Chemical biology studies on norrisolide

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ABSTRACT

The cellular activity of norrisolide (7), a novel Golgi-vesiculating agent, was dissected as function of its chemical structure. This natural product induces irreversible vesiculation of the Golgi membranes and blocks protein transport at the level of the Golgi. The Golgi localization and fragmentation effects of 7 depend on the presence of the perhydroindane core, while the irreversibility of fragmentation depends on the acetyl group of 7. We show that fluorescent derivatives of norrisolide are able to localize to the Golgi apparatus and represent important tools for the study of the Golgi structure and function.

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1. Introduction

The process of protein secretion through the secretory pathway is fundamental for the survival of cells and the development of every organism. In complex organisms specialized secretory cells carry out key functions, such as the release of neurotransmitters, the release of antibodies and the secretion of digestive enzymes.[1] Malfunctions of the secretory pathway can lead to a variety of diseases including asthma, Lowe's syndrome and cystic fibrosis.[2] Moreover, toxins and pathogens have been shown to exploit various steps of this pathway to gain access to the cytosol where they exert their function.[3]

Essential to the secretory pathway is the Golgi apparatus, an organelle that consists of organized stacks of flattened membranes, referred to as cisternae. This organelle is responsible for the modification and sorting of cargo proteins.[4] Within the Golgi, secretory proteins undergo complex post-translational modifications and are eventually sorted to their final destination. During protein transport, large amounts of membranes and proteins move across the Golgi complex; despite this dynamic trafficking, Golgi membranes are able to maintain their structural identity. The Golgi apparatus is therefore a dynamic structure whose organization is maintained by a balance of membrane input and output.[5]

Together with genetic screens[6] and in vitro assays,[7] pharmacological approaches based on small molecules have proven to be extremely helpful in studying the complex organization and membrane architecture of the Golgi apparatus. For example, studies with N-ethylmaleimide have led to the isolation and identification of a protein, termed N-ethylmaleimide sensitive factor, which is required for fusion of transport vesicles with Golgi.[8] Investigations with nocodazole (1, Fig. 1) have shown that polymerization of microtubules can lead to stacking of the Golgi membrane.[9] Screening of combinatorial libraries led to the identification of secramine (2), a small molecule that can block protein transport from Golgi to the plasma membrane,[10] and CCL-19 (4), an agent that blocks the exit of proteins from Golgi and induces Golgi fragmentation.[11]

Natural products can also affect the dynamics of the Golgi complex. For instance, ionophores such as monensin (6) can disrupt the pH gradient within the Golgi ultimately affecting protein transport.[12] The fungal metabolite brefeldin A (3) was found to cause fusion of Golgi with endoplasmic reticulum (ER) and helped in unraveling the Golgi to ER retrograde pathway.[13] The marine sesquiterpene ilimaquinone (5) 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.[14]

Screening of a natural products library for molecules that affect the secretory pathway led to the discovery of norrisolide (7),[15] a marine diterpene that induces irreversible fragmentation of the Golgi complex.[16] The chemical structure of norrisolide contains an uncommon fused γ-lactone-γ-lactol ring system pendant from a hydrophobic trans hydrindane core.[15] Inspired by these observations, we sought to characterize the cellular phenotype of norrisolide and explore its Golgi activity as a function of its structure. Here we report a detailed account of these studies.

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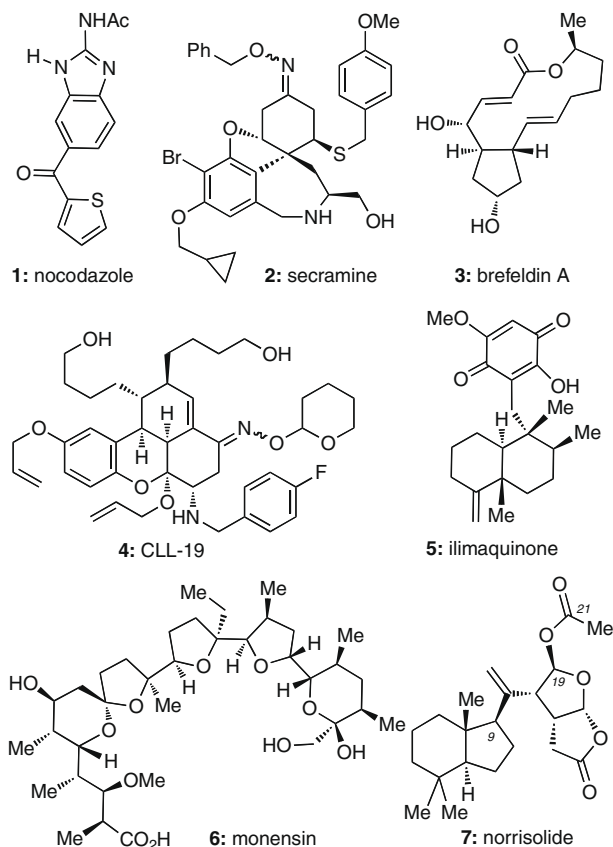† Centre for Genomic Regulation, C/Dr. Aiguader 88, 08003 Barcelona, Spain.

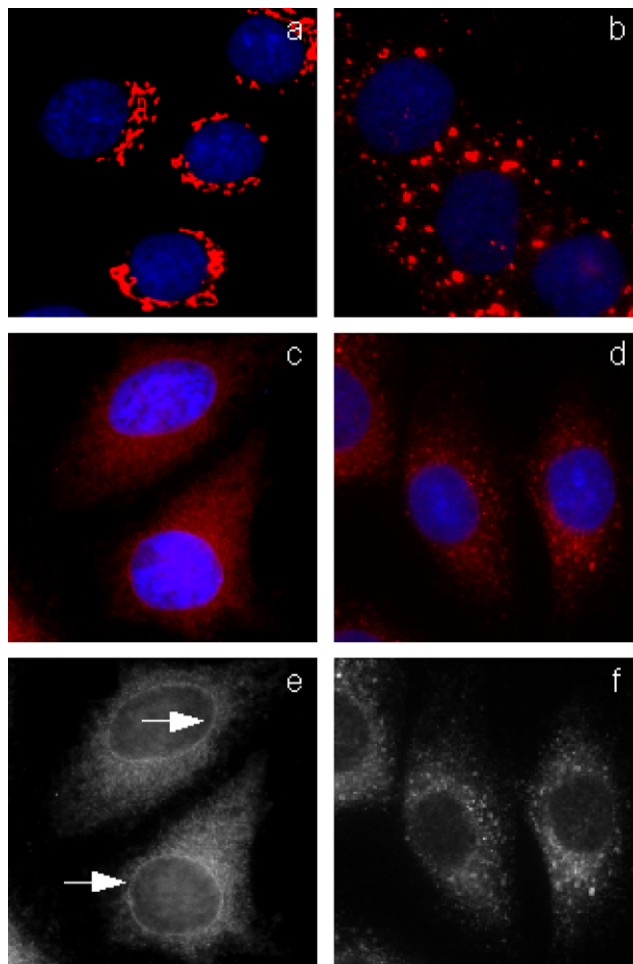**Figure 1.** Structures of selected Golgi-disturbing agents.

Figure 2. Phenotypic comparison of selected Golgi-disturbing agents. NRK cells (a) were treated with the following Golgi-disturbing agents: nocodazole (b), brefeldin A (c, e), ilimaquinone (d, f). Arrows indicate nuclear envelope staining. Golgi complex is shown in red, nuclei in blue.
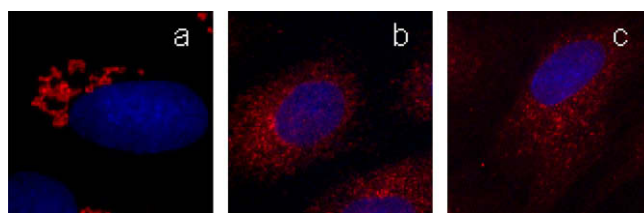

Figure 3. Norrisolide induces irreversible Golgi fragmentation. NRK cells plated on coverslips were treated with norrisolide for 0 (a) and 60 min (b). Cells were then washed and allowed to recover in fresh medium (c). Golgi complex is shown in red, nuclei in blue.

2. Results and discussion

2.1. Characterization of norrisolide-induced Golgi fragmentation

At the onset of this investigation we compared the phenotypic changes induced by norrisolide to that of other known Golgi-disturbing agents. It should be noted that different known Golgi-disturbing agents have different effects on Golgi morphology. These effects can be grouped in three main phenotypes: Golgi fragmentation in discrete 'ministacks' (e.g., Fig. 2b), Golgi fusion with the ER (e.g., Fig. 2c and e), and Golgi dispersion into a cytosolic haze (e.g., Fig. 2d and f).

The results of the phenotypic comparison studies are shown in Figure 2. Normal rat kidney (NRK) cells were chosen for these studies as they allow an optimal visualization of cellular organelles. Untreated cells (Fig. 2a) show the typical perinuclear Golgi stacks of cisternae. Application of 30 μM nocodazole for 60 min led to depolymerization of microtubules, which resulted in the breakdown of the Golgi apparatus to ministacks that were dispersed throughout the cytoplasm (Fig. 2b).[9] Brefeldin A, on the other hand, induced fusion of Golgi membranes with the ER (Fig. 2c); after brefeldin treatment (7 μM for 60 min), Golgi staining showed a typical ER pattern, as can be deduced by the presence of Golgi markers within the rim of the nuclear envelope (Fig. 2e).[10] A third group of Golgi-disturbing agents is represented by ilimaquinone (**5**) and norrisolide (**7**). As shown in Figure 2d and f, treatment of NRK cells with 30 μM of **5** for 60 min induced complete Golgi vesiculation without fusion with ER membranes.

Norrisolide induced a similar phenotype to that of ilimaquinone (**5**) (Fig. 3b). However, in contrast to **5**, the norrisolide-induced Golgi fragmentation was found to be irreversible. Specifically, at concentrations and times comparable to those of ilimaquinone, removal of norrisolide by extensive washing does not result in Golgi reassembly (Fig. 3c).[16]

It is known that the intact microtubule (MT) cytoskeleton is required to maintain the Golgi architecture.[17] For instance, depolymerization of MT by nocodazole, leads to fragmentation of the Golgi membranes into ministacks that subsequently disperse across the cytoplasm (Fig. 4a).[18] Two experiments were performed to test whether the norrisolide-induced Golgi fragmentation arises from disrupting the MT cytoskeleton. First, cells were treated with Taxol (10 μg/mL, 30 min), a compound known to stabilize the MT cytoskeleton.[19] We found that norrisolide can still induce Golgi fragmen-
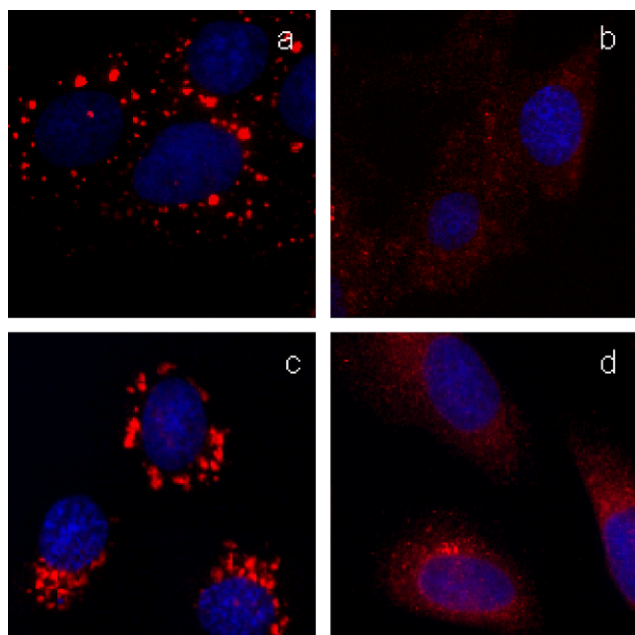
Figure 4. The norrisolide-induced Golgi fragmentation is independent on intact MT cytoskeleton and PKD activity. NRK cells pretreated for 60 min with nocodazole (a) were incubated with norrisolide for additional 60 min (b). NRK cells were treated for 60 min with IQ (c) or norrisolide (d) in presence of PKD inhibitor H89.

tation of the Taxol-treated cells.[16] Second, cells pretreated with nocodazole (Fig. 4a) were subsequently incubated with norrisolide. We found that norrisolide could further fragment the Golgi mini-stacks produced by nocodazole (Fig. 4b). These experiments allowed us to conclude that norrisolide induces Golgi vesiculation without affecting the MT cytoskeleton and that the intact MT cytoskeleton is not required for the norrisolide-induced Golgi fragmentation.

It has also been shown that the overactivation of the membrane fission process results in the complete conversion of Golgi stacks into transport vesicles.[20] Specifically, ilimaquinone (**5**) induces Golgi vesiculation by overstimulating Protein Kinase D (PKD), a protein required for Golgi membrane fission and production of secretory vesicles (Fig. 4d). With this in mind, we tested whether the norrisolide activity proceeds via a PKD-dependent membrane fission process. NRK cells were pretreated with PKD inhibitor H-89[21] (20 μM) for 30 min, then incubated with **5** (Fig. 4c) or norrisolide (Fig. 4d) for 1 h. As shown in Figure 4c, under these conditions ilimaquinone is no longer able to fragment the Golgi, since its downstream target, PKD, is no longer functional. On the other hand, H-89 does not affect norrisolide-mediated Golgi fragmentation (Fig. 4d). These results demonstrate an important difference between the mode-of-action of ilimaquinone and norrisolide. Although the changes in Golgi morphology induced by ilimaquinone and norrisolide are indistinguishable at the immunofluorescence level, at molecular level the two processes are different, as norrisolide activity is not sensitive to the PKD inhibitor H89, while ilimaquinone is.

We then tested whether norrisolide can affect protein secretion. For this experiment we used a temperature-sensitive variant of the vesicular stomatitis virus (VSV)-G protein. This protein is commonly used as it allows one to follow its transport through the secretory pathway in discrete steps by temperature blocks. For our experiments we used the tsO45 strain of VSV-G-GFP, which has a thermosensitive mutation causing it to unfold in the ER at the nonpermissive temperature of 39.5 °C.[22] In addition, the green fluorescent protein (GFP) is fused with VSV-G to allow its visualization within the cells. After shifting the cells to 20 °C, VSV-G

reaches the Golgi apparatus. At such temperature, protein transport is arrested at the level of the Golgi. When the temperature is raised to 32 °C, VSV-G finally reaches the plasma membrane.

We followed the transport of VSV-G from the ER to the Golgi and to the plasma membrane in control cells (Fig. 5a and c) and norrisolide-treated cells (Fig. 5b and d). Cells transfected with VSV-G were incubated at 40 °C to arrest VSV-G in the ER. The cells were then incubated with DMSO (control) or norrisolide for 1 h and then shifted to 20 °C, to allow VSV-G to exit the ER and reach the Golgi. In both DMSO (Fig. 5a) and norrisolide (Fig. 5b) treated cells, VSV-G was able to reach the Golgi apparatus, indicating that **7** does not affect protein transport from ER to Golgi. It is important to note that although Golgi structure is affected by norrisolide (Fig. 5b), the ability of the fragmented Golgi to receive protein cargo is not impaired (Fig. 5b: VSV-G, in green, enters norrisolide-induced Golgi fragments, in red). To measure the arrival of VSV-G at the plasma membrane, after arresting VSV-G in Golgi, cells were incubated with DMSO (control) or norrisolide, then shifted at permissive temperature (32 °C). As shown in Figure 5, while in DMSO-treated cells VSV-G reached the plasma membrane (Fig. 5c), in norrisolide-treated cells VSV-G (green) was permanently trapped in the Golgi vesicles (red) induced by **7** (Fig. 5d).

Based on the above results, we can make the following conclusions regarding the cellular characterization of norrisolide-induced Golgi fragmentation: (a) norrisolide affects the Golgi structure in a direct manner that is independent of the MT cytoskeleton architecture; (b) the norrisolide-induced Golgi fragmentation is independent of PKD, a protein previously shown to be involved in Golgi membrane fission and required for ilimaquinone-dependent Golgi vesiculation; (c) while norrisolide-induced Golgi fragments are able to receive protein cargo, they are not competent for the delivery of cargo molecules to their final destination. The latter effect results in a norrisolide-induced post Golgi block in protein transport.

2.2. Structure-function studies of norrisolide

To evaluate the biological activity of norrisolide as a function of its structure we studied the effect of analogues **8**–**13** (Fig. 6) on the
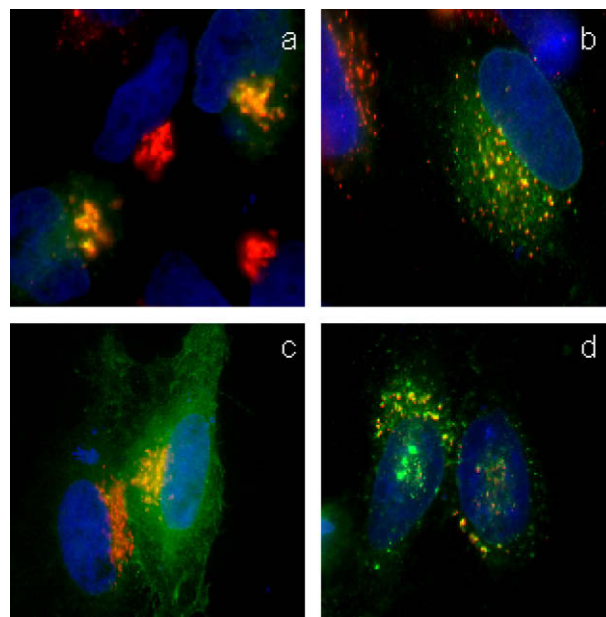

Figure 5. Protein transport in norrisolide-treated cells. Cells transfected with VSV-G (green) were incubated at 40 °C, then shifted to 20 °C (a, b) and 32 °C (c, d) in presence of DMSO (a, c) and norrisolide (b, d). VSV-G localizes in Golgi (a,b) and at the plasma membrane (c, d). Golgi complex is shown in red, VSV-G in green. Nuclei are in blue. Colocalization between Golgi and VSV-G is in yellow.
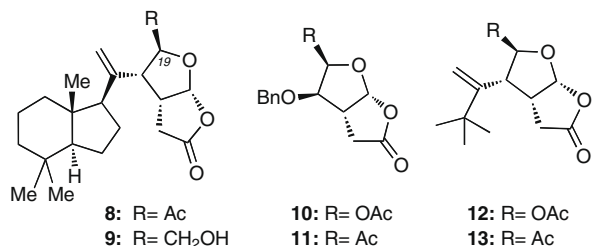
Figure 6. Synthetic analogues of norrisolide.

Golgi complex.[16] Compounds **8** and **9** are synthetic precursors of norrisolide[23] and contain the entire carbon framework of the natural product but lack the acetoxyl functionality. On the other hand, compounds **10–13** contain a variably functionalized γ-lactol-γ-lactone ring system of the natural product but lack the perhydroindane core.[24]

As shown in Figure 7, compound **8** exhibited partial Golgi vesiculation without inducing any microtubule disassembly (Fig. 7b). However, in contrast with norrisolide (see Fig. 3), the fragmentation was reversible and after a standard washing protocol the cells recovered completely (Fig. 7c). Similar results were obtained with **9**. In contrast, NRK cells treated with compounds **10–13** were indistinguishable from control untreated cells, suggesting that these analogues have no effect on Golgi membranes.

Evaluation of the above data indicates that the perhydroindane core of norrisolide is essential for norrisolide activity and appears to function as the target recognition element. This could explain why compounds **10–13**, lacking the perhydroindane core, have no effect on the Golgi membranes, while analogues **8–9** induce a visible although reversible fragmentation. In turn, the irreversible Golgi fragmentation observed with norrisolide may be due to a covalent modification of a target protein, presumably due to the highly electrophilic nature of the C21 acetyl group (norrisolide numbering). In other words, norrisolide may act as a selective acetylating reagent. Selective binding is therefore achieved by the perhydroindane core of norrisolide, which brings the lactol–lactone side chain in close proximity to a nucleophile, which can then undergo acetylation.

2.3. Intracellular localization studies of norrisolide

To further characterize the biological effect of norrisolide we examined the intracellular localization of norrisolide-derived fluorescent probes. The fluorophore coumarin was coupled to a norrisolide precursor that contains the entire carbon framework of the natural product, to the perhydroindane core and to the lac-
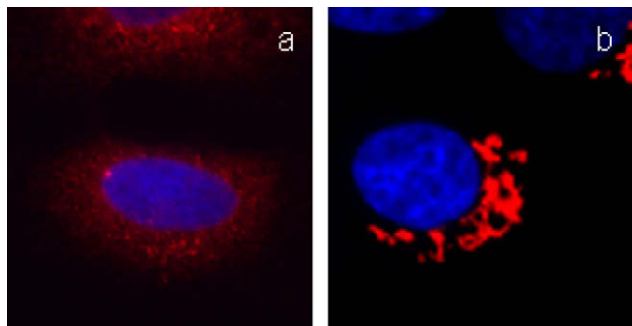
tol–lactone side chain, respectively, to generate probes **14**, **15** and **16** (Fig. 8).[25]

To assess the biological activity, NRK cells were incubated with probes **14–16** for 60 min, then processed for immunofluorescence to reveal Golgi structure. These probes had comparable stability with norrisolide in cells. While **15** had no activity (Fig. 9a), both **14** and **16** induced complete Golgi fragmentation in 60 min (results with **14** are shown in Fig. 9b). When live cells were imaged after 10 min incubation with **14** and **16**, both probes showed a perinuclear localization identical to a typical Golgi staining (Fig. 9c). To confirm that **14** and **16** indeed localized to the Golgi apparatus, NRK cells were fixed, labeled with a Golgi-specific antibody (Fig. 9d), and incubated with the fluorescent probes and imaged by light microscopy. Compound **15** showed no specific localization (data not shown), while compounds **14** and **16** both co-localized with Golgi markers (results with **14** are shown in Fig. 9e and f). To evaluate whether these compounds bind the same receptor as norrisolide, we performed the following competition experiment: NRK cells were fixed and incubated with analogue **14** in the presence of cell medium (Fig. 9g), DMSO (Fig. 9h) or norrisolide (Fig. 9i). We found that norrisolide (**7**) can displace **14** from the
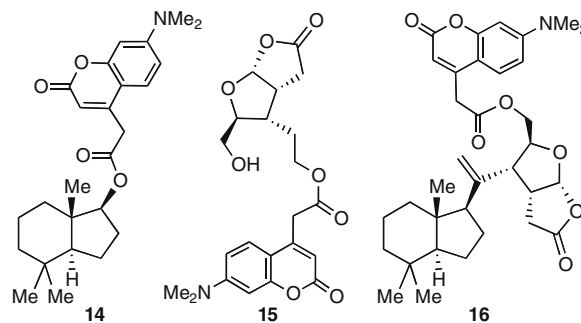

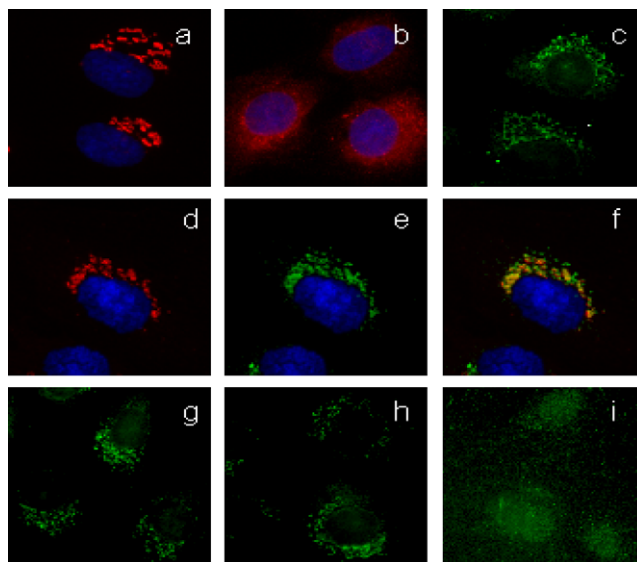**Figure 8.** Norrisolide-based fluorescent probes.

Figure 9. Fluorescent analogues of norrisolide induce Golgi fragmentation and localize on Golgi. NRK cells were treated with **15** (a) and **14** (b) for 60 min. Cells were treated with **14** for 10 min and imaged in live conditions (c). Fixed cells were labeled with a Golgi-specific antibody (d) then incubated with **14** (e); yellow color in (f) indicates co-localization of probe **14** and the antibody. Competition experiment: fixed NRK cells were incubated with **14** in presence of cell medium (g), DMSO (h) or **7** (i). Golgi complex is shown in red. Coumarin fluorescence is shown in green. Nuclei are in blue.

Figure 7. Effect of norrisolide analogue **8** on Golgi vesiculation. NRK cells were treated with **8** for 60 min (a) and allowed to recover for 60 min (b). Golgi complex is shown in red. Nuclei are in blue.

Golgi complex, as shown by the loss of green color from the Golgi (due to the coumarin fluorescence) in Figure 9i. Similar competition experiments showed that norrisolide could displace **16** from its target, indicating that compounds **7**, **14** and **16** share the same cellular receptor. These results indicate that **7** induces phenotypic changes in Golgi morphology by binding directly to the Golgi complex and further support the notion that the perhydroindane core of **7** is necessary and sufficient for binding.

2.4. Design of norrisolide-based irreversible Golgi-vesiculating agents

The above findings suggest that the C19 acetoxy group of norrisolide plays an essential role in the irreversibility of the fragmentation either by stabilizing the binding or by creating a covalent bond with its target protein. Inspired by a study that identified epoxides as suitable functionalities for protein labeling, we replaced the entire side chain of **7** with a bis-epoxide motif to form adduct **18**. Compound **17** was synthesized in a similar manner and used as a control (see Fig. 10).[25]

Figure 11 summarizes the effect of probes **17**–**20** on the Golgi membranes. Compounds **17** and **18** induced extensive Golgi vesiculation, as shown in Figure 11a and c, respectively. However, while the fragmentation induced by **17** was completely reversible (Fig. 11b), the Golgi fragmentation by **18** persisted even after washing (Fig. 11d). This level of irreversibility was the highest we had achieved with a norrisolide derivative. Similarly, iodinated analogue **19** and alkyne analogue **20** induced irreversible Golgi fragmentation in more than 90% of cells.[26] These findings indicate that it is possible to functionalize further the aromatic ring of probe **18** without affecting the cellular phenotype of norrisolide.

2.5. Design of norrisolide-based trifunctional probes

The above results suggest that the hydrocarbon-containing perhydroindane core of norrisolide binds to a receptor on the Golgi membranes and induces their reversible vesiculation. When this core is decorated with electrophilic residues, like the C19 acetoxy group (norrisolide numbering), the vesiculation becomes irreversible. To further explore this concept, we sought to synthesize and study trifunctional norrisolide probes that would contain the following groups: (a) the perhydroindane core for Golgi localization, (b) a crosslinking reagent for covalent binding to its target and, (c) a tag for subsequent protein purification and isolation.

An example of this design, that combines the perhydroindane motif with an epoxide unit and biotin, is shown with probe **21** (Fig. 12). Incubation of NRK cells with **21** induced less than 10% of Golgi fragmentation in the entire cell population (Fig. 13a). Moreover, this effect was fully reversible after cell washing (Fig. 13b). The extent of Golgi fragmentation was clearly below the level observed with norrisolide or probes **17**–**20** that, under identical conditions, led to complete and irreversible vesiculation in more than 90% of the cell population.[26]
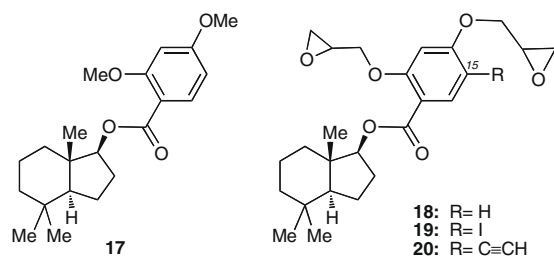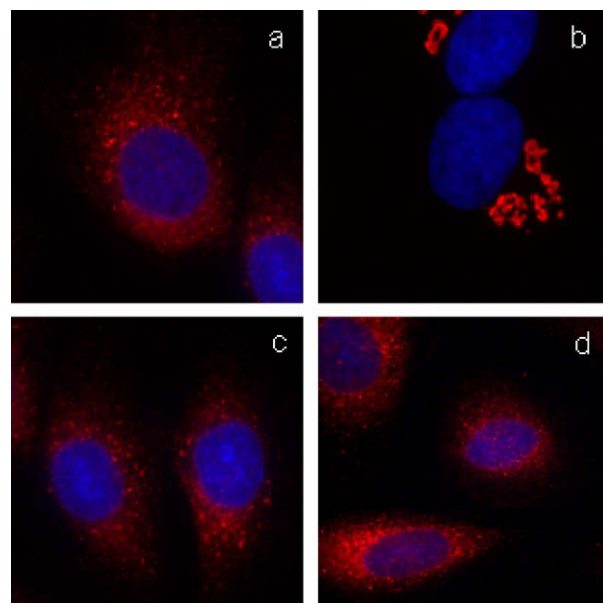

Figure 11. Effect of probes **17** and **18** on Golgi. NRK cells were treated with the probe **17** for 60 min (a) and then allowed to recover for additional 60 min (b). Cells were treated with **18** for 60 min (c) and then allowed to recover (b). Golgi complex is shown in red. Nuclei are in blue.
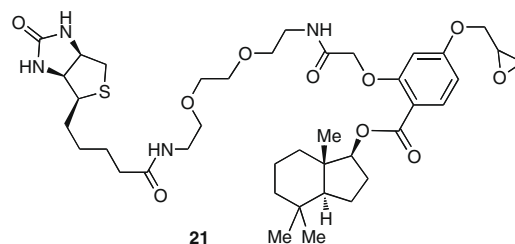

Figure 12. A norrisolide-based trifunctional probe **21** that combines the perhydroindane core with an epoxide and a biotin motif.
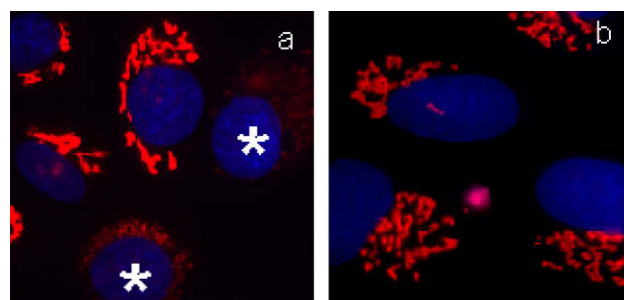

Figure 13. Effect of probe **21** NRK on Golgi. Cells were treated with **21** for 60 min (a); then washed and incubated in fresh medium for 60 min (b). Asterisks indicate cells with fragmented Golgi. Golgi is shown in red; nuclei in blue.

We hypothesized that the low extent of Golgi vesiculation induced by compound **21** is due to the presence of the biotin tag. It is possible that the steric hindrance caused by biotin in combination with its intrinsic affinity for cellular biotin receptors leaves insufficient amounts of these probes on the Golgi membranes thus dramatically reducing their effect on the Golgi complex. In other words, the biotin tag could interfere with the ability of the perhydroindane motif to localize these probes to the Golgi.[27] This analysis led us to reconsider our overall strategy and replace the biotin tag with a fluorescent probe. As the fluorophore, we selected

Figure 10. Norrisolide-based probes **17**–**20**.

the 7-dimethyl aminocoumarin-4-acetic acid (DMACA), since this probe displays desirable photophysical properties,[28] and shows negligible interference in cellular assays. Moreover, this probe has been used in combination with a monoclonal antibody (mAb) for immunoaffinity fluorescence studies.[29] We also felt that maintaining the bis-epoxide motif would ensure the irreversible Golgi vesiculation, as observed with probes 18–20. With this in mind, we synthesized trifunctional probe 22, by linking DMACA to 19 (Fig. 14).

As predicted, probe 22 localized efficiently to the Golgi complex in living cells (Fig. 15b) and induced complete Golgi vesiculation after a 60 min incubation period (Fig. 15c). However, the Golgi vesiculation proved to be reversible (Fig. 15d). We also performed competition experiments, which showed that the fluorescent staining of probe 22 could be displaced from the Golgi after incubation with an excess of norrisolide (data not shown), indicating that the binding of 22 to its target was not covalent. Similar results were obtained with derivatives of 22 in which the triple bond was partially or fully reduced, suggesting that the flexibility of the linker connecting the norrisolide core with the DMACA fluorophore does not contribute to the loss of irreversibility of the Golgi vesiculation.
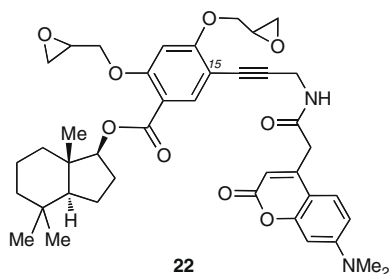

Figure 14. A norrisolide-based trifunctional probe that combines the perhydroindane core with two epoxides and a DMACA fluorophore.
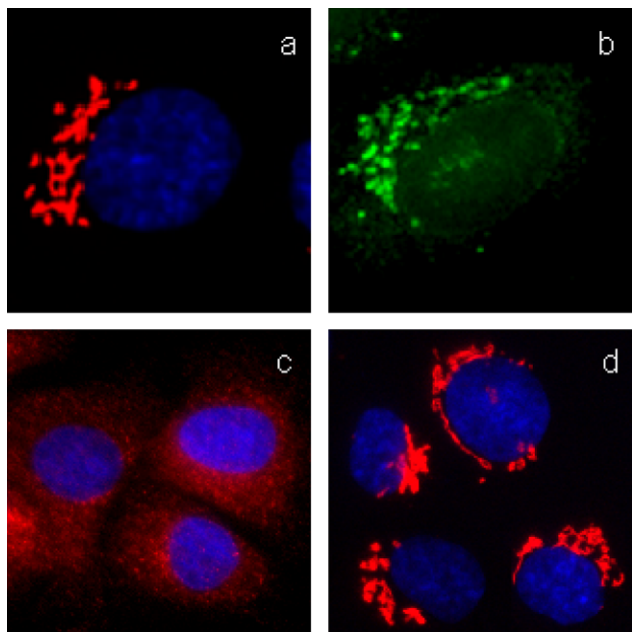

Figure 15. Effect of probe 22 on Golgi. Fixed NRK cells were labeled with Golgi-specific antibody (a); live cells were incubated with 22 for 10 min (b). Cells were treated with 22 for 60 min (c) and then allowed to recover for 60 min (d). Golgi complex is shown in red. DMACA fluorescence is shown in green. Nuclei are in blue.

The reversible Golgi vesiculation, induced by 22, was interesting, as compared to the irreversible vesiculation obtained with probes 18–20. One possible explanation is that the steric hindrance imposed by the added coumarin at the C15 center of 22 impairs the covalent interaction of the epoxide units with the norrisolide receptor. In contrast, smaller groups attached at C15, such as the alkyne motif of 20 or the iodide functionality of 19, do not affect significantly this interaction leading to presumed covalent binding. Alkyne-containing probe 20 is particularly interesting since it could be used to identify the cellular target of norrisolide under Click conditions.[30,31] Alternatively, probe 19, armed with a radioactive isotope ^{125}I at C15, could be used to isolate the protein target via radiochromatography.[32]

3. Conclusion

In conclusion, we report here a detailed study of norrisolide-induced Golgi vesiculation. We found that this natural product fragments Golgi membranes in an irreversible manner without affecting the microtubule cytoskeleton. This effect is distinctly different from that observed for ilimaquinone, a structurally related natural product that induces reversible Golgi fragmentation that is dependent on the activation of protein kinase D. Our studies also suggest that norrisolide-induced Golgi fragments are able to receive secretory proteins from the ER but they cannot deliver them to the plasma membrane, indicating that this compound specifically blocks protein transport at the level of the Golgi apparatus. Structure–activity studies indicate that the perhydroindane motif of norrisolide functions as the target recognition element, while the C19 acetoxy group is essential for the irreversibility of Golgi fragmentation. This group can be replaced with other electrophilic functionalities without significant loss of activity. Through the use of fluorescent derivatives of norrisolide, we were able to demonstrate that this compound binds directly to Golgi membranes, and that the perhydroindane core is necessary and sufficient for this binding. Moreover, the identification of norrisolide-based probes, such as 19 and 20, that reproduce the cellular phenotype of the natural product and are functionalized with appropriate tags, could be used for the isolation of its cellular target. In general, these studies attest to the significance of norrisolide and related designed analogues as biological tools for the evaluation of Golgi dynamics and protein trafficking.

4. Experimental

4.1. General chemical techniques

Compounds 1, 3 and taxol were purchased from Sigma–Aldrich, 5 was purchased from Alexis Biochemicals and H-89 was purchased from BIOMOL Research Laboratories. Norrisolide was synthesized according to Ref. 23 All other chemical reagents were commercially obtained (Aldrich, Sigma Acros) at highest commercial quality and used without further purification except where noted. The synthesis of compounds 8–13 is described in Refs. 16,23. The synthesis of compounds 14–18 is described in Ref. 25 The synthesis of compounds 19 and 21 is described in Ref. 26 Compounds 20 and 22 were synthesized by standard Pd(0)-catalyzed derivatization of iodinated compound 19.

4.2. Cell culture and transfection

NRK cells were grown in complete medium (alpha MEM-GIBCO-supplemented with 10% fetal calf serum, 2 mM L-glutamine and 25 mM Hepes pH 7.4), at 37 °C in a 7% CO_2 incubator. For

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immunofluorescence experiments, the cells were plated on 12 mm glass coverslips coated with Pronectin F (Sigma) and grown in complete medium (500 μL per coverslip).

The cells were transfected with VSV-G-GFP using FuGene 6 (Roche) following the manufacturer's recommendations.

4.3. Reagents

Stock solution (25 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 5 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, 8.4 mM Na_2HPO_4) and then incubated in fresh complete medium at 37 °C for 90 min. BFA, nocodazole and Taxol were used, respectively at 7 μM, 30 μM and 10 μg/mL. H-89 was used at 20 μM.

4.4. Immunofluorescence microscopy

For fluorescent labeling, cells were incubated in blocking buffer (PBS containing 2.5% fetal bovine serum and 0.1% Triton X-100) for 30 min at room temperature. The cells were then incubated for 1 h 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 1 h 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 60× magnification.

4.5. Golgi membranes fragmentation assay

Cells plated on coverslips were treated with norrisolide or analogues at the indicated concentrations. To control cells was added the same volume of DMSO as negative control. After 1 h incubation at 37 °C, the cells were either processed for immunofluorescence microscopy, or washed four times with phosphate-buffered saline (PBS) (150 mM NaCl, 1.8 mM NaH_2PO_4, 8.4 mM Na_2HPO_4) and then incubated in fresh complete medium at 37 °C for 90 min, then processed for immunofluorescence microscopy.

4.6. VSV-G transport assay

Cells were transfected with ts045VSV-G-GFP and cultured at 40 °C for 20 h. 100 mg/ml of cycloheximide was then added before a 2 h incubation at 20 °C. To follow the transport to the plasma membrane, the cells were then shifted at 32 °C for 1 h.

4.7. Golgi membrane localization assay

The cells were first fixed with 2% formaldehyde for 90 s. This mild fixation induces a minimal perturbation of cellular proteins, allowing the binding between the fluorescent derivatives and the cellular receptor. Cells were processed for immunofluorescence microscopy as described above. Before the coverslips were mounted onto glass slides, the fluorescent compounds were added at 25 μM for 30 min (Fig. 3d–f).

4.8. Competition experiments

Norrisolide was added after compound **14** at 100 μM for 30 min. Coverslips were then mounted onto glass slides and visualized using a Nikon micophot-FXA fluorescence microscope at 60× magnification. The intracellular visualization of compound **14** was accomplished using excitation at 370 nm and emission at 460 nm.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.02.007.

References and notes

1. For selected reviews on the secretory pathway see: Pelham, H. R. *Cell Struct. Funct.* **1996**, *5*, 413; Rothman, J. E.; Orci, L. *Nature* **1992**, *355*, 409; Pelham, H. R.; Munro, S. *Cell* **1993**, *75*, 603; Corda, D.; Hidalgo Carcedo, C.; Bonazzi, M.; Luini, A.; Spanò, S. *Cell. Mol. Life Sci.* **2002**, *59*, 1819; Luini, A.; Mironov, A. A.; De Matteis, M. A.; Griffiths, G. *Med. Secoli.* **2007**, *19*, 29; Lowe, M.; Nakamura, N.; Warren, G. *Trends Cell Biol.* **1998**, *8*, 40.
2. For selected reports on this topic see: Fahy, J. V. *Chest* **2002**, *122*, 320S; Suchy, S. F.; Olivos-Glander, I. M.; Nussabaum, R. L. *Hum. Mol. Genet.* **1995**, *4*, 2245; Cheng, S. H.; Gregory, R. J.; Marshall, J.; Paul, S.; Souza, D. W.; White, G. A.; O'Riordan, C. R.; Smith, A. E. *Cell* **1990**, *63*, 827.
3. For selected reports on this topic see: Spooner, R. A.; Smith, D. C.; Easton, A. J.; Roberts, L. M.; Lord, J. M. *Virol. J.* **2006**, *3*, 26; Sandvig, K.; van Deurs, B. *Annu. Rev. Cell Dev. Biol.* **2002**, *18*, 1; Ludwig, J. W.; Richards, M. L. *Curr. Top. Med. Chem.* **2006**, *6*, 165.
4. For selected reports on this topic see: Roth, J. *Chem. Rev.* **2002**, *102*, 285; Arnold, S. M.; Kaufman, R. J. *New Compr. Biochem.* **2003**, *38*, 411; van Vliet, C.; Thomas, E. C.; Merino-Trigo, A.; Teasdale, R. D.; Gleeson, P. A. *Prog. Biophys. Mol. Biol.* **2003**, *83*, 1; Schulein, R. *Rev. Physiol. Biochem. Pharmacol.* **2004**, *151*, 45; Sifers, R. N. *Science* **2003**, *299*, 1330.
5. For selected reports on this topic see: Warren, G.; Malhotra, V. *Curr. Opin. Cell. Biol.* **1998**, *10*, 493; Bard, F.; Malhotra, V. *Annu. Rev. Cell Dev. Biol.* **2006**, *22*, 439.
6. Bard, F.; Casano, L.; Mallabiabarrena, A.; Wallace, E.; Saito, K.; Kitayama, H.; Guizzunti, G.; Hu, Y.; Wendler, F.; Dasgupta, R.; Perrimon, N.; Malhotra, V. *Nature* **2006**, *439*, 604.
7. Balch, W. E.; Dunphy, W. G.; Braell, W. A.; Rothman, J. E. *Cell* **1984**, *39*, 405.
8. Balch, W. E.; Glick, B. S.; Rothman, J. E. *Cell* **1984**, *39*, 525; Malhotra, V.; Orci, L.; Glick, B. S.; Block, M. R.; Rothman, J. E. *Cell* **1988**, *54*, 221.
9. Thyberg, J.; Moskalewski, S. *Exp. Cell Res.* **1985**, *159*, 1; Turner, J. R.; Tartakoff, A. M. *J. Cell Biol.* **1989**, *109*, 2081.
10. Pelish, H. E.; Peterson, J. R.; Salvarezza, S. B.; Rodriguez-Boulan, E.; Chen, J. L.; Stammes, M.; Macia, E.; Feng, Y.; Shair, M. D.; Kirchhausen, T. *Nat. Chem. Biol.* **2006**, *2*, 39; Pelish, H. E.; Westwood, N. J.; Feng, Y.; Kirchhausen, T.; Shair, M. D. *J. Am. Chem. Soc.* **2001**, *123*, 6740.
11. Goess, B. C.; Hannoush, R. N.; Chan, L. K.; Kirchhausen, T.; Shair, M. D. *J. Am. Chem. Soc.* **2006**, *126*, 5391.
12. Tartakoff, A. M.; Vassalli, P. *J. Exp. Med.* **1977**, *146*, 1332.
13. Pelham, H. R. *Cell* **1991**, *67*, 449; Schiaky, N.; Presley, J.; Smith, C.; Zaal, K. J. M.; Cole, N.; Moreira, J. E.; Terasaki, M.; Siggia, E.; Lippincott-Schwartz, J. *J. Cell. Biol.* **1997**, *139*, 1137.
14. Takizawa, P. A.; Yucel, J. K.; Veit, B.; Faulkner, J. D.; Deernick, T.; Soto, G.; Ellisman, M.; Malhotra, V. *Cell* **1993**, *73*, 1079; Jamora, C.; Takizawa, P. A.; Zaarour, R. F.; Denesvre, C.; Faulkner, D. J.; Malhotra, V. *Cell* **1999**, *91*, 617; Bossard, C.; Bresson, D.; Polishchuk, R. S.; Malhotra, V. *J. Cell. Biol.* **2007**, *179*, 1123.
15. Hochlowski, J. E.; Faulkner, D. J.; Matsumoto, J.; Clardy, J. *J. Org. Chem.* **1983**, *48*, 1141.
16. Brady, T. P.; Wallace, E. K.; Kim, S. H.; Guizunnti, G.; Malhotra, V.; Theodorakis, E. A. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5035.
17. Storrie, B.; Yang, W. *Biochim. Biophys. Acta* **1998**, *1404*, 127.
18. Thyberg, J.; Moskalewski, S. *Exp. Cell Res.* **1999**, *246*, 263.
19. Wehland, J.; Henkart, M.; Klausner, R.; Sandoval, I. V. *PNAS U.S.A.* **1983**, *80*, 4286.
20. Jamora, C.; Yamanouye, N.; Van Lint, J.; Laudenslager, J.; Vandenheede, J. R.; Faulkner, D. J.; Malhotra, V. *Cell* **1999**, *98*, 59.

21. Johannes, F. J.; Prestle, J.; Dieterich, S.; Oberhagemann, P.; Link, G.; Pfizenmaier, K. *Eur. J. Biochem.* **1995**, *227*, 303.
22. Ward, T. H.; Lippincott-Schwartz, J. *Methods Biochem. Anal.* **2006**, *47*, 305.
23. Brady, T. P.; Kim, S. H.; Wen, K.; Theodorakis, E. A. *Angew. Chem., Int. Ed.* **2004**, *43*, 739; Brady, T. P.; Kim, S. H.; Wen, K.; Kim, C.; Theodorakis, E. A. *Chem. Eur. J.* **2005**, *11*, 7175.
24. Kim, C.; Hoang, R.; Theodorakis, E. A. *Org. Lett.* **1999**, *1*, 1295.
25. Guizzunti, G.; Brady, T. P.; Malhotra, V.; Theodorakis, E. A. *J. Am. Chem. Soc.* **2006**, *128*, 4190.
26. Guizzunti, G.; Brady, T. P.; Malhotra, V.; Theodorakis, E. A. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 320.
27. Kirkeby, S.; Moe, D.; Bøg-Hansen, T. C.; van Noorden, C. J. *Histochemistry* **1993**, *100*, 415.
28. Song, H. Y.; Ngai, M. H.; Song, Z. Y.; MacAry, P. A.; Hobley, J.; Lear, M. J. *Org. Biomol. Chem.* **2009**, *7*, 3400; Jarzeba, W.; Walker, G. C.; Johnson, A. E.; Kahlow, M. A.; Barbara, P. *J. Phys. Chem.* **1988**, *92*,

7039; Trenor, S. R.; Shultz, A. R.; Love, B. J.; Long, T. E. *Chem. Rev.* **2004**, *104*, 3059.
29. Rodriguez, A. D.; Lear, M. J.; La Clair, J. J. *J. Am. Chem. Soc.* **2008**, *130*, 7256; Hughes, C. C.; MacMillan, J. B.; Gaudencio, S. P.; Fenical, W.; La Clair, J. J. *Angew. Chem., Int. Ed.* **2009**, *48*, 728; Alexander, M. D.; Burkart, M. D.; Leonard, M. S.; Portonovo, P.; Liang, B.; Ding, X.; Joullie, M. M.; Gulledge, B. M.; Aggen, J. B.; Chamberlin, R. A.; Sandler, J.; Fenical, W.; Cui, J.; Gharpure, S. J.; Polosukhin, A.; Zhang, H.-R.; Evans, P. A.; Richardson, A. D.; Harper, M. K.; Ireland, C. M.; Vong, B. G.; Brady, T. P.; Theodorakis, E. A.; La Clair, J. J. *ChemBioChem* **2006**, *7*, 409.
30. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004; Lutz, J. F. *Angew. Chem., Int. Ed.* **2007**, *46*, 1018.
31. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596; Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192; Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686.
32. Sun, P.; Wang, G. X.; Furuta, K.; Suzuki, M. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2433; Hashimoto, M.; Kato, Y.; Hatanaka, Y. *Tetrahedron Lett.* **2006**, *47*, 3391.