Interactions of (–)-ilimaquinone with methylation enzymes: implications for vesicular-mediated secretion

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Background: The marine sponge metabolite (–)-ilimaquinone has antimicrobial, anti-HIV, anti-inflammatory and antimitotic activities, inhibits the cytotoxicity of ricin and diptheria toxin, and selectively fragments the Golgi apparatus. The range of activities demonstrated by this natural product provides a unique opportunity for studying these cellular processes.

Results: Affinity chromatography experiments show that (–)-ilimaquinone interacts with enzymes of the activated methyl cycle: *S*-adenosylmethionine synthetase, *S*-adenosylhomocysteinase and methyl transferases. Known inhibitors of these enzymes were found to block vesicle-mediated secretion in a manner similar to (–)-ilimaquinone. Moreover, the antisecretory effects of (–)-ilimaquinone and inhibitors of methylation chemistry, but not brefeldin A, could be reversed in the presence of the cellular methylating agent *S*-adenosylmethionine. Of the enzymes examined in the activated methyl cycle, *S*-adenosylhomocysteinase was specifically inhibited by (–)-ilimaquinone. Consistent with these observations, (–)-ilimaquinone was shown to obstruct new methylation events in adrenocorticotrophic hormone (ACTH)-secreting pituitary cells.

Conclusions: (–)-Ilimaquinone inhibits cellular methylations through its interactions with *S*-adenosylhomocysteinase. Furthermore, these studies indicate that the inhibition of secretion by ilimaquinone is the result of the natural product's antimethylation activity. It is likely that the ability to fragment the Golgi apparatus, as well as other activities, are also related to ilimaquinone's influence on methylation chemistry.

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Key words: *S*-adenosylhomocysteinase, affinity chromatography, ilimaquinone, methylation, secretion

Received: **22 February 1999**
Revisions requested: **22 March 1999**
Revisions received: **6 May 1999**
Accepted: **8 June 1999**

Published: **12 August 1999**

Chemistry & Biology September 1999, **6**:639–647
http://biomednet.com/elecref/1074552100600639

1074-5521/99/$ – see front matter

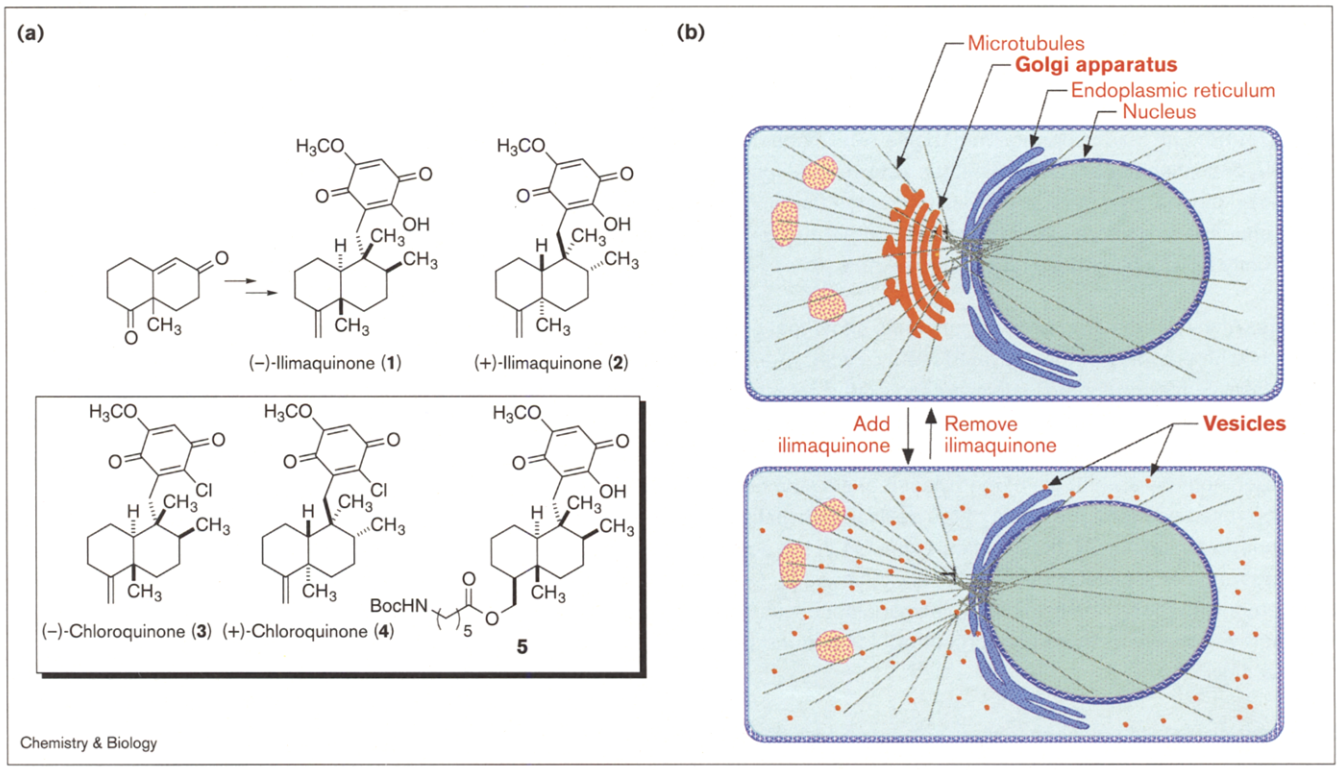
Introduction

The broad range of chemical reactions performed by eukaryotes requires extensive compartmentalization of the cell. Accompanying this structural complexity is the need to communicate between the various spatially segregated organelles [1,2]; the endoplasmic reticulum, Golgi apparatus and plasma membrane all exchange their cellular cargoes using small, lipid bilayer enclosed vesicles. Although the general events of this vesicle-mediated transport between the organelles and the cell membrane are becoming clearer [3–6], an understanding of the molecular machinery involved in regulating vesicle budding [7], fission [8], targeting [9,10], docking and fusion [11–19] is still largely in question.

The discovery that the marine sponge metabolite, (–)-ilimaquinone [20,21] (**1**) selectively breaks down the Golgi apparatus into small vesicles blocking cellular secretion in a reversible manner provides a unique opportunity for studying these important Golgi and endosomal trafficking events (Figure 1b) [22–25]. In addition, ilimaquinone has been reported to have other interesting biological effects including mild antimicrobial, anti-HIV, anti-inflammatory and antimitotic activities [26–33], and the inhibition of the cytotoxicity of ricin and diphtheria toxin [34]. To provide insight into these activities, as well as processes regulating vesicle-mediated secretory events, we have prepared structural variants of ilimaquinone that could be useful for uncovering the cellular interactions of the natural product.

(–)-Ilimaquinone (**1**), the unnatural enantiomer (+)-ilimaquinone (**2**) and other variants of the natural product were prepared through total synthesis (Figure 1a) [35]. By evaluating their antisecretory effects, analogs were selected that influence vesicle-mediated processes of the Golgi apparatus in a manner similar to the natural product [36]. Specifically, compounds **3** and **5** were shown to inhibit alkaline phosphatase secretion in Jurkat cells at concentrations comparable to **1**, whereas related compounds in the enantiomeric series (**2** and **4**) demonstrated much less antisecretory activity. This dependence of activity on absolute stereochemistry suggested that the antisecretory activities of **1**, **3** and **5** resulted from direct interactions with specific cellular target(s). Furthermore, the protected amine functionality

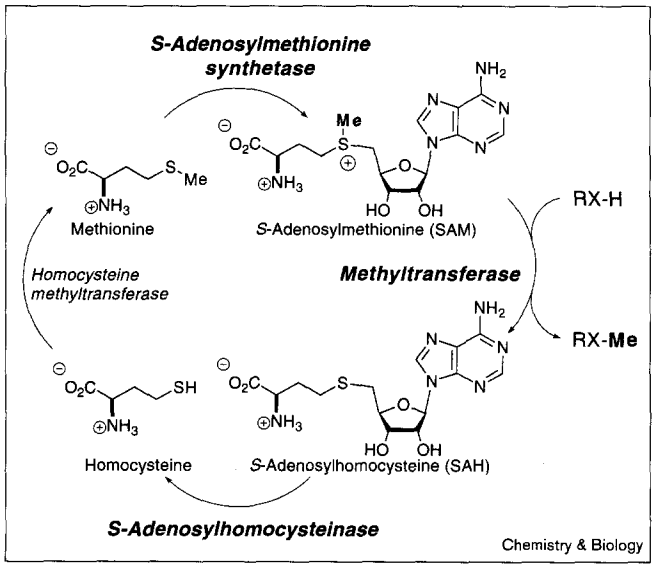
Figure 1



(a) (–)-Ilimaquinone and related analogs. **(b)** (–)-Ilimaquinone's influence on Golgi structure and function.

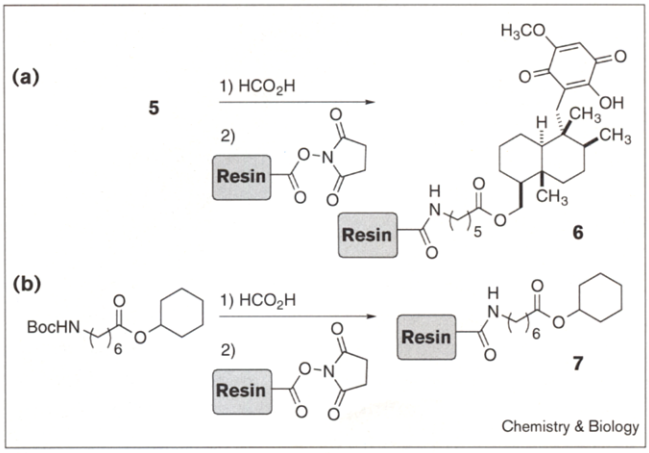
of compound **5** offered a versatile handle for preparing reagents that might be useful for identifying the cellular targets of the natural product. Through the use of these reagents, we report here our findings on the interactions of (–)-ilimaquinone and related analogs with proteins of the activated methyl cycle (Figure 2), as well as preliminary investigations on the relevance of these interactions to vesicle-mediated processes of the Golgi apparatus, such as secretion.

Figure 2



The activated methyl cycle.

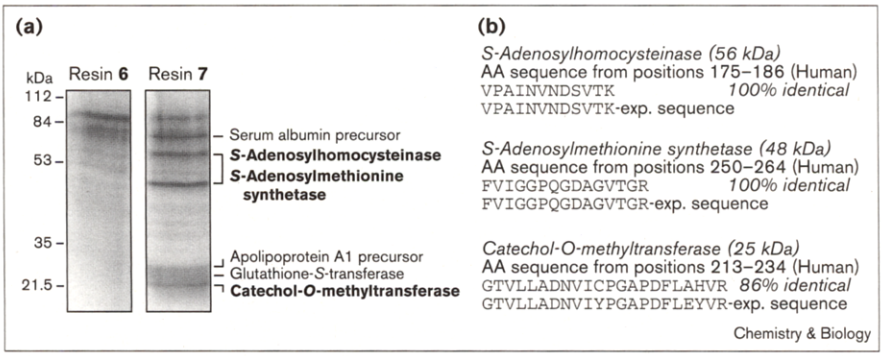
Figure 3



Syntheses of **(a)** ilimaquinone affinity resin (**6**) and **(b)** control resin (**7**).

Figure 4

(a) Bovine liver proteins eluted from control resin (**7**) and ilimaquinone resin (**6**). Samples were resolved using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and visualized through silver staining. **(b)** Representative sequencing data of isolated proteins.



Results and discussion

Affinity chromatography reveals cellular targets of *S*-adenosylhomocysteinase

As shown in Figure 3, treatment of analog **5** with formic acid followed by capture of the resulting free amine with an activated resin (Affigel-10) afforded the ilimaquinone affinity resin **6** (Figure 3a). Similarly, control resin **7** was generated from a tethered cyclohexanol derivative (Figure 3b). Tissue samples from a variety of sources were homogenized and incubated with these ilimaquinone and control resins. The resins were then examined for proteins that remained bound after extensive washings.

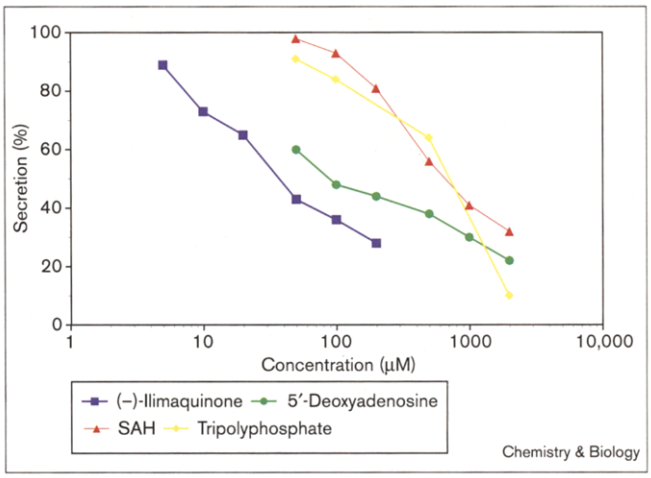
Figure 4a illustrates a representative example of the crude bovine liver proteins that were specifically retained on the ilimaquinone resin **6**, in comparison with the control resin **7**. A competition experiment in the presence of excess **1** significantly reduced the binding of these proteins to resin **6**. Sequencing of the major bands that were selectively observed on the ilimaquinone resin **6** identified several known proteins. In addition to some targets of undetermined interest (including a polipoprotein A1 precursor, serum albumin precursor and glutathione-*S*-transferase), three enzymes from the activated methyl cycle were isolated (Figure 4b). Specifically, *S*-adenosylmethionine synthetase (SAM synthetase; EC2.5.1.6) [37–40], *S*-adenosylhomocysteinase (SAHase) [41–43], and a protein that might be catechol-*O*-methyl transferase (COMT) [44–47] all bind selectively to the immobilized analog resin (**6**).

Inhibitors of enzymes of the activated methyl cycle inhibit secretion

Intrigued by the collective role these enzymes play in mediating cellular methylations, we examined their relevance to vesicle trafficking. Specifically, we asked whether blocking the function of these enzymes with known inhibitors of methylation chemistry will influence secretion. We found that cycloleucine and tripolyphosphate both obstruct alkaline phosphatase secretion in Jurkat cells [36] at concentrations consistent with their known inhibitory affects on SAM synthetase [48,49]. Similarly, as illustrated in Figure 5, *S*-adenosylhomocysteine (SAH), an inhibitor of methyltransferases [50,51], and 5′-deoxyadenosine, an inhibitor of *S*-adenosylhomocysteinase [52], also block secretion in a concentration dependent fashion. These results suggest that, as inhibitors of methylation enzymes also influence secretion, methylation events are involved in some aspect of vesicular-mediated secretion.

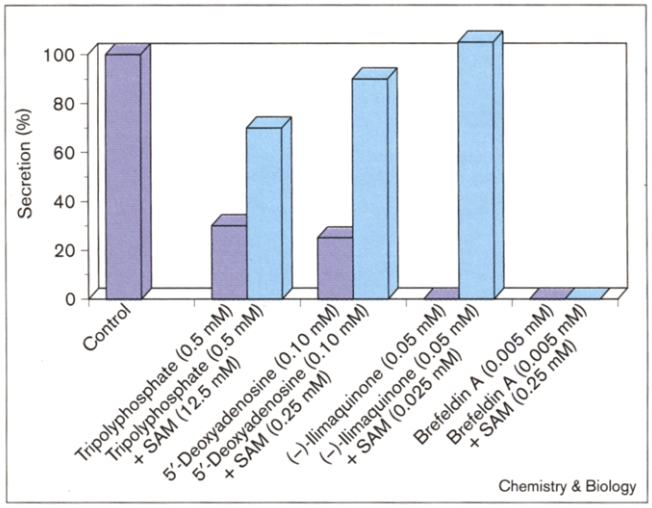
Further support for ilimaquinone's interference with methylation being the basis of its antisecretory effects was found by examining the influence of the methylating agent *S*-adenosylmethionine (SAM, the product of SAM synthetase) on secretion in the presence of various antimethylation and antisecretory agents. As illustrated in Figure 6, the addition of SAM to cells treated with tripolyphosphate, 5′-deoxyadenosine or (–)-ilimaquinone returned some or all secretory activity to these cells (control experiments

Figure 5



Secreted levels of alkaline phosphatase the presence of ilimaquinone and various inhibitors of methylation.

Figure 6



Exogenous SAM overrides the inhibitory effects of antimethylation agents and ilimaquinone on alkaline phosphatase secretion in Jurkat cells.

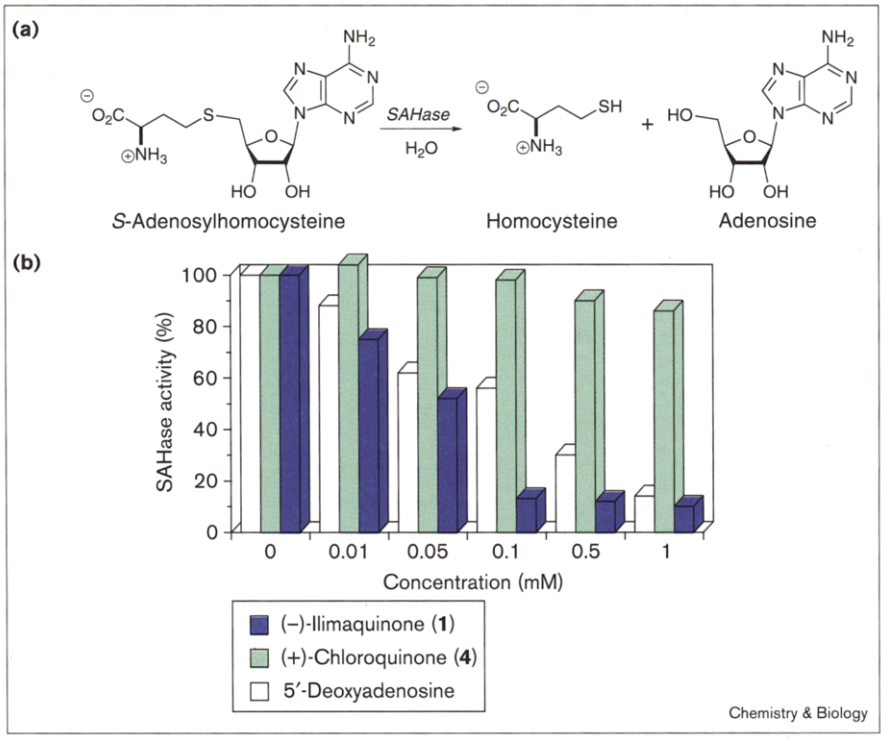
show that SAM does not react with ilimaquinone under the assay conditions). On the other hand, cells treated with brefeldin A, an unrelated inhibitor of Golgi structure and function [53–57], did not recover secretory activity upon the addition of SAM. As the antisecretory effects of ilimaquinone, as well as the other inhibitors of methylation can be reversed by adding the product of the active methyl cycle, SAM, a functional role for methylation in vesicle-mediated secretion gains further credibility.

(–)-Ilimaquinone inhibits *S*-adenosylhomocysteinase

Whether (–)-ilimaquinone has a specific influence on any one of the methylation enzymes was addressed next. Activity assays for each of the isolated enzymes of the activated methyl cycle were examined in the presence and absence of the natural product (**1**). The methyl-transfer activity of COMT was not inhibited by ilimaquinone [58] and **1** did not serve as a substrate for this enzyme [59]. Although the natural product did appear to limit SAM synthetase activity in crude liver cytosol [60,61], it did not have a profound influence in cytosol derived from the pancreas, brain, kidney, heart or pituitary cells, nor did it inhibit the function of SAM synthetase isozymes isolated from bovine liver (for isolation of SAM synthetase isozymes see [62–64]). (–)-Ilimaquinone was found to inhibit the SAHase-catalyzed hydrolysis of *S*-adenosylhomocysteine (Figure 7a), however, in a fashion similar to the known inhibitor, 5′-deoxyadenosine [65–67]. As illustrated in Figure 7, the inactive (+)-chloroquinone analog **4** had little influence on the enzyme. Preliminary kinetic analyses indicate that **1** is a competitive inhibitor of SAHase; additional studies with bovine sources of the enzyme are required, however, to confirm this observation [68,69].

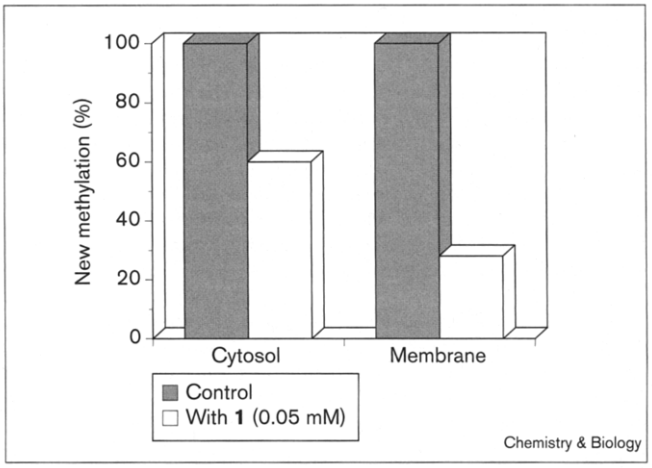
A consequence of interfering with SAHase should be an intracellular accumulation of *S*-adenosylhomocysteine.

Figure 7



(–)-Ilimaquinone inhibits *S*-adenosylhomocysteinase (SAHase).

Figure 8



(–)-Ilimaquinone (1) hinders new methylations.

This agent is known to be a potent feedback inhibitor of methyl transferases [50,51]. The addition of ilimaquinone could, therefore, interfere with new methylation events by increasing the intracellular levels of *S*-adenosylhomocysteine. As shown in Figure 8, mouse adrenocorticotrophic hormone (ACTH)-secreting pituitary cells treated for 1 hour with [^{3}H]-SAM (or [^{3}H]-methionine), in the presence of 1, showed a marked reduction of new methylations relative to the cells that are exposed to only [^{3}H]-SAM. This inhibition was particularly evident within the crude membrane fraction. The influence of ilimaquinone on SAHase and therefore cellular methylation events, as well as the ability of SAM to reverse the natural product's effects on secretion, support a role for methylation in mediating cellular secretory events.

Significance

We present here several advances in understanding the cellular activities of the natural product ilimaquinone. The ability of ilimaquinone to block secretion is shown to be due to its inhibition of methylation chemistry. A connection between the natural product's antisecretory activity and its influence on cellular methylation comes in several forms. Affinity chromatography experiments show that the methylation enzymes *S*-adenosylmethionine (SAM) synthetase, *S*-adenosylhomocysteinase, and possibly catechol-*O*-methyltransferase (COMT) interact either directly or indirectly with (–)-ilimaquinone [70]. Known inhibitors of these methylation enzymes were found to inhibit secretion. Moreover, SAM, the cellular methylating agent, reverses the antisecretory effects of these inhibitors, as well as ilimaquinone. In addition, we show that ilimaquinone inhibits the activity of *S*-adenosylhomocysteinase and disrupts new methylation events occurring within the cell. Although a clearer picture of the cellular activities of ilimaquinone is emerging, questions still remain.

The specific relationship between the natural product's influence on cellular methylations, Golgi morphology and vesicle fusion has not been determined [71]. Protein and lipid methylation events are known to play a role in vesicle-mediated processes [72–74]. For example, carboxyl methylation of the rab family GTPases have been implicated as a regulatory step in intracellular vesicular trafficking [75-77]. Determining the effects of (–)-ilimaquinone on the methylation state of these proteins could provide insight in the natural product's influence on vesicle fusion. Similarly, determining whether SAM antagonizes ilimaquinone's ability to vesiculate the Golgi apparatus is another question that remains. Furthermore, identifying the methylation event that leads to the inhibition of vesicle-mediated secretion and the nature of ilimaquinone's inhibition of *S*-adenosylhomocysteinase are of principal importance.

The findings detailed above identify *S*-adenosylhomocysteinase as a cellular target of (–)-ilimaquinone. These observations provide a clearer understanding of ilimaquinone's influence on vesicle-mediated processes of the cell. Moreover, new opportunities for viral infection and atherothrombosis therapies might also be possible considering the involvement of *S*-adenosylhomocysteinase in these diseases [78–83].

Materials and methods

General

Starting materials and reagents were purchased from commercial suppliers and used without further purification. CH_2Cl_2 and dimethylsulfoxide (DMSO) were distilled over calcium hydride under an atmosphere of nitrogen. Methanol and isopropanol were predried over 4 Å sieves. Syntheses were carried out under N_2 or Ar in oven-dried (140°C ≥ 4 h) glassware. Air- or moisture-sensitive liquids were transferred by syringe and were introduced into the reaction flasks through rubber septa. Air- or moisture-sensitive solids were transferred in a glove bag under N_2. Unless otherwise stated, reactions were stirred with a Teflon covered stir bar and carried out at room temperature. ^{3}[H]-Methionine (25 Ci/mmol) and [8-C^{14}]-5′ chlorodeoxyadenosine (250 μCi/mmol) were obtained from Movarek Laboratories. Packard Sovable and Packard Formula-989 LSC cocktail were obtained from DuPont-New England Nuclear. Affigel-10, polyvinylidene difluoride (PVDF) membrane, and polyprep columns (10 ml) were purchased from BioRad. Centrifugation was carried out in a Beckman L-70 Ultracentrifuge using a Type 70 Ti rotor. Proteins were separated using SDS–PAGE. Fresh tissue samples were obtained from Research 87 (Hopkinton, MA) and were homogenized in a Waring Blender. Protein sequencing was performed by the Harvard Microchemistry Laboratories, Harvard University, Cambridge, MA. Comparisons of isolated protein sequences to known proteins in the SwissProt database were carried out on the Computational Biochemistry server at Eidgenbssische Technische Hochschule Zürich (ETHZ).

Preparation of ilimaquinone affinity chromatography resins

Ilimaquinone analog **5** (3.0 mg, 5.1 μmol) was dissolved in formic acid (88%, 0.10 ml). After 1 h of stirring, the solvent was removed under vacuum to yield the amine as a purple oil (2.4 mg, 98%), which was used immediately in the subsequent reaction. Affigel-10 (1.2 ml) was placed in a poly-prep column and washed with isopropanol (3× bedvolume). The amine dissolved in DMSO (120 μl) and isopropanol (1.8 ml) was added. The slurry was shaken gently at 4°C. After 6 h the resin

was filtered and washed with isopropanol (3× bedvolume). The resin was then capped with ethanolamine (30 µl) in isopropanol (1.8 ml) for 2 h at 4°C. The purple ilimaquinone resin **6** was filtered, washed with isopropanol (3× bedvolume) and stored in isopropanol at −78°C until further use.

Control resin **7** was prepared as follows. Cyclohexanol (0.217 g, 2.17 mM), N-BOC-heptanoic acid (0.532 g, 2.6 mmoles), and DMAP (0.106 g, 0.87 mmoles) were dissolved in CH_2Cl_2 (2.0 ml) and cooled to 0°C. EDCI (0.500 g, 2.6 mmoles) dissolved in CH_2Cl_2 (3.0 ml) was added dropwise to the cooled stirred solution. After the addition was complete, the reaction mixture was stirred for another 2 h and then allowed to warm up to room temperature. After 12 h the reaction was quenched with H_2O and 5% HCl and extracted with ether (3×). The organic layer was washed with saturated NH_4Cl, brine, dried over Na_2SO_4, and concentrated to yield the tethered cyclohexanol derivative as a clear oil (0.35 g, 98%), which was used without further purification. ^1H NMR ($CDCl_3$, 300 MHz) δ 4.75 (1H, m), 4.51 (1H, br s), 3.11 (2H, dt, $J = 6.1$, 6.4 Hz), 2.28 (2H, t, $J = 7.3$ Hz), 1.83 (3H, m), 1.70 (4H, m), 1.66–1.49 (6H, m), 1.44 (9H, s), 1.39–1.30 (5H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 207.6, 197.6. 153.7, 156.6, 73.1, 41.0, 35.2, 32.3, 29.4, 27.1, 26.9, 25.6, 24.4; IR (thin film, NaCl) 3383, 2936, 2861, 1722, 1527, 1363, 1243, 1187 cm^{-1}.

The tethered cyclohexanol derivative (4.1 mg, 12 µmol) was dissolved in formic acid (88%, 0.10 ml). After 1 h of stirring, the solvent was removed under vacuum to yield the amine as a green oil (3.0 mg, 97%), which was used immediately in the subsequent reaction. Affigel-10 (1.5 ml) was placed in a poly-prep column and washed with isopropanol (3× bedvolume). The amine dissolved in isopropanol (2.0 ml) was added. The slurry was shaken gently at 4°C. After 6 h the resin was filtered and washed with isopropanol (3× bedvolume). The resin was capped with ethanolamine (30 µl) in isopropanol (2.0 ml) for 2 h at 4°C. The colorless resin was filtered, washed with isopropanol (3× bedvolume) and stored in isopropanol at −78°C until further use.

General procedure for affinity chromatography

Calf tissue was cut into cubes (2 cm^3) and homogenized in a blender (homogenizing buffer: 4 M glycerol, 100 mM piperazine-1,4-*bis* (PIPES), 1 mM dithiothreitol (DTT), 2 mM EDTA, 5 µg/ml pepstatin, 75 µg/ml phenylmethyl sulfonyl fluoride (PMSF), 2 µg/ml aprotinin, 2 mg/ml leupeptin pH 6.9, 1:3 g tissue:ml buffer). The crude extract was centrifuged at 48,000 rpm (100,000 × g) for 1.5 h to yield a clear supernatant. Aliquots were frozen in liquid nitrogen and stored at −78°C until further use.

Tissue supernatant (6 ml) was incubated with control resin **7** (20 µl) at 4°C to remove abundant proteins binding indiscriminately to the resin and the linker. After 6 h the mixture was filtered. The filtrate was divided into two batches: (i) ilimaquinone resin **6** (20 µl) was incubated with supernatant (3 ml) and (ii) a control experiment containing control resin **7** (20 µl) with the remaining supernatant (3 ml). After 12 h the resin of each experiment was filtered and washed three times with homogenizing buffer (750 µl) for 1 h. The resins were then washed with SDS sample buffer (25 µl) to collect the proteins that were still bound to the resin. These proteins were separated using SDS–PAGE and visualized with Commassie Blue or silver nitrate.

To obtain sufficient sample for sequencing, liver supernatant (550 ml) was incubated with control resin **7** (200 µl) at 4°C to remove abundant proteins binding to the resin and the linker. After 6 h the mixture was filtered. The filtrate was divided into 2 batches: (i) ilimaquinone resin **6** (100 µl) was incubated with supernatant (500 ml) and (ii) a control experiment containing control resin **7** (20 µl) with supernatant (50 ml). After 12 h the resin of each experiment was filtered and washed with homogenizing buffer (750 µl, 3×) for 1 h. Resins were washed with SDS sample buffer (50 µl) to collect bound proteins. These proteins were separated by SDS–PAGE gel electrophoresis and visualized with Commassie Blue.

The SDS–PAGE gel was placed along with a PVDF membrane (PVDF membrane is pre-equilibrated in MeOH for 2 min and then washed in H_2O for 20 min before use) into BioRad blotting apparatus. Protein transfer was carried out for 40–120 min at 100 V in transfer buffer (10% MeOH, 192 mM Tris, 25 mM Gly). After protein transfer, PVDF membrane was stained with Panceau (1% Panceau, 3% acetic acid) and subsequently washed with H_2O (10 ml). Stained protein bands were cut out and again washed with H_2O (2 ml). The membranes were stored at −20°C until sequencing. Several proteins were sequenced directly from the polyacrylamide gel. Here, the protein bands were cut out of the gel and washed with 50% CH_3CN (1 ml, 2×) for 10 min. After washings, the protein bands were stored at −20°C until sequenced.

Evaluation of methylation inhibitors on secretory activity

All plasticware and glassware were pre-sterilized or purchased sterile. Cell culture media was filtered through Nalgene sterile filters with 0.2 µm nylon membrane filters before use. The plasmid encoding for secreted alkaline phosphatase was obtained from the Schreiber laboratory at Harvard University and used with permission. Incubation refers to storing cells in tissue culture flasks with canted necks and vent caps in a CO_2 incubator at 37°C, 5.0% CO_2 and 100% humidity. Centrifugation, pelleting, or spinning down refers to using a swinging bucket centrifuge at room temperature at 1000 rpm (165 × g) for 10 min then aspirating the spent media. Cell counts were obtained using a hemacytometer with cells stained with trypan blue (0.4%), counts were done in duplicate and averaged. Cell morphology was monitored using a phase-contrast cell culture light microscope at 40×.

Jurkat cells were incubated in RPMI 1640 media (without phenol red), 10% fetal bovine serum (FBS), and 5% penicillin/streptomycin. For normal cell maintenance, cells were grown until saturation ($1.5–2.5 \times 10^6$ cells/ml), then diluted tenfold with fresh media. To run the assay, 1×10^7 cells were pelleted. (Amount required for two compounds assayed in duplicate). Cells were rinsed with RPMI 1640 media and resuspended in 500 µl of RPMI 1640 media. The cells were pipetted into a 4 mm cuvette containing plasmid (20 µg). Equilibration (20 min, room temperature), was then followed by electroporation (960 µF, 300 V, room temperature). The cells were reequilibrated (20 min, room temperature), then diluted with unselected media (10.0 ml, RPMI 1640, 10% FBS) and incubated for 48 h. Under these conditions, a 50% viability of cells was obtained. The cells were then pelleted and resuspended in fresh media (4 ml, RPMI 1640, 10% FBS, 5% penicillin/streptomycin).

Compounds to be tested for effects on secretion were introduced in a range of concentrations into 96-well plates. Unactivated cells (100 µl, 1.2×10^6 cells/ml) were pipetted into the wells. To activate the cells for alkaline phosphatase secretion, ionomycin and phorbol myristate acetate (PMA) (to a 2 µM and 100 ng/ml concentration, respectively) were added. The total volume in each well was diluted to 200 ml with fresh media. The plate was incubated overnight (37°C, 5% CO_2, 100% humidity) and then heated (68°C, 1.5 h) to denature heat-sensitive phosphatases. A *p*-nitrophenylphosphate solution (100 µl, 1 mg/ml of *p*-nitrophenylphosphate in 2 M diethanolamine bicarbonate, pH 10.0) was added to the wells of a new plate. The heat-treated cells and media (100 µl) were added to the corresponding *p*-nitrophenylphosphate-containing wells and incubated for 12–24 h. The absorption (410 nm) of each well was measured using a microplate spectrophotometer. Compounds tested include: cycloleucine, tripolyphosphate, 5′-deoxyadenosine, *S*-adenosylhomocysteine, (+)-ilimaquinone, (−)-ilimaquinone and brefeldin A.

Secretion assay in the presence of SAM

The methylation inhibitors were introduced into the 96-well plate in the following concentrations: (−)-ilimaquinone (50 µM), tripolyphosphate (1.0 mM), *S*-adenosylhomocysteine (2.0 mM), 5′-deoxyadenosine (1.0 mM), (+)-ilimaquinone (0.1 mM) and brefeldin A (5 µM). A range of SAM concentrations (6 µM–25 mM) were also added into the 96-well

plates containing the methylation inhibitor. The secretion assay was then run as described above.

Effects of ilimaquinone on enzymes of the activated methyl cycle (SAM synthetase)

SAM synthetase activity was measured using either crude bovine liver cytosol or purified isoforms from bovine liver. The samples (50 µl) were diluted with assay buffer (300 mM KCl, 15 mM $MgSO_4$, 4 mM DTT, 100 mM HEPES, 10 mM ATP, [^{3}H]-methionine (0.75 µg, 5 pmol, 0.25 µCi), pH 7.5) to yield a total volume of 100 µl. The reaction mixtures were incubated at 37°C. Enzyme dilution was adjusted so that no more than 20% of [^{3}H]-methionine was converted to SAM. After 1 h, 80 µl of the reaction mixture was spotted on a Whatman P-81 phosphocellulose disk and placed in ammonium formate (0.1 M, 10 ml). The ammonium formate was decanted and the disk was washed with ammonium formate (0.1 M, 10 ml), ethanol (10 ml), and diethyl ether (10 ml). The disk was allowed to air dry and the amount of SAM produced was determined through scintillation methods. This procedure was carried out in the presence and absence of several inhibitors such as (–)-ilimaquinone, (+)-ilimaquinone, 5′-deoxyadenosine and *S*-adenosylhomocysteinase at various concentrations.

Catechol-O-methyltransferase

COMT was assayed with slight modifications to the procedure of Rivett *et al.* [58]. Buffer A (2.5 mM $MgCl_2$, 1.0 mM paraglyine, 1.0 mM SAM, 4.0 µM, 1.5 × 10^4 cpm/nM dopamine) was prepared (total volume 4 ml) and 300 µl was added by pipette into six separate 1.5 ml eppendorfs. Ilimaquinone (0.625 µl, 0.04 M) was added by pipette into two of the eppendorfs, pyrogallol (0.625 µl, 5 mM) into another two eppendorfs, and double-distilled water (ddw 0.625 µl) were added by pipette into the last two. The solutions were incubated (37°C, 100% humidity, 5% CO_2, 40 min) The reactions were terminated with potassium borate (1 ml, 0.05 M). Methylated dopamine was isolated by extraction (3:2 toluene/isoamyl alcohol, 5 ml). The mixture was vortexed for 15 s and centrifuged (800 × g, 5 min). An aliquot (1 ml) of the organic phase was added to scintillation fluid (5 ml). Radioactivity of the methylated dopamine was quantified by liquid scintillation methods.

S-*adenosylhomocysteinase*

[8-^{14}C]-*S*-Adenosylhomocysteine (5.2 µg, 14 nM, 12 µCi) was incubated in the presence of enzyme (0.5 µg) at 37°C for 15 min in a total reaction volume of 0.1 ml. The incubation buffer was 15 mM Hepes, 5 mM $Mg(OAc)_2$, 150 mM KCl, 2 mM 2-mercaptoethanol, 0.25% bovine serum albumin, pH 7.0 including adenosine deaminase (50 units/ml). The incubations were stopped by the addition of formic acid (5 N, 0.1 ml) and the reaction mixture was poured onto a SP-Sephadex-C25 column (50 mm × 150 mm) pre-equilibrated with 0.1 N formic acid. [^{14}C]-Inosine was eluted with formic acid (0.1 N, 1.5 ml) and collected in a scintillation vial with scintillation fluid (5 ml). Inhibitors such as (–)-ilimaquinone, 5′-deoxyadenosine, (+)-chloroquinone, methionine and *S*-adenosylhomocysteine were added to assay at concentrations ranging between 0.05–1.0 mM.

Ilimaquinone's influence on new methylations

Cells (2.7×10^7) were pelleted, rinsed with methionine-free F12K media, repelleted and suspended in 1.9 ml methionine-free F12K media. Tritiated SAM (100 µl, 2.6 µCi, 10 Ci/mmole) was added to the cell suspension (1.9 ml, 1.4×10^7 cells/ml). (–)-Ilimaquinone (0.63 µl, 0.04 M) was then pipetted into two sterile eppendorfs, whereas ddw (0.63 µl) was pipetted into another two eppendorfs. After several minutes, equal amounts of radiolabeled cell suspension (500 µl, 1.4×10^7 cells/ml) were pipetted into the four eppendorfs and mixed thoroughly. All solutions were warmed (1 h, 37°C, 5% CO_2, 100% humidity) and shaken every 15 min. After the incubation was complete, nonincorporated SAM was removed via centrifugation (100 × g, 4°C, 10 min). Supernatant was removed and counted to quantify amount of nonincorporated SAM. The cellular pellet was lysed with 2 ml of lysis buffer (3.0 M $MgCl_2$, 7.6 mM TrisCl, 1.1 mM β-mercaptoethanol pH 7.6; titrated with HCl). Lysis was carried out with 100 strokes in a glass homogenizer. Lysed cells were removed and the homogenizer was rinsed (3×) with ddw (1 ml) and the cell suspension (5 ml total) was placed in a polypropylene ultracentrifuge tube. The following protease inhibitors were used during the lysis and throughout the remainder of the assay and for storage purposes: lysis buffer with 2.9 µM pepstatin-A, 2.1 µM leupeptin, 1.0 mg/l aprotinin, 2.0 mM EDTA, 0.43 M PMSF. Ultracentrifugation (15,000 × g, 4°C, 30 min) was carried out to remove unbroken debris, mitochondria and lysosomes. The supernatant was pipetted into a clean ultracentrifuge tube and pelleted (100,000 × g, 4°C, 60 min) to separate the cytosol from the membranes. The pellet from previous centrifugation was dissolved and counted. Membrane and cytosol were separated and crude counts were measured. Scintillation techniques were used to count 10% of the total volume of a solution.

Acknowledgements

The National Institutes of Health (CA66617) is gratefully acknowledged for financial support. In addition, we thank George D Markham (Fox Chase Cancer Center) for a gift of *S*-adenosylmethionine synthetase and Clinton A Krueger for preliminary *S*-adenosylmethionine synthetase assay results. C.A.D. and R.L.C. are Department of Education GAANN fellows. M.L.S. is a Lilly Grantee, Alfred P. Sloan Fellow, Camille Dreyfus Teacher-Scholar, DuPont Young Professor, and Glaxo Wellcome Chemistry Scholar. Protein sequencing was performed by the Harvard Microchemistry Laboratories, Harvard University, Cambridge, MA. Sequence comparisons to known protein in the SwissProt databases were carried out on the Computational Biochemistry server at Eidgenbssische Technische Hochschule Zürich (ETHZ).

References

1. Hurtley, S.M. (1996). *Protein Targeting.* pp. 214, IRL Press, Oxford, UK.
2. Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science* **279**, 519-526.
3. Rothman, J.E. & Söllner, T.H. (1997). Throttles and dampers: controlling the engine of membrane fusion. *Science* **276**, 1212-1213.
4. Riezman, H., Woodman, P.G., van Meer, G. & Marsh, M. (1997). Molecular mechanisms of endocytosis. *Cell* **91**, 731-738.
5. Lowe, S.L., Peter, F., Subramaniam, V.N., Wong, S.H. & Hong, W. (1997). A SNARE involved in protein transfort through the Golgi apparatus. *Nature* **389**, 881-884.
6. Kuehn, M.J., Hermann, J. M. & Schekman, R. (1998). COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature* **391**, 187-190.
7. Muñiz, M., Martîn, M.E., Hidalgo, J. & Velasco, A. (1997). Protein kinase A activity is required for the budding of constitutive transport vesicles from the trans-Golgi network. *Proc. Natl Acad. Sci. USA* **94**, 14461-14466.
8. Jones, S.M., Howell, K.E., Henley, J.R., Cao, H. & McNiven, M.A. (1998). Role of dynamin in the formation of transport vesicles from the trans-Golgi network. *Science* **279**, 573-577.
9. Mermall, V., Post, P.L. & Mooseker, M.S. (1998). Unconventional myosins in cell movement, membrane traffic, and signal transduction. *Science* **279**, 527-533.
10. Echard, A., *et al.*, & Goud, B. (1998). Interaction of a Golgi-associated kinesin-like protein with Rab6. *Science* **279**, 580-585.
11. Nichols, B.J., Ungermann, C., Pelham, H.R.B., Wickner, W.T. & Haas, A. (1997). Homotypic vacuolar fusion mediated by t- and v-SNAREs. *Nature* **387**, 199-202.
12. Lupashin, V.V. & Waters, M.G. (1997). t-SNARE activation through transient interaction with a Rab-like guanosine triphosphatase. *Science* **276**, 1255-1258.
13. Kondo, H., *et al.*, & Warren, G. (1997). p47 is a cofactor for p97-mediated membrane fusion. *Nature* **388**, 75-78.
14. Schweizer, F.E., Dresbach, T., DeBello, W.M., O'Conner, V., Augustine, G.J. & Betz, H. (1998). Regulation of neurotransmitter release kinetics by NSF. *Science* **279**, 1203-1205.
15. Südhof, T.C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375**, 645-653.
16. Pfanner, N., Glick, B.S., Arden, S.R. & Rothman, J.E. (1990). Fatty acylation promotes fusion of transport vesicles with Golgi cisternea. *J. Cell Biol.* **110**, 955-961.
17. Weber T., *et. al.*, & Rothman, J.E. (1998). SNAREpins: Minimal machinery for membrane fusion. *Cell* **92**, 759-772.

18. Fasshauer, D., Otto, H., Eliason, W.K., Jahn, R. & Brünger, A.T. (1997). Structural changes are associated with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. *J. Biol. Chem.* **272**, 28036-28041.
19. Mayer, A., Wickner, W. & Haas, A. (1996). Sec18p (NSF)-driven release of Sec17p (a-SNAP) can preceed docking and fusion of yeast Vacuoles. *Cell* **85**, 83-94.
20. Luibrand, R.T., Erdman, T.R., Vollmer, J.J., Scheuer, P.J., Finer, J. & Clardy, J.C. (1979). Ilimaquinone, a sesquiterpenoid quinone from a marine sponge. *Tetrahedron* **35**, 609-612.
21. Capon, R.J. & MacLeod, J.K. (1987). A revision of the absolute stereochemistry of ilimaquinone. *J. Org. Chem.* **52**, 5059-5060.
22. Talizawa, P.A., *et. al.*, & Malhotra, V. (1993). Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite, ilimaquinone. *Cell* **73**, 1079-1090.
23. Veit, B., Yucel, J.K. & Malhotra, V. (1993). Microtubule independent vesiculation of Golgi membranes and the reassembly of vesicles into Golgi stacks. *J. Biol. Chem.* **122**, 1197-1206.
24. Jamora, C., Takizawa, P.A., Zaarour, R.F., Denesvre, C., Faulkner, D.J. & Malhotra, V. (1997). Regulation of Golgi structure through heterotrimeric G proteins. *Cell* **91**, 617-626.
25. Wang, H.J., Benlimame, N. & Nabi, I.R. (1997). The AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the endoplasmic-reticulum. *J. Cell Sci.* **110**, 3043-3053.
26. Loya, S., Tal, R., Kashman, Y. & Hizi, A. (1990). Illimaquinone, a selective inhibitor of the RNase H activity of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **34**, 2009-2012.
27. Kushlan, D.M., Faulkner, D.J., Parkanyi, L. & Clardy, J.C. (1989). Metabolites of the Palauan sponge *Dactylospongia* sp. *Tetrahedron* **45**, 3307-3312.
28. Bourguet-Kondracki, M.-L., Longeon, A., Morel, E. & Guyot, M. (1991). Sesquiterpene quinones as immunomodulating. *Agents Int. J. Immunopharmacol.* **13**, 393-399.
29. Schröder, H.C., *et. al.*, & Müller, W.E.G. (1989). Suppression of the modulatory effects of the antileukemic and anti-human immunodeficiency virus compound avarol on gene expression by tryptophan. *Cancer Res.* **49**, 2069-2076.
30. Sarin, P.S., Sun, D., Thornton, A. & Müller, W.E.G. (1987). Inhibition of replication of the etiologic agent of acquired immune deficiency syndrome (Human T-Lymphotropic Retrovirus/Lymphadenopathy-Associated Virus) by avarol and avarone. *J. Natl Cancer Inst.* **78**, 663-666.
31. Belisario, M.A., Maturo, M., Pecce, R., De, R.S. & Villani, G.R.D. (1992). Effect of avarol and avarone on in vitro-induced microsomal lipid peroxidation. *Toxicology* **72**, 221-233.
32. Müller, W.E.G., *et. al.*, & Schroeder, H.C. (1985). Inhibition of mitosis by avarol, a natural product isolated from the sponge *Dysidea avara*. *Basic Appl. Histochem.* **29**, 321-330.
33. Rangel, H.R., Dagger, F. & Compagnone, R.S. (1997). Antiproliferative effect of illimaquinone on Leishmania mexicana. *Cell Biol. Int.* **21**, 337-339.
34. Nambiar, M.P. & Wu, H.C. (1995). Ilimaquinone inhibits the cytotoxicities of ricin, diphtheria toxin, and other protein toxins in vero cells. *Exp. Cell Res.* **219**, 671-678.
35. Bruner, S.D., Radeke, H.S., Tallarico, J.A. & Snapper, M.L. (1995). Total synthesis of (–)-ilimaquinone. *J. Org. Chem.* **60**, 1114-1115.
36. Radeke, H.S., Digits, C.A., Bruner, S.D. & Snapper, M.L. (1997). New tools for studying vesicular-mediated protein trafficking: synthesis and evaluation of ilimaquinone analogs in a non-radioisotope-based anti-secretory assay. *J. Org. Chem.* **62**, 2823-2831.
37. Takusagawa, F., Kamirtori, S., Misaki, S. & Markham, G.D. (1996). Crystal structure of *S*-adenosylmethionine synthetase. *J. Biol. Chem.* **271**, 136-147.
38. Tabor, C.W. & Tabor, H. (1984). Methionine adenosyltransferase (*S*-adenosylmethionine synthetase) and *S*-adenosylmethionine decarboxylase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **56**, 251-282.
39. Alvarez, L., Corrales, F., Martín-Duce, A. & Mato, J.M. (1993). Characterization of a full-length cDNA encoding human liver *S*-adenosylmethionine synthetase: tissue-specific gene expression and mRNA levels in hepatopathies. *Biochem. J.* **293**, 481-486.
40. Mitsui, K., Teraoka, H. & Tsukada, K. (1988). Complete purification and immunochemical analysis of *S*-adenosylmethionine synthetase from bovine brain. *J. Biol. Chem.* **263**, 11211-11216.
41. de la Haba, G. & Cantoni, G.L. (1959). The enzymatic synthesis of *S*-adenosyl-L-homocysteine from adenosin and homocysteine. *J. Biol. Chem.* **234**, 603-608.
42. Cantoni, G.L. (1986). Experimental and clincial roles of *S*-adenosylmethionine in biological methylation and drug design. (Borchardt, R.T., Creveling, C.R. & Ueland, P.M., eds), pp. 227-238, Humana Press, Clifton, NJ.
43. Fujioka, M. & Takata, Y. (1981). *S*-Adenosylhomocysteine hydrolase from rat liver. Purification and some properties. *J. Biol. Chem.* **256**, 1631-1635.
44. Bruice, T.C. & Zheng, Y.-J. (1997). A theoretical examination of the factors controlling the catalytic efficiency of a transmethylation enzyme: catechol-O-methyltransferase. *J. Am. Chem. Soc.* **119**, 8137-8145.
45. Kundström, K., Tenhunen, J., Tilgmann, C., Karhunen, T., Panula, P. & Ulmanen, I. (1995). Cloning, expression and structure of catechol-O-methyltransferase. *Biochim. Biophys. Acta* **1251**, 1-10.
46. Creveling, C.R. & Inoue, K. (1994). Catechol-O-methyltransferase: factors relating to the carcinogenic potential of catecholestrogens. *Polycycl. Aromat. Comp.* **6**, 253-259.
47. Cheng, X. (1995). Structure and function of DNA methyltransferases. *Annu. Rev. Biophys. Biomol. Struct.* **24**, 293-318.
48. Okada, G., Teraoka, H. & Tsukada, K. (1981). Multiple species of mammalian *S*-adenosylmethionine synthetase. Partial purification and characterization. *Biochemistry* **20**, 934-940.
49. Katb, M. & Kredich, N.M. (1985). *S*-Adenosylmethionine synthetase from human lymphocytes. *J. Biol. Chem.* **260**, 3923-3930.
50. Ueland, P.M. & Saebø, J. (1979). *S*-Adenosylhomocysteinase from mouse liver. Effect of adenine and adenine nucleotides on the enzyme catalysis. *Biochemistry* **18**, 4130-4135.
51. Kredich, N.J. & Martin, D.W. (1977). Role of *S*-adenosylhomocysteine in adenosine mediated toxicity in cultured mouse T lymphoma cells. *Cell* **12**, 931-938.
52. Palmer, J.L. & Abeles, R.H. (1979). The mechanism of action of *S*-adenosylhomocysteinase *J. Biol. Chem.* **254**, 1217-1225.
53. Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., Tamura, G. & Ikehara, Y. (1986). Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* **261**, 11398-11403.
54. Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., Ikehara, Y. (1988). Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Bio. Chem.* **263**, 18545-18552.
55. Donaldson, J.G., Finazzi, D. & Klausner, R.D. (1992). Brefeldin A inhibits Golgi membrane-catalyzed exchange of guanine nucleotide onto ARF Protein. *Nature* **360**, 350-352.
56. Doms, R.W., Russ, G. & Yewdell, J.W. (1989). Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* **109**, 61-72.
57. Orci, L., *et al.*, & Rothman, J.E. (1991). Brefeldin A, a drug the blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell* **64**, 1183-1195.
58. Rivett, A.J., Eddy, B.J. & Roth, J.A. (1982). Contribution of sulfate conjugation, deamination, and O-methylation to metabolism of dopamine and norepinephrine in human brain. *J. Neurochem.* 1009-1016.
59. Merriam, G.R., MacLusky, N.J., Picard, M.K. & Naftolin, F. (1980). Comparative properties of the catechol estrogens, I: methylation by catechol-O-methyltransferase and binding to cytosol estrogen receptors. *Steroids* **36**, 1-11.
60. Chou, T.C. & Lombardini, J.B. (1972). Rapid assay procedure for ATP:L-methionine adenosyl-transferase. *Biochim. Biophys. Acta* **276**, 399-406.
61. Markham, G.D., Hafner, E.W., Tabor, C.W. & Tabor, H. (1983). *S*-Adenosylmethionine synthetase (methionine adensoyltransferase) (*Esherichia coli*). *Methods Enzymol.* **94**, 219-222.
62. Suma, Y., Shimizu, K. & Tsukada, K. (1986). Isozymes of *S*-adenosylmethionine synthetase form rat liver: isotation and characterization. *J. Biochem.* **100**, 67-75.
63. Hoffman, J.L. (1983). Fractionation of methionine adenosyl transferase isoenzymes (rat liver). *Methods Enzymol.* **94**, 223-228.
64. Sullivan, D.M. & Hoffman, J.L. (1983). Fractionation and kinetic properties of rat liver and kidney methionine adenosyltransferase isozymes. *Biochemistry* **22**, 1636-1641.
65. Ramalingam, K. & Woodard, R.W. (1984). An improved synthesis of *S*-adenosylhomocysteine and related compounds. *J. Org. Chem.* **49**, 1291-1293.
66. Kikugawa, K. & Ichino, M. (1971). Direct halogenation of sugar moiety of nucleosides. *Tetrahedron Lett.* **2**, 87-90. For assay information, see:
67. Chiang, P.K., Richards, H.H. & Cantoni, G.L. (1977). *S*-Adenosyl-L-homocysteine hydrolase: analogues of *S*-adenosyl-L-homocysteine as potential inhibitors. *Mol. Pharmacol.* **13**, 939-937.

68. Radeke, H.S. (1998). Total synthesis of ilimaquinone and analogs: isolation of cellular target(s). Ph.D. Thesis, Boston College, MA, USA.
69. Digits, C.A. (1998). Ilimaquinone; a probe to study vesicular mediated protein transport. M.S. Thesis, Boston College, MA, USA.
70. Radeke, H.S. & Snapper, M.L. (1998) Photoaffinity study of the cellular interactions of ilimaquinone. *Bioorg. Med. Chem.* **6**, 1227-1223.
71. Phelouzat, M.-A., *et al.*, & Robert-Gero, M. (1992). *Leishmania donovani*: antagonistic effect of *S*-adenosyl methionine on ultrastructural changes and growth inhibition induced by sinefungin. *Exp. Parasit.* **74**, 177-187.
72. Diliberto, Jr., E.J., Viveros, O.H. & Axelrod, J. (1976). Subcellular distribution of protein carboxymethylase and its endogenous substrate in the adrenal medulla: possible role in excitation-secretion coupling. *Proc. Natl Acad. Sci. USA* **73**, 4050-4054.
73. Hirata, F. & Axelrod, J. (1980). Phospholipid methylation and biological signal transmission. *Science* **209**, 1082-1090.
74. Kim, S., Galletti, P. & Paik, W.K. (1980). *In vivo* carboxyl methylation of human erythrocyte membrane proteins. *J. Biol. Chem.* **255**, 338-341.
75. Valentijn, J.A. & Jamieson, J.D. (1998). Carboxyl methylation of rab3D is developmentally regulated in the rat pancreas: correlation with exocrine function. *Eur. J. Cell Biol.* **76**, 204-211.
76. Li, G. & Stahi, P.D. (1993). Post-translational processing and membrane association of the two early endosome-associated rab GTP-binding proteins (rab4 and rab5). *Arch. Biochem. Biophys.* **304**, 471-478.
77. Giannakouros, T., Newman, C.M.H., Craighead, M.W., Armstrong, J. & Magee, A.I. (1993). Post-translational processing of *Schizosaccharomyces pombe* YPT5 protein. *J. Biol. Chem.* **268**, 24467-24474.
78. Wnuk, S.F., Liu, S., Yuan, C.-S., Borchardt, R.T. & Robins, M.J. (1996). Inactivation of *S*-adenosyl-L-homocysteine hydrolase by amide and ester derivatives of adenosine-5′-caroxylic acid. *J. Med. Chem.* **39**, 4162-4166.
79. Siddiqi, S.M., Chen, X. & Schneller, S.W. (1994). Antiviral enantiomeric preference for 5′-noraristeromycin. *J. Med. Chem.* **37**, 551-554.
80. Wnuk, S.F., Dalley, N.K. & Robins, M.J. (1993). Nucleic acid related compounds. 76. Synthesis of 5′(E and Z)-chloro-4′,5′-didehydro-5′-deoxyadenosines via chlorination and thermolysis of adenosine 5′-sulfoxides. Mechanism-based inhibition of *S*-adenosyl-L-homocysteine hydrolase. *J. Org. Chem.* **58**, 111-117.
81. Wolfe, M.S., Lee, Y., Bartlett, W.J., Borcherding, D.R. & Borchardt, R.T. (1992). 4′-Modified analogues of aristeromycin and neplanocin A: synthesis and inhibitory activity toward *S*-adenosyl-L-homocysteine hydrolase. *J. Med. Chem.* **35**, 1782-1791.
82. Samano, V. & Robins, M.J. (1991). Nucleic acid related compounds. 70. Synthesis of 2′ (and 3′)-deoxy-2′ (and 3′)-methyleneadenosines and bis(methylene)furan 4′,5′-didehydro-5′-deoxy-2′ (and 3′)-methyleneadenosines. Inhibitors of *S*-adenosyl-L-homocysteine hydrolase and ribonucleotide reductase. *J. Org. Chem.* **56**, 7108 7113.
83. Welch, G.N. & Loscalzo, J. (1998). Homocysteine and atherothrombosis. *N. Engl. J. Med.* **338**, 1042-1050.

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