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Bioorganic & Medicinal Chemistry Letters 14 (2004) 5035–5039
Bioorganic & Medicinal Chemistry Letters

Fragmentation of Golgi membranes by norrisolide and designed analogues

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Received 19 July 2004; revised 3 August 2004; accepted 4 August 2004
Available online 28 August 2004

Abstract—The effect of norrisolide (**4**) and designed analogues on the Golgi membranes is presented. We found that **4** is the first compound known to induce an irreversible vesiculation of these membranes. To investigate the chemical origins of this new effect we synthesized and evaluated a series of norrisolide analogues in which the chemical functionalities present in the parent structure were altered. Such structure/function studies suggest that the perhydroindane core of **4** is critical for binding to the target protein, while the C21 acetate unit is essential for the irreversible vesiculation of the Golgi membranes.

The transfer of ribosome-synthesized proteins from endoplasmic reticulum to their ultimate destination (referred to as secretory pathway)[1] requires a multitude of signals and involves passage through the Golgi complex.[2] The Golgi complex consists of an assembly of membranes that play a key role in the final structural modifications of proteins, their sorting and transport to the relevant location.[3]

Ultrastructural analysis has revealed that the Golgi apparatus is composed of stacks of flattened cisternae, referred to as *cis*, medial and *trans*, and the *trans* Golgi network (TGN).[4] Transport vesicles originating at the rough endoplasmic reticulum (ER) bring newly synthesized and correctly folded proteins (cargo) into the *cis* Golgi cisternae. The cargo then travels across the Golgi stack in a *cis* to TGN direction. Within each cisterna, the cargo undergoes specific modification such as trimming and addition of oligosaccharides, phosphorylation, and sulfation. The appropriately decorated proteins are then transferred from TGN to their respective destination, such as endosomes and plasma membranes, or are secreted from the cell. It is thus obvious that the Golgi complex is a dynamic structure whose organization is maintained by a balance of membrane input and output. These observations have raised several issues related to the organization of the Golgi complex and its function during cell cycle. For example, the mechanism by which Golgi membranes are organized into stacks of cisternae as well as their fragmentation during mitosis and reassembly in each daughter cells are important and contentious issues.[2,5]

In the absence of genetic screens, chemical reagents, and natural products have proven to be highly useful in elucidating the function and dynamics of the Golgi apparatus (Fig. 1).[6] For example, studies with nocodazole (**1**) have revealed that microtubules are essential for the maintenance of Golgi apparatus in the percentriolar position.[7] Brefeldin A (**2**) was found to cause fusion of the Golgi membranes with the ER and helped in unraveling a retrograde (Golgi to ER) pathway.[8] Ilimaquinone (**3**) was shown to induce a microtubule independent and reversible fragmentation of the Golgi membranes into small vesicles.[9] The latter may be attributed to inhibition of methylation of a Golgi-specific protein, which is involved in activation of trimeric G-protein and its downstream target Protein Kinase D (PKD).[10] These components are required for formation of transport carriers from the trans Golgi network.

Herein we report our studies on the Golgi fragmentation using norrisolide (**4**), a natural product containing an

Keywords: Natural product; Acetylation; Golgi complex; Secretory pathway.
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doi:10.1016/j.bmcl.2004.08.003

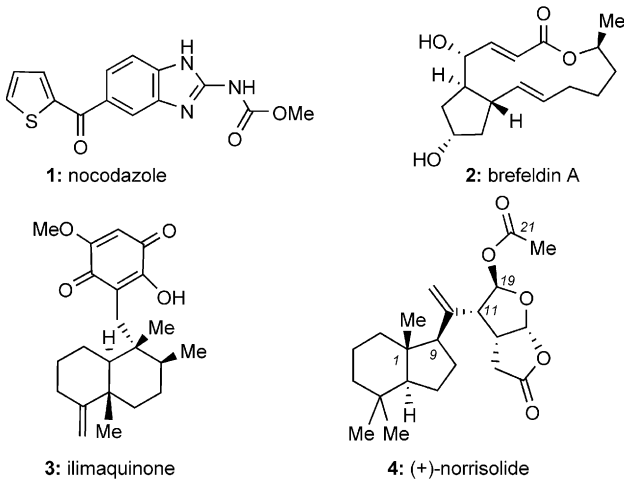


Figure 1. Chemical structures of selected Golgi-disturbing agents.

uncommon rearranged spongiane skeleton.[11] We have found, and report herein, that treatment of Normal Rat Kidney (NRK) cells with norrisolide causes an irreversible fragmentation of Golgi complex, which is independent of the microtubule structure. Using a series of designed norrisolide analogues, shown in Figure 2, we determined that the observed irreversibility stems from the unique reactivity of the fused γ-lactone-γ-lactol motif of **4**. A hypothesis regarding the mode of action of norrisolide based on our observations is proposed.

Evaluation of the structure of norrisolide as a function of its activity in Golgi vesiculation was performed with analogues **5**–**14**, in which crucial functionalities of the parent structure were partially deleted (Fig. 2). Compounds **5** and **6** have an identical structural motif to **4** but lack the acetoxy functionality at the C19 center. In compound **7** the unusual fused γ-lactone-γ-lactol motif of the natural product has been truncated to a monocyclic system, while analogue **8** was further modified by replacing the acetoxy unit with a more stable carbonyl group. In compounds **9** and **10** the perhydroindane core of norrisolide has been replaced by a *tert*-butyl group. Analogues **11**, **12** and **13**, **14** representing the two hemispheres of the natural product were also synthesized and screened.

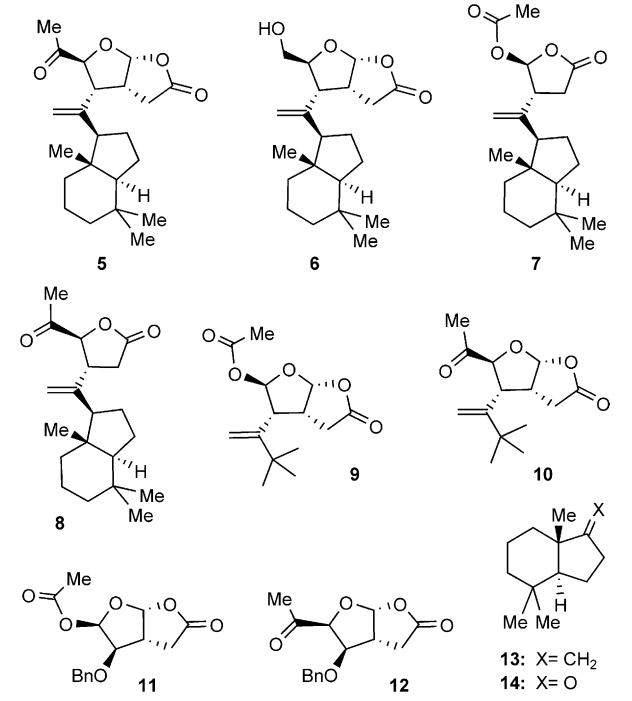
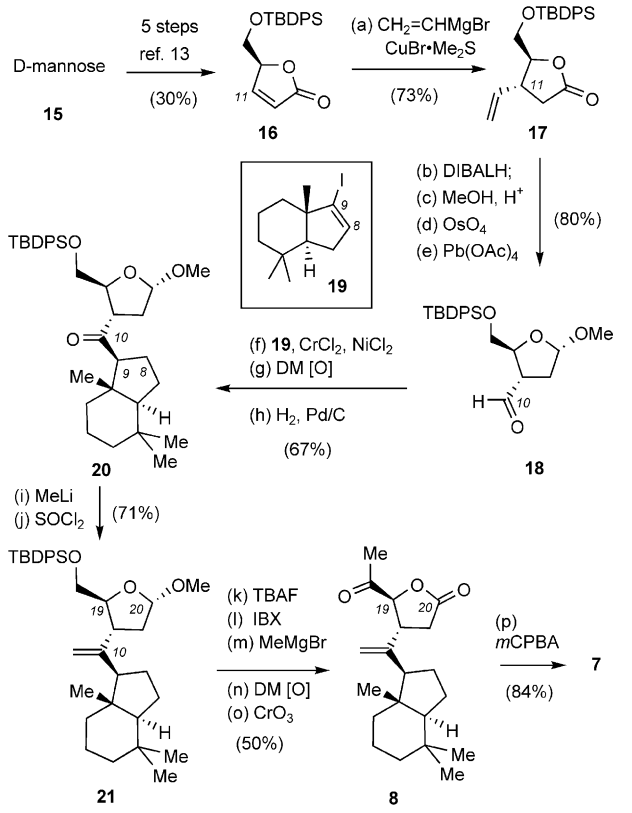


Figure 2. Structures of designed norrisolide analogues.

Compounds **5**, **6**, and **11**–**14** were synthesized during our campaign toward the synthesis of norrisolide.[12] The synthesis of analogues **7** and **8** is highlighted in Scheme 1. Crucial to this effort was the synthesis of aldehyde **18** representing the precursor of the truncated side chain of the natural product. Compound **18** was prepared from butenolide **16**[13] via a vinylcuprate addition[14] at the C11 center (norrisolide numbering) followed by



Scheme 1. Reagents and conditions: (a) vinylmagnesium bromide (1.1 equiv), CuBr·Me_2S (0.15 equiv), THF, −78 °C, 1 h, 73%; (b) DIBAL-H (1.1 equiv), CH_2Cl_2, −78 °C, 30 min, 99%, (c) MeOH, HCl (cat), 25 °C, 15 min, 99%; (d) OsO_4 (2.5%), NMO (1.2 equiv), pyridine (cat), acetone, 25 °C, 12 h, 92%; (e) $Pb(OAc)_4$ (1 equiv), CH_2Cl_2, 25 °C, 20 min, 89%; (f) $NiCl_2$ (cat), $CrCl_2$ (10 equiv), DMF, 1 h, 25 °C, 71%; (g) Dess–Martin periodinane (1.5 equiv), CH_2Cl_2, 25 °C, 8 h, 95%; (h) 10% Pd/C, H_2 (1 atm), MeOH, 25 °C, 12 h, 99%; (i) MeLi (10 equiv), DME, 0 °C, 30 min; (j) $SOCl_2$ (3 equiv), pyridine (10 equiv), CH_2Cl_2, 25 °C, 30 min, 71% (over two steps); (k) TBAF (1.1 equiv), THF, 25 °C, 6 h, 99%; (l) IBX (2 equiv), CH_3CN, reflux, 2 h, 99%; (m) methylmagnesium bromide (5 equiv), THF, 0 °C, 30 min, 65%; (n) Dess–Martin periodinane (1.5 equiv), CH_2Cl_2, 25 °C, 12 h, 95%; (o) CrO_3 (10 equiv) acetic acid/H_2O/acetone (8/1/1), 25 °C, 2 h, 83%; (p) *m*CPBA (1.3 equiv), $NaHCO_3$, CH_2Cl_2, 0 °C, 45 min, 84%.

reduction/protection at the C20 and oxidative cleavage of the pendant alkene of **17**. Nozaki coupling[15] between **18** and **19**,[12] followed by oxidation of the resulting alcohol and hydrogenation of the C8–C9 alkene produced compound **20**, which after a two step olefination procedure gave rise to adduct **21**.[16] The latter was functionalized at the C19 and C20 centers to furnish methyl ketone **8** that, after Baeyer–Villiger oxidation,[17] produced adduct **7**.[18] Analogues **9** and **10** were synthesized in an analogous manner.[19]

The effects of norrisolide (**4**) and analogues **5**–**14** in the Golgi apparatus were investigated using normal rat kidney cells (NRK cells).[20] Cells growing on coverslips in complete growth medium were treated with a 30 μM stock solution of the compounds in DMSO, and then incubated for 60 min at 37 °C. The cells were then divided in two groups. One set of coverslips (Fig. 3, column 2) was fixed after 60 min of incubation and processed for immunofluorescence. The other set (Fig. 3, column 3) was subjected to washout with PBS, to eliminate all traces of the compounds. The cells were then allowed to recover in fresh growth medium for 90 min at 37 °C prior to the staining procedure for fluorescence microscopy. Control cells (Fig. 3, column 1) were treated only with DMSO and processed as above. All cells were visualized by immunofluorescence microscopy with antibodies specific to Golgi membranes and microtubules. The nuclei were visualized using the DNA specific dye Hoechst (H3334, Molecular Probes). Under these conditions the Golgi membranes appear red (Alexa fluor 594 goat anti rabbit), the microtubules green (Alexa fluor 488 goat anti mouse) and the nucleus blue.[21]

The results of the above study are summarized in Figure 3. Within 60 min of incubation with norrisolide (**4**) the Golgi membranes were fragmented with little change in microtubule organization (Fig. 3, column 2, row 1).[22] The fragmentation of the Golgi apparatus was irreversible since removal of norrisolide by extensive washing did not promote its reassembly (Fig. 3, column 3, row 1). Under these conditions the cells died, presumably due to irreversible Golgi fragmentation.

Compounds **5**–**8** exhibited partial Golgi vesiculation without inducing any microtubule disassembly (Fig. 3, column 2, row 2). However, in contrast with norrisolide, in these cases the fragmentation was reversible and after the washing protocol the cells recovered completely (Fig. 3, column 3, row 2). NRK cells treated with compounds **9**–**14** were indistinguishable from control

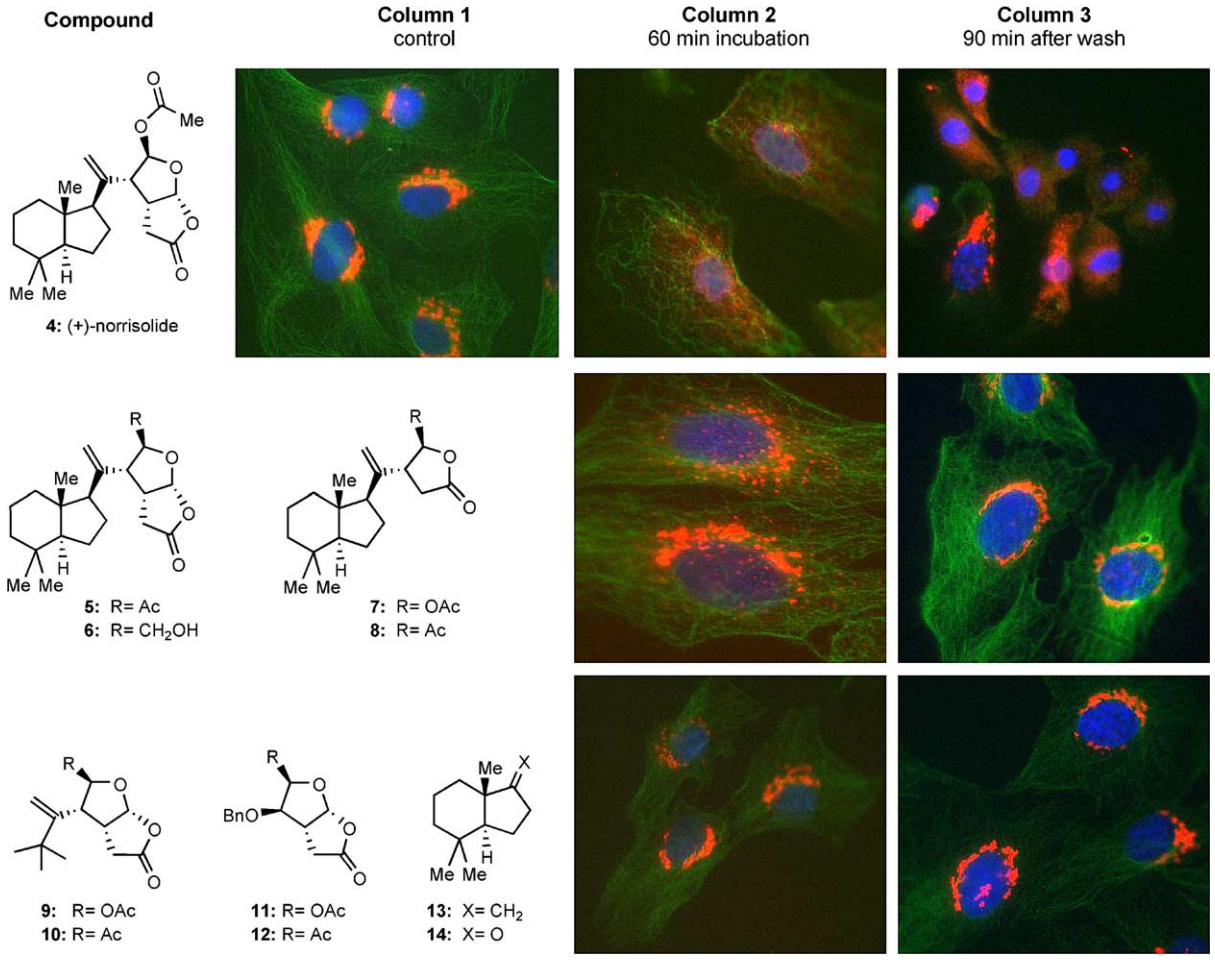


Figure 3. Effect of norrisolide and analogues on Golgi membranes. Column 1: untreated NRK cells; column 2: NRK cells treated with norrisolide and analogues and incubated for 60 min; column 3: NRK cells treated as in column 2 and then washed with buffer and incubated for an additional 90 min. The Golgi apparatus is shown in red color, microtubules in green, and nucleus in blue.

untreated cells, suggesting that these analogues have no effect on Golgi membranes (Fig. 3, columns 2 and 3, row 3).

The Golgi fragmentation induced by norrisolide (**4**) and analogues **5**–**8** is independent of the microtubule structure, as evidenced by fluorescence microscopy. Nonetheless, to exclude the possibility of undetectable changes in the microtubules structure, NRK cells were preincubated for 30 min with 10 μg/mL of Taxol™, a microtubule stabilizing agent, and then treated with norrisolide and analogues as described above. Under these conditions, compounds **4**–**8** still cause Golgi membranes to vesiculate (data not shown). Thus, norrisolide (**4**) and analogues **5**–**8** break down Golgi membranes even in the presence of taxol-stabilized microtubules.

Evaluation of the above data suggests that the perhydroindane core of **4** is essential for activity and appears to function as the target recognition element. This could explain why compounds **9**–**14**, lacking the perhydroindane core, have no effect on the Golgi membranes, while analogues **4**–**8** induce a visible fragmentation. Among the latter analogues, the fragmentation is more pronounced with compounds **4**–**6** that contain the entire C1–C20 backbone of the natural product, and is somewhat attenuated with compounds **7** and **8** that lack the γ-lactone subunit (data not shown). Of particular significance is the finding that norrisolide (**4**) induces an irreversible fragmentation of the Golgi membranes, while the partially reduced analogues **5**–**8** induce a reversible fragmentation. This observation invites the hypothesis that a covalent modification of the target protein occurs in the case of norrisolide (**4**), presumably due to the highly electrophilic nature of the C21 acetyl group.

A possible scenario that rationalizes these findings would involve selective binding of the perhydroindane core of **4** into a hydrophobic pocket of a protein that is involved in the signal transduction related to the Golgi fragmentation. This binding could bring the γ-lactol-γ-lactone side chain in close proximity to a nucleophile, which can then undergo acetylation. The identification of norrisolide dependent acetylation of a Golgi specific protein will help reveal the significance of acetylation dependent regulation of Golgi organization.

In conclusion, we present herein the effects of norrisolide (**4**) and designed analogues on the Golgi membranes. We found that the natural product induces an irreversible vesiculation of these membranes without affecting the microtubules structure. To the best of our knowledge, this is the first compound to have such an effect. We have also synthesized a series of norrisolide analogues in which several functionalities found in the structure of this natural product were sequentially deleted or altered. This allowed us to investigate the chemical origins of the irreversible fragmentation of the Golgi membranes that is produced by **4**. Such structure/function studies suggest that the perhydroindane core of **4** is critical for binding to the target protein, while the C21 acetyl group unit is essential for the irreversible vesiculation of the Golgi membranes.

Acknowledgements

Financial support from the NIH (CA 086079) is gratefully acknowledged.

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18. Spectroscopic and analytical data for compounds **7**–**8**. Compound **7**: R_f = 0.4 (50% ether in hexanes); $[\alpha]_D^{25}$ +1.67, (*c* 0.3, CH_2Cl_2); IR (film) ν_{max} 2924, 1802, 1765, 1646, 1458, 1370, 1260, 1213, 1142, 1014, 704 cm^{-1}; ^1H NMR (400 MHz, C_6D_6) δ 6.45 (d, *J* = 1.2 Hz, 1H), 4.82 (s, 1H), 4.68 (s, 1H), 2.68 (d, *J* = 8 Hz, 1H), 2.41 (dd, *J* = 8.4, 17.6 Hz, 1H), 2.11 (dd, *J* = 2.8, 17.6 Hz, 1H), 1.90 (t, *J* = 10.4 Hz, 1H), 1.48 (s, 3H), 1.39–0.77 (m, 11H), 0.80 (s, 3H), 0.79 (s, 3H), 0.43 (s, 3H); ^{13}C NMR (100 MHz, C_6D_6) δ 174.1, 168.2, 146.7, 112.3, 99.1, 58.3, 58.0, 46.3, 43.7, 41.6, 39.6, 33.3, 33.2, 32.6, 30.2, 26.7, 25.1, 20.7, 20.3, 14.1; HRMS calcd for $C_{20}H_{30}O_4$ ($M+Na^+$) 357.2042, found 357.2019. Compound **8**: R_f = 0.6 (50% ether in hexanes); $[\alpha]_D^{25}$ +2.9, (*c* 0.7, CH_2Cl_2); IR (film) ν_{max} 2945, 2855, 1791, 1723, 1459, 1362, 1147, 1063, 899 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.13 (s, 1H), 5.05 (s, 1H), 4.56 (d, *J* = 4.4 Hz, 1H), 3.07 (m, *J* = 4.4 Hz, 1H), 2.68 (dd, *J* = 9.2, 18 Hz, 1H), 2.52 (dd, *J* = 5.2, 18 Hz, 1H), 2.28 (s, 3H), 2.07 (t, *J* = 8 Hz, 1H), 1.72–0.85 (m, 11H), 0.85 (s, 6H), 0.64 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.8, 175.3, 148.5, 112.0, 88.0, 58.5, 58.1, 43.6, 43.2, 41.4, 41.3, 39.6, 34.2, 33.2, 33.1, 26.7, 25.3, 20.5, 19.9, 14.1; HRMS calcd for $C_{20}H_{30}O_3$ ($M+H^+$) 319.2273, found 319.2298.
19. Spectroscopic and analytical data for compounds **9**–**10**. Compound **9**: R_f = 0.5 (60% ether in hexanes). $[\alpha]_D^{25}$ +13.7, (*c* 0.3, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 6.24 (d, *J* = 5.6 Hz, 1H), 5.42 (s, 1H), 4.87 (s, 1H), 4.48 (d, *J* = 10.8 Hz, 1H), 3.25 (m, 1H), 3.07 (t, *J* = 10.4 Hz, 1H), 2.68 (dd, *J* = 3.2, 18.8 Hz, 1H), 2.52 (dd, *J* = 10.8, 19.2 Hz, 1H), 2.23 (s, 3H), 1.03 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.2, 174.5, 151.2, 113.1, 107.7, 85.1, 45.6, 42.8, 36.6, 30.3, 28.8, 27.2, 19.9, 14.1; HRMS calcd for $C_{14}H_{20}O_4$ ($M+Na^+$) 275.1260, found 275.1281. Compound **10**: R_f = 0.4 (50% ether in hexanes); $[\alpha]_D^{25}$ +9.18, (*c* 0.43, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 6.44 (d, *J* = 5.2 Hz, 1H), 5.65 (d, *J* = 6 Hz, 1H), 4.84 (s, 1H), 4.57 (s, 1H), 2.75 (m, 1H), 2.22 (m, 1H), 2.13 (dd, *J* = 5.2, 18 Hz, 1H), 1.65 (dd, *J* = 10.8, 18.8 Hz, 1H), 1.54 (s, 3H), 0.69 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.7, 168.6, 151.5, 136.1, 112.1, 106.8, 102.1, 47.3, 40.8, 36.3, 30.1, 28.4, 27.0, 20.3; HRMS calcd for $C_{14}H_{20}O_5$ ($M+Na^+$) 291.1209, found 291.1228.
20. Similar results were obtained with experiments in HeLa cells.
21. Reagents and cells: NRK cells were plated on 12 mm glass coverslips coated with Pronectin F (Sigma) and grown in complete medium (250 μL per coverslip), consisting of alpha MEM medium (GIBCO) with 10% fetal calf serum, 2 mM L-glutamine and 25 mM Hepes pH 7.4, at 37 °C in a 5% CO_2 cell incubator. Stock solution (5 mg/mL) of norrisolide and analogues were made in DMSO and stored at −20 °C. The working concentration of the compounds was 30 μM for each coverslip. To half of the coverslips (70% confluent) were added norrisolide or analogues (2.5 μL of the stock solutions). To the other half were added 2.5 μL of DMSO as negative control. Both groups of cells were incubated at 37 °C for 60 min. Part of the treated cells (Fig. 1, column 2) and part of the control cells (Fig. 1, column 1) were then fixed with 4% formaldehyde and processed for immunofluorescence microscopy. The remaining cells were washed four times with phosphate-buffered saline (PBS) (150 mM NaCl, 1.8 mM NaH_2PO_4, 8.4 mM Na_2HPO_4). The cells were incubated in fresh complete medium at 37 °C for 90 min, then fixed with 4% formaldehyde and processed for immunofluorescence microscopy (Fig. 3, column 3). 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 1 h at room temperature in primary antibody diluted in blocking buffer. Rat tubulin antibody (1:75) (Accurate Chemicals) was used to detect microtubules; 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 1 h at room temperature. Alexa fluor 488 goat anti mouse (1:500) and Alexa Fluor 594 goat anti rabbit (1:500) from Molecular Probes were 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 60× magnification.
22. The fragmentation of the Golgi membranes is evidenced by the appearance of Golgi vesicles (shown as red spots) in the cytoplasm.