}_3 \)) \( \delta \) 166.0, 163.1, 160.1, 136.5, 136.0, 133.8, 128.5, 128.4, 128.1, 127.6, 127.4, 126.3, 112.9, 105.9, 101.3, 70.4, 70.1, 51.6; HRMS, calcd for \( \text{C}_{22}\text{H}_{20}\text{O}_4 \) [M+Na\(^+\)]: 371.1259, found 371.1263.
**Carboxylic acid 8:** Ester 7a (3.8 g, 11 mmol) was stirred in a 1/1 mixture of 1N NaOH/THF 20 mL at 50 °C for 4 hours. The reaction was cooled to 0 °C and neutralized with 1N HCl (~10 mL). The aqueous phase was extracted with EtOAc (3 x 25 mL). The combined organics were dried over magnesium sulfate and the solvent was removed on the rotary evaporator to afford 3.6 g of carboxylic acid 8 as a white solid (99%). 8: R_f = 0.2 (75% ether in hexanes); ¹H NMR (400 MHz, CDCl₃) δ 8.14 (d, J= 8.8 Hz, 1H), 7.44–7.36 (m, 5H), 6.73 (dd, J= 8.8, 2.4 Hz, 1H), 6.69 (d, J= 2 Hz, 1H), 5.22 (s, 2H), 5.11 (s, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 166.02, 163.1, 160.1, 133.8, 128.6, 128.4, 128.1, 127.6, 126.3, 112.9, 105.9, 101.3, 70.4, 70.1, 51.6; HRMS, calcd for C₂₁H₁₈O₄ [M+Na⁺]: 357.1103, found 357.1103.

**Benzyl ether 8a:** Carboxylic acid 6 (2 g, 6 mmol), alcohol 7 (0.64 g, 3.5 mmol), EDC (1.2 g, 6 mmol), and DMAP (0.73g, 6 mmol) were stirred in CH₂Cl₂ (10 mL) at 25 °C for 24 hours. The solvent was removed on the rotary evaporator and the residue was purified by silica gel chromatography (25% ether in hexanes) to afford 2.5 g of benzyl ether 8a (92%). 8a: [α]₂₅^D = +19.3 (c= 1.2, CH₂Cl₂); R_f = 0.8 (50% ether in hexanes); ¹H NMR (400 MHz, CDCl₃) δ 7.87 (d, J= 8.4 Hz, 1H), 7.45 (m, 2H), 7.41–7.36 (m, 8H), 6.60 (m, 2H), 5.12 (s, 2H), 5.07 (s, 2H), 4.73 (t, J= 8.8 Hz, 1H), 2.22 (m, 1H), 1.64–1.03 (m, 10H), 0.87 (s, 3H), 0.86 (s, 3H), 0.84 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 165.7, 162.9, 160.1, 136.4, 136.2, 133.7, 128.6, 128.5, 128.2, 127.8, 127.5, 127.4, 113.7, 105.7, 101.1, 83.3, 70.5, 70.1, 52.7, 42.6, 41.4, 37.4, 33.0, 32.8, 26.5, 20.7, 20.3, 19.4, 12.8; HRMS, calcd for C₃₃H₃₈O₄ [M+Na⁺]: 521.2668, found 521.2671.

**Ester 8b:** Benzyl ether 8a (2.0 g, 4 mmol) and 10% Pd/C (0.4 g) were stirred in EtOAc (10 mL) under 1 atmosphere of H₂ (via balloon) at 25 °C for 6 hours. The Pd/C solids were removed by filtration through a plug of celite. The solvent was removed on the rotary evaporator and the residue was purified by silica gel chromatography (25% ether in hexanes) to afford 1.1 g of ester 8b (92%). 8b: [α]₂₅^D = +21.4 (c= 0.76, CH₂Cl₂); R_f = 0.8 (50% ether in hexanes).
ether in hexanes); $^1$H NMR (400 MHz, CDCl$_3$) δ 11.12 (s, 1H), 7.74 (d, J= 8.8 Hz, 1H), 6.38 (m, 2H), 5.41 (s, 1H), 4.76 (t, J= 8.8 Hz, 1H), 2.21 (m, 1H), 1.69-1.03 (m, 10H), 0.99 (s, 3H), 0.91 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 169.8, 163.6, 161.6, 131.8, 107.6, 106.4, 103.1, 83.8, 52.7, 42.9, 41.3, 37.6, 33.1, 32.8, 26.5, 20.7, 20.3, 19.3, 12.9; HRMS, calcd for C$_{19}$H$_{26}$O$_4$ [M+Na$^+$]: 341.1729, found 341.1723.

**Ester 10**: Diphenol 8b (0.5 g, 1.6 mmol), epibromohydrin (1.1 g, 8 mmol), and CsCO$_3$ (0.98 g, 3 mmol) were stirred in DMF (5 mL) at 25 °C for 12 hours. The solution was diluted with H$_2$O (50 mL) and extracted with EtOAc (3 x 10 mL). The combined organics were dried over magnesium sulfate, and the solvent was removed on the rotary evaporator. The resulting residue was applied to silica gel chromatography (20% ether in hexanes) to afford 0.53 g of ester 10 (75%). 10: [α]$_{25}^D$ = +7.9 (c= 0.3, CH$_2$Cl$_2$); R$_f$ = 0.3 (50% ether in hexanes); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.85 (d, J= 8.4 Hz, 1H), 6.5 (m, 2H), 4.74 (m, 1H), 4.30-4.24 (m, 2H), 4.06-4.01 (m, 1H), 3.97-3.93 (m, 1H), 3.40-3.34 (m, 2H), 2.93-2.90 (m, 2H), 2.86 (m, 1H), 2.76 (m, 1H), 2.24 (m, 1H), 1.75-1.34 (m, 6H), 1.26-1.01 (m, 4H), 0.99 (s, 3H), 0.89 (s, 3H), 0.86 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 162.6, 160.0, 133.8, 113.9, 105.9, 105.8, 100.9, 83.4, 69.5, 68.9, 52.7, 50.1, 49.9, 44.9, 44.6, 42.8, 41.4, 37.7, 33.1, 32.8, 26.6, 20.8, 20.3, 19.4, 13.1; HRMS, calcd for C$_{25}$H$_{34}$O$_6$ [M+Na$^+$]: 453.2253, found 453.2252.

**Ester 11**: 2,4-Dimethoxy benzoic acid (15 mg, 0.09 mmol), alcohol 9 (11 mg, 0.06 mmol), EDC (17 mg, 0.09 mmol), and DMAP (11 mg, 0.09 mmol) were stirred in CH$_2$Cl$_2$ (1 mL) at 25 °C for 24 hours. The solvent was removed on the rotary evaporator and the residue was purified by silica gel chromatography (25% ether in hexanes) to afford 20 mg of ester 11 (96%). 11: [α]$_{25}^D$ = +13.2 (c=0.4, CH$_2$Cl$_2$); R$_f$ = 0.7 (50% ether in hexanes); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.88 (d, J= 8.8 Hz, 1H), 6.49 (d, J= 2 Hz, 1H), 6.47 (s, 1H), 4.74 (t, J= 9.2 Hz, 1H), 3.87 (s, 3H), 3.84 (s, 3H), 2.23 (m, 1H), 1.69-1.03 (m, 10H), 0.99 (s, 3H),
0.90 (s, 3H), 0.87 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 165.5, 164.1, 161.4, 133.7, 112.9, 104.4, 98.9, 83.2, 55.8, 55.4, 52.7, 42.8, 41.5, 37.7, 33.1, 32.9, 26.7, 20.8, 20.4, 19.4, 13.0; HRMS, calcd for C$_{21}$H$_{30}$O$_4$ [M+Na$^+$]: 369.2042, found 369.2044.

**Ester 12:** Methyl-2,4-dihydroxy benzoate (0.2 g, 1.2 mmol), epibromohydrin (0.8 g, 6 mmol) and CsCO$_3$ (0.78 g, 2.4 mmol) were stirred in DMF (3 mL) at 25 °C for 12 hours. The solution was diluted with H$_2$O (30 mL) and extracted with EtOAc (3 x 15 mL). The combined organics were dried over magnesium sulfate, and the solvent was removed on the rotary evaporator. The resulting residue was applied to silica gel chromatography (20% ether in hexanes) to afford 0.26 g of the methyl ester 12 (78%). 12: $R_f$ = 0.3 (50% ether in hexanes); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.83 (1H, d, J= 8.8 Hz), 6.50 (2H, m), 4.32-4.24 (2H, m), 4.06-4.01 (1H, ddd, J= 11.2, 5.2, 1.6 Hz), 3.97-3.93 (1H, ddd, J= 11.2, 6, 2.4 Hz), 3.85 (3H, s), 3.41-3.32 (2H, m), 2.96-2.90 (3H, m), 2.86 (1H, m), 2.76 (1H, dd, J= 4.8, 2.4 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 165.8, 162.8, 160.0, 133.9, 113.2, 106.0, 101.1, 68.9, 51.7, 50.1, 44.6; HRMS, calcd for C$_{14}$H$_{16}$O$_6$ [M+Na$^+$]: 303.0845, found 303.0847.