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Bioorganic & Medicinal Chemistry Letters 17 (2007) 320–325
Bioorganic & Medicinal Chemistry Letters

Trifunctional norrisolide probes for the study of Golgi vesiculation

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Received 17 August 2006; accepted 23 October 2006
Available online 6 November 2006

Abstract—Inspired by the effect of norrisolide on the Golgi complex, we synthesized norrisolide probes that contain: the perhydroindane core of the parent natural product for Golgi localization, a crosslinking unit (aryl azide or epoxide) for covalent binding to the target, and a tag (biotin or iodine) for subsequent target purification. We found that biotin-containing probes **14**, **20** and **24** induced inefficient Golgi vesiculation. However, the iodinated probe **25** induced extensive and irreversible Golgi fragmentation. This probe can be used for the isolation of the cellular target of norrisolide.

The intracellular transport and sorting of biomolecules, a process referred to as the secretory pathway, is essential to cell function and survival.[1] Central to this process is the Golgi complex, an assembly of membranes, whose function involves the post-translational modification of newly synthesized proteins and lipids, their sorting into secretory vesicles, and their trafficking to the sites of function.[2] It has also been shown that during mitosis the Golgi membranes undergo complete fragmentation to small vesicles.[3] However, the mechanisms governing these vesiculation processes are currently poorly understood.[4] Small molecules that affect Golgi vesiculation can be useful as probes for exploring this event and as leads for controlling disease pathogenesis.[5] For instance, brefeldin A (**1**, Fig. 1) was found to cause fusion of Golgi with endoplasmic reticulum (ER) and helped in unraveling the Golgi to ER retrograde pathway.[6] Secramine (**2**) was shown to block protein transport from Golgi to the plasma membrane,[7] while CCL-19 (**4**) blocks the exit of proteins from Golgi and induces Golgi fragmentation.[8] The marine sesquiterpene ilimaquinone (**3**) was found to induce a reversible vesiculation of the Golgi and led to the identification of Protein kinase D as a component of the secretion machinery.[9] More recently, norrisolide (**5**)[10] was shown to fragment the Golgi complex in an irreversible manner.[11]

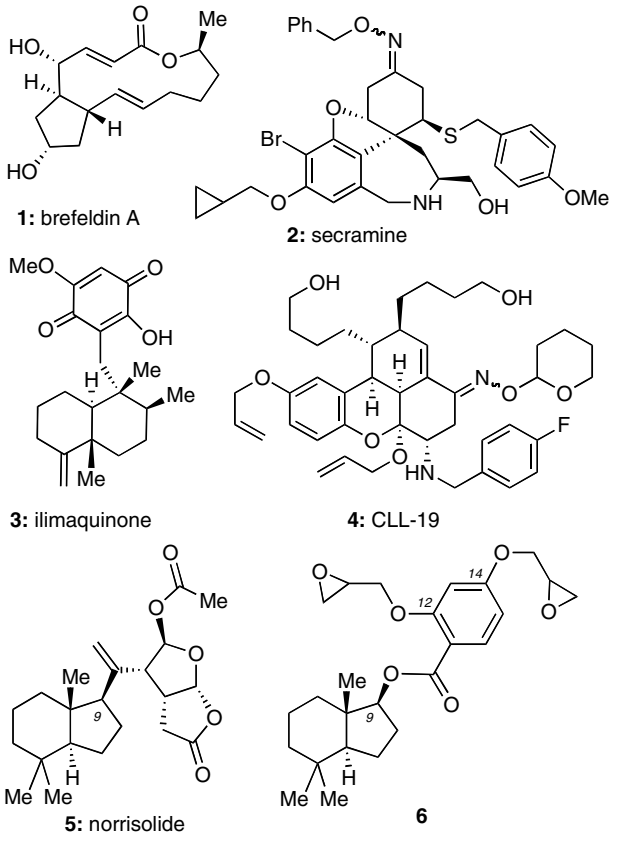

Figure 1. Structures of selected Golgi-disturbing agents.

Keywords: Natural products; Biotin; Crosslinking; Secretion; Golgi.
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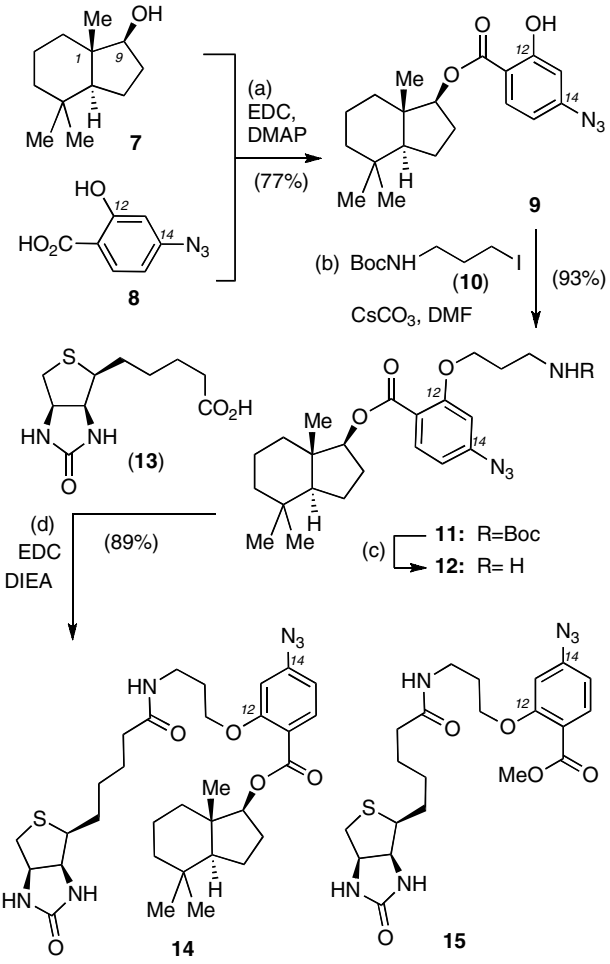
doi:10.1016/j.bmcl.2006.10.101

Recent studies with norrisolide have suggested that the hydrocarbon-containing perhydroindane core of this natural product binds to a receptor at the Golgi membranes and induces reversible vesiculation.[12] The vesiculation becomes irreversible when this core is decorated with electrophilic residues. Our efforts to explore this concept led to identification of compound **6** (Fig. 1) that was shown to induce an identical phenotype with **5**. We proposed that this effect is due to covalent reaction of the epoxide units of **6** with its biological receptor.[12] Based on these findings, we sought to synthesize and study trifunctional norrisolide probes that would contain the following functional groups: (a) the perhydroindane core for Golgi localization, (b) a crosslinking reagent for covalent binding to its putative target and, (c) a tag for subsequent protein purification and isolation.

The first probe that we evaluated was compound **14** in which the perhydroindane motif is attached to an aryl azide unit and biotin. It was expected that light-activation of the aryl azide would produce a reactive nitrene intermediate that would react covalently with its receptor and thus mimic the irreversible Golgi vesiculation observed with the natural product.[13] The biotin tag could then allow the isolation and purification of the target via affinity chromatography on resin-bound streptavidin. The synthesis of **14** is summarized in Scheme 1.[14] Coupling of **7**[15] with **8**[16] in the presence of EDC and DMAP produced ester **9** in 77% yield. The phenol functionality of **9** was then alkylated with *N*-Boc iodopropane (**10**) in the presence of $CsCO_3$ in DMF to form compound **11** (93% yield). TFA-induced Boc deprotection of **11**, followed by coupling of the resulting amine with biotin (**13**), gave rise to trifunctional probe **14**.[17] The bifunctional probe **15**,[17] containing the aryl azide functionality and the biotin but not the perhydroindane motif, was prepared in a similar manner and was used as a control.



Scheme 1. Reagents and conditions: (a) 7 (1.0 equiv), 8 (1.1 equiv), EDC (1.2 equiv), DMAP (1.2 equiv), CH_2Cl_2, 25 °C, 24 h, 77%; (b) 10 (1.3 equiv), $CsCO_3$ (2.0 equiv), DMF, 25 °C, 4 h, 93%; (c) TFA (5.0 equiv), CH_2Cl_2, 25 °C, 0.5 h, then; (d) 13 (1.3 equiv), DIEA (3.0 equiv), EDC (1.4 equiv), DMF/CH_2Cl_2 1:1, 25 °C, 4 h, 89%.

The effect of compounds **14** and **15** in Golgi vesiculation was evaluated using normal rat kidney (NRK) cells. Cells grown on coverslips in complete growth medium were treated with **14** and **15** (30 μM) for 60 min at 37 °C.[18] The cells were then fixed and processed for immunofluorescence microscopy.[18] The Golgi complex is shown in red and the cell nucleus is shown in blue. Figure 2 highlights the results of this study. Comparison of Figure. 2a and b indicates that compound **14** caused Golgi fragmentation in 50% of the cell population. In contrast, compound **15** did not lead to any fragmentation (data not shown), supporting the notion that the perhydroindane motif is essential for norrisolide activity. As expected, the fragmentation induced by **14** was completely reversible upon washing when the photoactivation of the aryl azide unit did not take place (Fig. 2c). UV-irradiation for 15 min caused an irreversible Golgi fragmentation in a small percent of the cells (Fig. 2d).[18]

Despite the low extent of irreversibility, we attempted isolation of the protein target of compound **14**. After UV irradiation, the cells were lysed and analyzed by Western blot to identify proteins containing biotin.[18] As a negative control, we used probe **15**, containing biotin but not the norrisolide core. The results, shown in Figure 3, indicate that both compounds **14** and **15** led to isolation of the same protein bands. This suggests that these protein bands are due to non-specific interactions of biotin with cellular proteins.[19]

In parallel with the above studies, we considered an alternative design of trifunctional probes in which the photoactivated aryl azide unit would be replaced by an epoxide functionality. This design was inspired by the observation of compound **6** inducing an identical phenotype to that of norrisolide in Golgi membranes.[12] We hypothesized that a probe that combines the perhydroindane motif with an epoxide unit and biotin would ensure the irreversible Golgi vesiculation and allow the isolation of its target protein. This hypothesis led us to synthesize and evaluate probes **20** and **24**.

The synthesis of compound **20** is shown in Scheme 2. Alkylation of **16** with 1,3-dibromopropane (1.1 equiv) in refluxing acetone using K_2CO_3 as the base produced

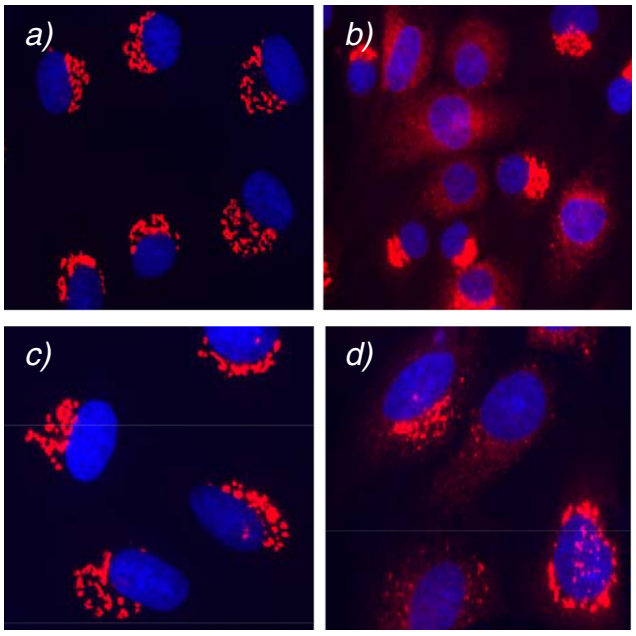


Figure 2. Effect of **14** on Golgi membranes. NRK cells were treated with **14** for 0 min (a) and 60 min (b). Cells were then exposed to photoactivation (d) and subsequently washed and incubated for 60 min. Control cells not subjected to UV irradiation are shown in (c). Golgi is shown in red; nuclei in blue.

selectively the C14-monoalkylated adduct that, after treatment with NaN_3, gave rise to azide **17** (83% yield over two steps). The C-12 phenolic oxygen was then benzylated and the adjacent methyl ester was saponified to afford carboxylic acid **18** (94% yield over two steps). Coupling of **18** with alcohol **7**, followed by Staudinger reduction[20] of the pendant azide group, produced amine **19** (75% combined yield) that was coupled with biotin (**13**). Hydrogenolysis of the C12 benzyl ether of **19**, followed by alkylation of the resulting alcohol with epibromohydrin using $CsCO_3$ as the base, produced probe **20** (59% yield over three steps).[21]

A similar strategy was devised for the construction of probe **24** (Scheme 3).[21] Compound **21** was selectively alkylated first at the C14 phenol (epibromohydrin, K_2CO_3) and then at the C12 center using EZ-link PEO-iodoacetylbiotin (**23**)[22] and $CsCO_3$, to form **24** in 55% yield (over two steps).

Evaluation of probes **20** and **24** in NRK cells was performed using the conditions discussed above. Incubation of NRK cells with **20** induced less than 10% of Golgi fragmentation in the entire cell population (Fig. 4a). Moreover, this effect was fully reversible after cell washing (Fig. 4b). Similar results were obtained with compound **24** (data not shown). The Golgi fragmentation induced by probes **20** and **24** was clearly less effective than that observed using norrisolide and compound **6** that, under identical conditions, led to complete and irreversible vesiculation in more than 90% of the cell population.[12]

We hypothesized that the low extent of Golgi vesiculation induced by probes **20** and **24** is due to the presence

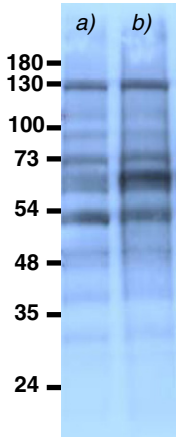
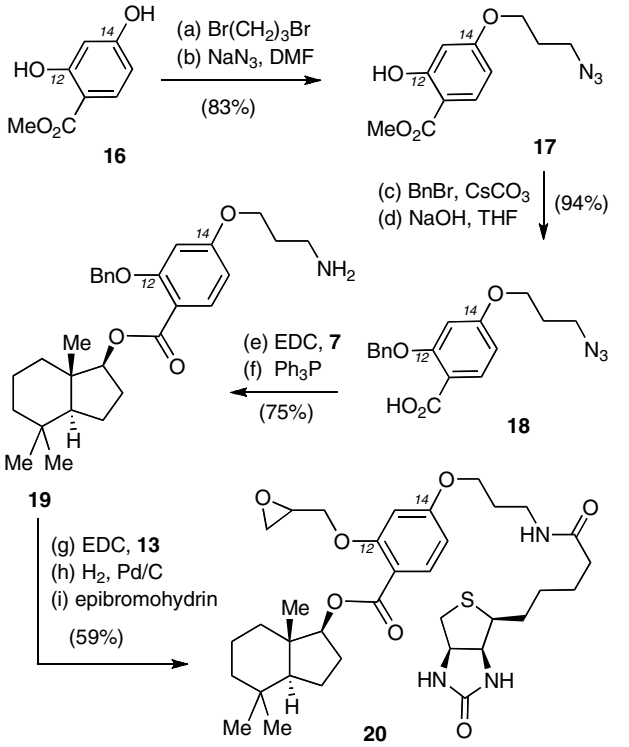
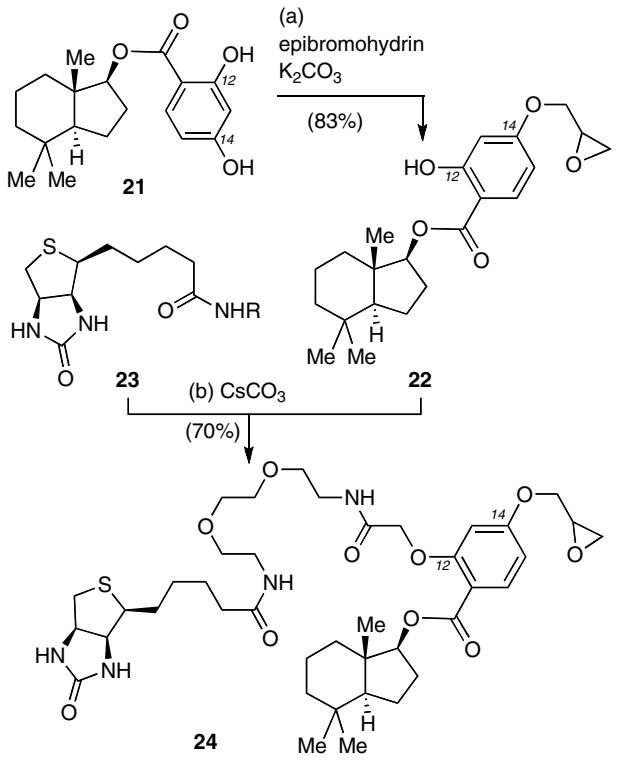


Figure 3. Immunoblot of total protein lysate of cells treated with **14** (a) and **15** (b), and subjected to photoactivation. Biotinylated proteins are visualized by Streptavidin–HRP.

of biotin tag. It is possible that the steric hindrance caused by biotin in combination with its intrinsic affinity for biotin receptors leaves insufficient amounts of these probes on the Golgi membranes thus dramatically reducing their effect on the Golgi complex. In other



Scheme 2. Reagents and conditions: (a) 1,3-dibromopropane (1.1 equiv), K_2CO_3 (2.0 equiv), acetone, 60 °C, 4 h, 87%; (b) NaN_3 (1.5 equiv), DMF, 25 °C, 16 h, 95%; (c) BnBr (1.6 equiv), $CsCO_3$ (2.0 equiv), DMF, 25 °C, 16 h, 95%; (d) NaOH (1 N) in THF (1:1) 50 °C, 4 h, 99%; (e) **7** (1.0 equiv), **18** (2.0 equiv), EDC (2.0 equiv), DMAP (2.0 equiv), CH_2Cl_2, 25 °C, 24 h, 83%; (f) PPh_3 (1.3 equiv), H_2O (1.3 equiv), THF, 25 °C, 24 h, 90%; (g) **13** (1.3 equiv), EDC (1.3 equiv), CH_2Cl_2/DMF 1:1, 25 °C, 12 h, 92%; (h) H_2 (1 atm), 10% Pd/C (0.2 equiv), AcOEt, 25 °C, 6 h, 79%; (i) epibromohydrin (1.3 equiv), $CsCO_3$ (1.3 equiv), DMF, 25 °C, 12 h, 81%.



Scheme 3. Reagents and conditions: (a) epibromohydrin (1.3 equiv), K_2CO_3 (1.3 equiv), acetone, 60 °C, 2 h, 83%; (b) **22** (1.0 equiv), **23** (R=$(CH_2CH_2O)_2CH_2CH_2NHCOCH_2I$) (1.3 equiv), $CsCO_3$ (1.5 equiv), DMF, 25 °C, 16 h, 70%.

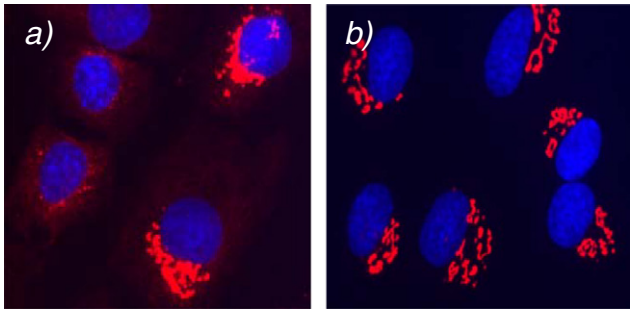
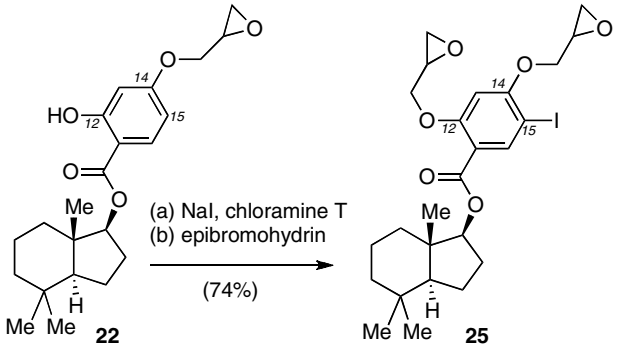


Figure 4. NRK cells treated with **20** for 60 min (a); then washed and incubated in fresh medium for 60 min (b). Golgi is shown in red; nuclei in blue. Similar results are obtained with probe **24**.



Scheme 4. Reagents and conditions: (a) NaI (1.2 equiv), chloramine T (1.1 equiv), CH_3CN/H_2O 10:1, 25 °C, 0.5 h, 89%; (b) epibromohydrin (2.0 equiv), $CsCO_3$ (1.5 equiv), DMF, 25 °C, 12 h, 83%.

words, the biotin tag could interfere with the ability of the perhydroindane motif to localize these probes on the Golgi. This hypothesis is also supported by the results obtained with probe **14** that again led to a decreased extent of Golgi vesiculation. This analysis led us to reconsider our overall strategy and design an alternative probe in which the biotin unit would be replaced by a radioisotope. Isolation of the norrisolide target(s) could then be pursued via radiolabeling.

The structure of epoxide **22** offers the unique advantage of facile incorporation of radioactive iodine at the aromatic ring at the C15 center. The synthesis of the non-radioactive probe **25**[21] is highlighted in Scheme 4 and involves iodination of **22** with NaI and chloramine T,[23] followed by alkylation of the C12 phenol with epibromohydrin (74% yield over two steps).

Incubation of NRK cells with compound **25** induced Golgi vesiculation at concentrations similar to those used for norrisolide and **6**. Importantly, this fragmentation was found to occur in more than 80% of cells and was irreversible after standard washing (Fig. 5). In fact, probe **25** induced comparable Golgi vesiculation to that obtained with analogue **6**. This result suggests that a probe **25** armed with a radioactive isotope ^{125}I could be used to tag the protein target and lead to its isolation by radiochromatography.[24]

In conclusion, we present herein the results of a study aiming to develop norrisolide-based trifunctional probes that could be used to identify the cellular target of this unusual natural product. Inspired by the finding that norrisolide and its analogue **6** induce an irreversible fragmentation of the Golgi membranes, we attempted to conjugate this structure with biotin and isolate its tar-

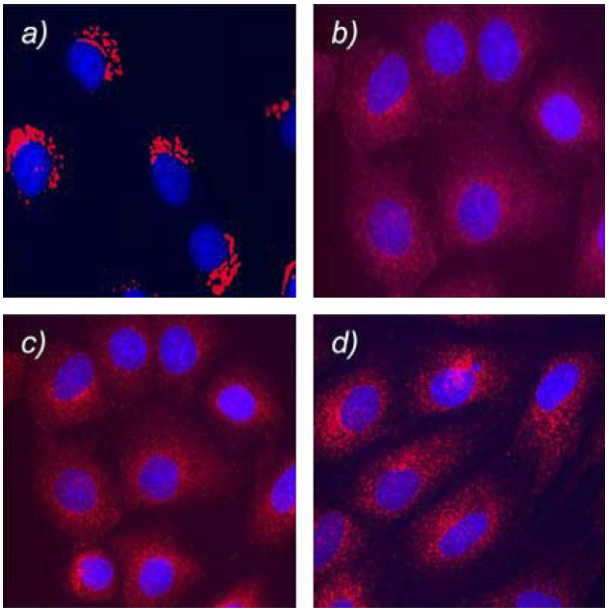


Figure 5. Effect of **6** and **25** on Golgi membranes. Untreated NRK cells (a) and NRK cells treated for 60 min with **6** (b) and **25** (c). Cells treated as in (c) are then washed and allowed to recover for 60 min in fresh medium (d).

get by affinity chromatography. None of the biotin-based probes induced significant Golgi fragmentation to justify further studies. The only proteins isolated with these probes were non-specific biotin targets. However, iodinated probe **25** induces sufficient Golgi vesiculation at comparable concentrations to that of the natural product. Thus, this probe represents an appropriate reagent for further biological studies aiming to isolate the cellular target of norrisolide.

Acknowledgment

Financial support by the NIH (CA 086079 to EAT, and GM 46224 and GM 53747 to V.M.) is gratefully acknowledged.

References and notes

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17. Spectroscopic and analytical data for compounds **14**–**15**. Compound **14**: $[\alpha]_D^{25}$ +48.7 ($c = 0.45$, CH_2Cl_2); $R_f = 0.2$ (3% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (m, 1H), 7.95 (d, $J = 8.4$ Hz, 1H), 6.67 (d, $J = 8.4$ Hz, 1H), 6.54 (s, 1H), 5.92 (br s, 1H), 4.68 (t, $J = 8$ Hz, 1H), 4.84 (m, 1H), 4.29 (m, 1H), 4.12 (m, 2H), 3.51 (m, 2H), 3.12 (m, 1H), 2.89 (m,1H), 2.86 (m, 1H), 2.72 (d, $J = 12.4$ Hz, 1H), 2.38 (t, $J = 7.2$ Hz, 2H), 2.25–2.20 (m, 2H), 2.05 (t, $J = 4.8$ Hz, 2H), 1.78–1.42 (m, 11H), 1.25–1.03 (m, 4H) 0.99 (s, 3H), 0.90 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9, 164.4, 160.5, 146.0, 133.7, 115.2, 110.4, 103.2, 83.7, 69.4, 61.7, 55.4, 52.8, 42.9, 41.4, 40.5, 38.4, 37.9, 35.3, 33.1, 32.9, 28.5, 28.2, 28.1, 27.0, 26.7, 25.9, 20.8, 20.4, 19.4, 13.1; HRMS, calcd for $C_{32}H_{46}N_6O_5S$ [M+Na$^+$]: 649.31478, found: 649.31469. Compound **15:** $R_f = 0.2$ (3% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, $J = 8.4$ Hz, 1H), 7.82 (t, $J = 4.8$ Hz, 1H), 6.65 (dd, $J = 8.4$, 2 Hz, 1H), 6.53 (d, $J = 1.6$ Hz, 1H), 6.10 (br s, 1H), 5.57 (br s, 1H), 4.56 (m, 1H), 4.27 (m, 1H), 4.11 (t, $J = 5.2$ Hz, 2H), 3.85 (s, 3H), 3.51 (q, $J = 5.2$ Hz, 2H), 3.12 (m, 1H), 2.86 (dd, $J = 12.8$, 5.2 Hz, 1H), 2.72 (d, $J = 12.8$ Hz, 1H), 2.35 (t, $J = 7.6$ Hz, 2H), 2.04 (m, 2H), 1.77–1.60 (m, 4H), 1.44 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.8, 165.1, 163.7, 160.4, 146.1, 133.7, 114.7, 110.4, 103.2, 69.2, 61.7, 60.1, 55.4, 51.9, 40.5, 38.2, 35.4, 28.5, 28.2, 28.1, 25.9; HRMS,

calcd for $C_{21}H_{28}N_6O_5S$ [M+Na^+]: 499.17396, found: 499.1742.

18. *Reagents and cell*: NRK cells were grown on coverslips as described in Ref. 11. Stock solutions (5 mg/ml) of norrisolide analogues were made in DMSO and stored at −20 °C. The working concentration of the compounds was 30 μM for each coverslip and cells were incubated at 37 °C for 60 min. An equal volume of DMSO was used as a negative control for each compound. For the crosslinking reaction, cells were irradiated for 15 min with UV light using a UVL-21 hand lamp (1 mW/cm^2 at 366 nm) placed at 2 cm distance from the cells. To test the irreversibility of Golgi fragmentation, cells were washed 4 times with phosphate-buffered saline (PBS) (150 mM NaCl, 1.8 mM NaH_2PO_4, and 8.4 mM Na_2HPO_4) and then incubated in fresh complete medium at 37 °C for 90 min.
Immunofluorescence microscopy was performed as described in Ref. 11.
Immunoblot: Cells treated with **14** and **15** for 60 min and irradiated with UV light were collected and lysed with loading buffer (60 mM Tris, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 0.01% Bromophenol blue, and 10% glycerol). Proteins in the lysate were separated by SDS–PAGE using a 10% running gel. Proteins were transferred on a nitrocellulose membrane (Western blot) (60 min, 350 mA) that was then kept in blocking buffer (50 mN, Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 1% BSA) for 30 min. The membrane was incubated for 1 h at room temperature with Streptavidin–HRP (BD Pharmingen) diluted in blocking buffer. After washing 3 times with PBS, it was added the reagent for ECL (Perkin-Elmer). Kodak Biomax films were used for exposure.
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21. Compound **23** is commercially available from Pierce Biotechnology, Inc. Rockford, IL.
22. Spectroscopic and analytical data for compounds **20**, **24**, and **25**. Compound **20**: R_f = 0.4 (5% MeOH in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, J = 8.8 Hz, 1H), 6.50 (m, 2H), 6.11 (br s, 1H), 5.78 (s, 1H), 5.10 (s, 2H), 4.86 (d, J = 8.8 Hz, 2H), 4.75 (t, J = 6.8 Hz, 1H), 4.50 (m, 2H), 4.31 (m, 2H), 4.29 (m, 2H), 4.04 (m, 2H), 3.42 (m, 2H), 3.14 (m, 1H), 2.91 (m, 3H), 2.71 (m, 1H), 2.34 (t, J = 6.8 Hz, 2H), 2.20 (m, 2H), 2.01 (m, 2H), 1.70–1.01 (m, 11H), 0.99 (s, 3H), 0.89 (s, 3H), 0.86 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1, 165.4, 163.2, 162.9, 160.1, 133.7, 107.5, 106.0, 100.7, 83.4, 69.6, 61.8, 61.7, 60.0, 55.3, 55.2, 52.7, 50.2, 41.4, 40.5, 37.7, 36.8, 35.9, 33.6, 31.4, 29.7, 28.9, 28.3, 27.9, 26.5, 25.5, 20.8, 20.3, 19.4, 13.1; HRMS, calcd for $C_{35}H_{51}N_3O_7S$ [M+Na^+]: 680.33454, found: 680.2249. Compound **24**: $[\alpha]_D^{25}$ +17.9 (c = 0.3, CH_2Cl_2); R_f = 0.3 (70% ether in hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (br s, 1H), 7.88 (d, J = 8.8 Hz, 1H), 6.82 (m, 1H), 6.56 (dd, J = 8.8, 2.4 Hz, 1H), 6.48 (d, J = 2.4 Hz, 1H), 6.36 (s, 1H), 5.37 (s, 1H), 4.69 (t, J = 8 Hz, 1H), 4.57 (s, 2H), 4.48 (m, 1H), 4.32 (dd, J = 11.2, 2.8 Hz, 1H), 4.29 (m, 1H), 3.95 (dd, J = 10.8, 5.6 Hz, 1H), 3.67–3.53 (m, 12H), 3.43–3.34 (m, 2H), 3.11 (m, 1H), 2.95–2.86 (m, 3H), 2.78–2.68 (m, 2H) 2.21 (m, 2H), 2.01 (br s, 2H) 1.75–1.01(m, 13H), 0.99 (s, 3H), 0.90 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 168.5, 164.6, 163.2, 159.6, 133.6, 112.5, 106.9, 100.8, 83.2, 70.2, 70.1, 69.9, 69.4, 69.1, 67.9, 61.7, 60.1, 55.2, 52.8, 49.9, 44.5, 43.8, 41.4, 40.5, 39.1, 38.9, 37.8, 35.9, 33.1, 32.9, 28.2, 28.0, 26.6, 25.6, 20.7, 20.3, 20.2, 19.4, 13.1; HRMS, calcd for $C_{40}H_{60}N_4O_{10}S$ [M+H^+]: 789.41084, found: 789.4107. Compound **25**: $[\alpha]_D^{25}$ +22.2 (c = 0.1, CH_2Cl_2); R_f = 0.3 (70% ether in hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (s, 1H), 6.60 (d, J = 6.4 Hz, 1H), 4.73 (t, J = 7.6 Hz, 1H), 4.44–4.33 (m, 2H), 4.09–3.99 (m, 2H), 3.38 (m, 2H), 2.95–2.87 (m, 4H), 2.83 (m, 1H), 2.22 (m, 1H), 1.75–1.01 (m, 11H), 0.99 (s, 3H), 0.90 (s, 3H), 0.86 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.2, 160.8, 160.5, 142.3, 115.7, 99.3, 83.7, 70.3, 69.8, 52.7, 50.1, 50.0, 49.9, 44.7, 44.5, 41.4, 37.7, 33.0, 32.8, 29.6, 26.5, 20.7, 20.3,19.4, 13.1; HRMS, calcd for $C_{25}H_{33}IO_6$ [M+H^+]: 557.1400, found: 557.1412.
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