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Photoaffinity Study of the Cellular Interactions of Ilimaquinone

Heike S. Radeke and Marc L. Snapper *

Eugene F. Merkert Chemistry Center, Boston College, Chestnut Hill, MA 02167-3860, USA

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Abstract—The marine sponge metabolite ilimaquinone (**1**) displays a broad range of biological activities. To better understand the effects of this natural product, a photoaffinity analogue was synthesized and used to probe the cellular interactions of ilimaquinone. Irradiation of photoaffinity probe **5** with liver cytosol in the presence and absence of excess competitive inhibitor **2** suggests that *S*-adenosylhomocysteinase is an important intracellular target of ilimaquinone.

Introduction

Ilimaquinone[1] (**1**), a novel sponge metabolite isolated from *Hippiospongia metachromia*, has been reported to possess a variety of potentially useful biological properties including anti-HIV, antimitotic, and antiinflammatory activities.[2] This natural product has also been reported to protect the cell against the harmful effects of ricin and diphtheria toxin.[3] In addition, recent studies have shown that in the presence of ilimaquinone the Golgi apparatus,[4] as well as portions of the endoplasmic reticulum,[5] are reversibly converted into small vesicles causing a breakdown of intracellular protein and lipid trafficking.

Vesicle-mediated trafficking events, such as those occurring at the Golgi apparatus, have been implicated in a number of health-related issues including: pathogen entry into cells, targets for bacterial toxins, as well as the progression of Alzheimer disease.[6] Developing a molecular understanding of the influence of ilimaquinone on vesicular trafficking[7] could yield new targets for combating these diseases. Our first goal for achieving a better understanding of intracellular trafficking, as well as the basis for the various other activities of ilimaquinone, was to identify the cellular target(s) of this compound. In this regard, we developed the first total synthesis of ilimaquinone,[8] which was then used to prepare structural variants of the natural product.[9] Figure 1 illustrates several analogues that retain the antisecretory activity of ilimaquinone. Herein we report on the preparation of photoaffinity probe **5** and its use in identifying the cellular interactions of ilimaquinone.

Results

Synthesis of photoaffinity probe. Photolabel **5** was prepared from analogue **4** by removing the BOC group with formic acid.[10] The resulting amine was then acylated with azido benzoic acid *N*-hydroxysuccinimide ester (HSAB).[11] Evaluation of analogue **5** in a secretion assay indicated that this compound retains nearly all of the anti-secretory activity of the natural product (**1**).[9] The tritiated version of **5** was prepared in an analogous manner (Scheme 1).

Optimization of photolysis conditions. To optimize the photoaffinity labeling conditions, irradiation of alcohol dehydrogenase at 254 nm with the nontritiated photoaffinity reagent **5** was examined as a function of time. Experiments were set up in minimal volumes (100 μL) to conserve radiolabel **5**. As indicated in Figure 2, minimal decomposition of protein occured with photolyses in a Rayonet reactor for 2 min or less. Furthermore, UV-Vis spectroscopy showed that complete consumption of the aryl azide functionality occured within this time frame. This preliminary study indicated that a maximum irradiation of 2 min (254 nm) would provide sufficient

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*Corresponding author. Tel.: (617) 552-8096; Fax: (617) 552-0230; E-mail: marc.snapper@bc.edu

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opportunity for activation of the photoaffinity label (**5**) without causing extensive degradation of the sample.

Photolabeling of cellular proteins. Using the optimized photolysis conditions, liver supernatant (100,000 *g*) was incubated with photolabel **5** (1 mM) in the presence and absence of excess ilimaquinone analogue **2** (100 mM). After a 2 min irradiation, efforts then focused on purifying and isolating the proteins that were selectively radiolabeled in the absence of competitive inhibitor **2**. The crude cytosol from the experiments with and without the competing analogue was separated first by ion exchange chromatography. The collected fractions of both experiments were then evaluated individually for tritium incorporation. The relative levels of radioactivity for the fractions are illustrated in Figure 3(a). Fractions that showed the greatest difference in radioactivity (#16–26) were pooled and purified further.

The combined fractions from the ion exchange chromatography were then subjected to size exclusion chromatography. A comparison of radiolabel incorporation for the isolated fractions from each experiment indicated that fractions #12–44 contain proteins selectively labeled during photolysis without competitive inhibitor **2** (Fig. 3(b)). This section was pooled, concentrated and subjected to SDS gel electrophoresis (Fig. 4). As shown in Figure 5, the relative radioactivity of comparable portions of the gel indicated that two bands (48 and 55 kD) were specifically labeled by the photoaffinity probe (**5**). Analysis of multiple digestion fragments from the 48 kD band indicated that the known enzyme *S*-adenosylhomocysteinase [EC 3.3.1.1][12] was a target of the ilimaquinone photoaffinity probe.[13]

By examining the antisecretory activity of known inhibitors of *S*-adenosylhomocysteinase, a connection to

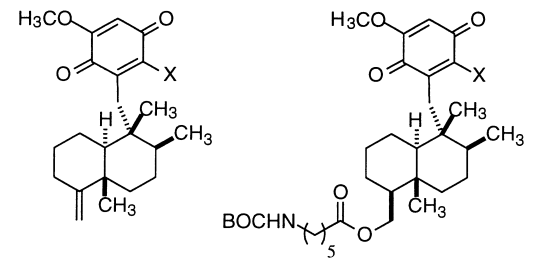
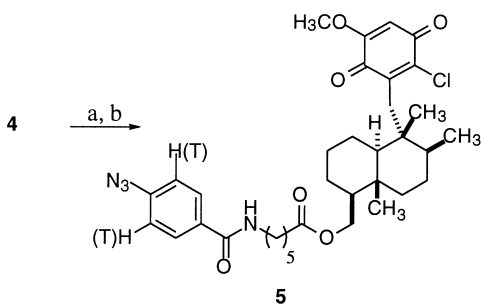


Figure 1. Ilimaquinone and biologically active structural variants.



Scheme 1. Reagents: (a) formic acid, 98%; (b) HSAB, DMAP, pyridine, 68%.

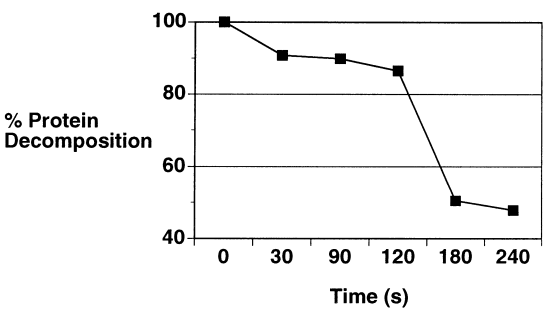


Figure 2. Photolysis of alcohol dehydrogenase.

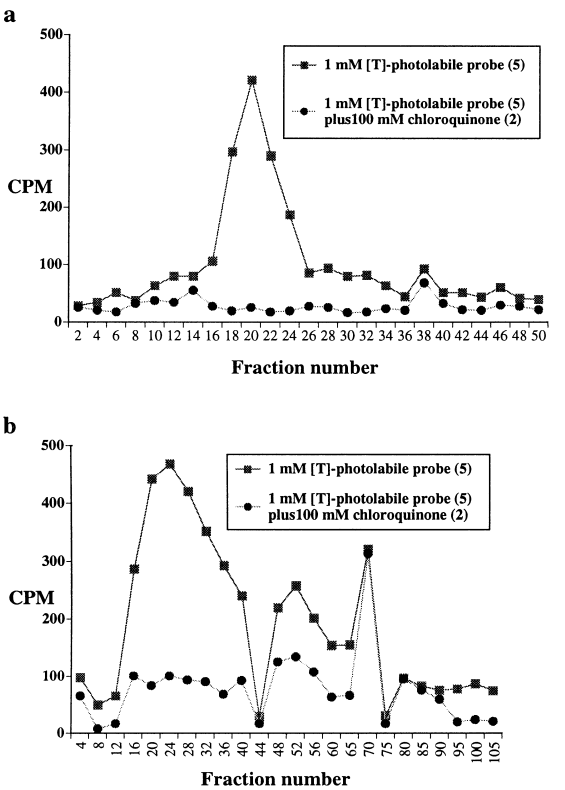


Figure 3. Purification of radiolabeled proteins through (a) ion-exchange, and (b) size exclusion chromatography.

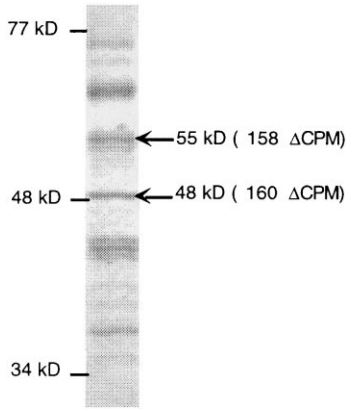


Figure 4. 10% SDS–PAGE of pooled fractions 12–44.

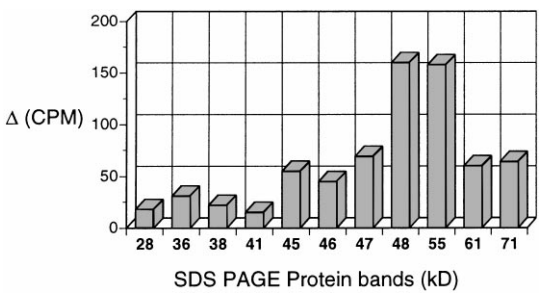


Figure 5. Radioactivity of labeled proteins.

vesicle-mediated events might be established. This analysis might also provide a functional relevance for the interaction between ilimaquinone and this enzyme. As illustrated in Figure 6, we found that 5′-deoxyadenosine[14] does indeed block the export of alkaline phosphatase in our secretion assay.[9] Furthermore, the antisecretory effects of 5′-deoxyadenosine can be overcome by adding excess *S*-adenosylmethionine (SAM) to the assay.

Discussion

In photoaffinity studies intracellular proteins present in high concentration, as well as those proteins that bind specifically with the probe molecule, are targets that are usually radiolabeled.[15] An excess of an active, nonradiolabeled analogue can compete effectively for the high affinity targets and can, therefore, be used to differentiate between the indiscriminately labeled abundant macromolecules and those less concentrated entities of interest. This was the strategy used to determine the proteins that bind selectivity to photoaffinity probe **5**. While active variants of ilimaquinone display antisecretory effects at much lower concentrations ($<20\,\mu M$), our photoaffinity experiments were run at sufficiently high concentration of compound **5** (1 mM) to ensure complete binding of relevant cellular targets. The control photolysis was run under similar conditions, but a 100-fold excess (100 mM) of a nonradiolabeled active ilimaquinone analogue **2** was used to compete for binding to the high affinity targets of ilimaquinone. Identification of the specific cellular interactions of ilimaquinone was then possible by comparing the relative levels of radiolabel incorporation for the various isolated proteins in these two experiments. Utilizing optimized photolysis conditions, radiolabeling of liver cytosol with photoaffinity probe **5** in the presence and absence of ilimaquinone analogue **2** identified *S*-adenosylhomocysteinase as a major target for ilimaquinone. This finding was supported by affinity chromatography experiments with immobilized analogue **3** (data not shown).[16]

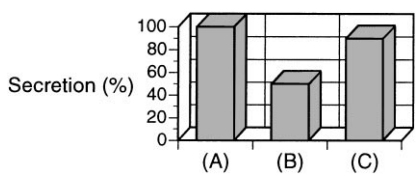


Figure 6. Alkaline phosphatase secretion assay: (A) Control; (B) 5′-deoxyadenosine (0.1 mM) and (C) 5′-deoxyadenosine (0.1 mM) and SAM (0.25 mM).

S-Adenosylhomocysteinase plays a key role in cellular methylation chemistry. Specifically, this enzyme catalyzes the breakdown of *S*-adenosylhomocysteine (SAH) to homocysteine and adenosine.[12] SAH, the methylation nucleofuge, functions to regulate the activity of methyltransferases such that elevated levels of SAH impede most methylation events in the cell.[17] Inhibition of methyltransferases by SAH can be overcome by increasing the cellular levels of *S*-adenosylmethionine (SAM).[12b] Since methylation events are known to play a role in cellular secretion,[18] it is possible that the antisecretory activity of ilimaquinone is directly related to the natural product's interaction with *S*-adenosylhomocysteinase. Given the importance of *S*-adenosylhomocysteinase as a target for antiviral chemotherapy,[19] studies to characterize further the interactions and influence of ilimaquinone on this enzyme are in progress.

Conclusions

Photoaffinity probe **5**, prepared through total synthesis, was used to identify a cellular target of the marine sponge metabolite ilimaquinone (**1**). Purification of proteins labeled preferentially in the absence of a nonradiolabeled competitive analogue (**2**) indicated that *S*-adenosylhomocysteinase has a specific interaction with ilimaquinone. Inhibition of secretion by a known inhibitor of *S*-adenosylhomocysteinase supported this enzyme's

interaction with ilimaquinone as having relevance to vesicular trafficking. Additional studies are underway to gain insight into the role of methylation in regulating vesicle-mediated processes of the Golgi apparatus.

Experimental

General

Starting materials and reagents were purchased from commercial suppliers and used without further purification. Syntheses were carried out under N_2 or Ar atm 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. Unless otherwise stated, reactions were stirred with a Teflon covered stir bar and carried out at rt.

^{3}[H]-Azidobenzoic acid *N*-hydroxysuccinimide ester (47 Ci/mmol) was obtained from New Life Science Products. Packard Sovable and Packard Formula-989 LSC cocktail were obtained from DuPont-New England Nuclear. Preparative TLC purifications were performed using Analtech Uniplate silica gel GF Plates (20 cm×20 cm). Sephacel and Sepharose were purchased from Pharmacia. Fresh liver samples were obtained from Research 87. Centrifugation was carried out in a Beckman L-70 Ultracentrifuge using a Type 70 Ti rotor. Proteins were isolated via liquid chromatography using the Bio-Rad Econo System. Photolysis reactions were carried out at 0 °C in a Rayonet Photochemical Reactor Model RPR-100 equipped with 17 RPR-2537 Å Lamps (35 W/lamp). Samples were photolyzed in a quartz condenser.

Proton nuclear magnetic resonance spectra (^{1}H NMR) were measured at 400 MHz on a Varian Gemini-400 instrument. Chemical shifts are reported in ppm down field from tetramethylsilane. Infrared spectra (IR) were measured on a Nicolet 510 FT-IR spectrometer and are reported in wave numbers (cm^{-1}). Optical rotations ($[\alpha]^{25}_D$) were measured on a Perkin–Elmer 241 polarimeter using a 1 dm cell. UV-Vis absorbances were measured on a Varian Cary 1 E and are reported as λ (log ε), nm. Elemental analyses were performed by Robertson Microlit Laboratories, Inc. (Madison, NJ, USA) and are reported in percent atomic abundance.

Synthesis of photoaffinity reagent (5)

(+)-2-Methoxy-5-chloro-6-[[*trans*-octahydro-5β,6β,8aβ-trimethyl-1β-[[6-(4-azidobenzoate-6-amino)hexanoy]oxy]-methyl]-5α-naphthyl]methyl]-1,4-cyclohexadiene-2,5-dione (5). Chloroquinone **4** (4.1 mg, 6.7 mmol) was dissolved in formic acid (88%, 0.15 mL). After 1 h of stirring, the solvent was removed under vacuum to yield the amine as a yellow oil (3.3 mg, 98%). 4-Azidobenzoic acid *N*-hydroxysuccinimide ester (HSAB, 1.3 mg, 5.5 mmol) and DMAP (0.6 mg, 4.7 mmol) dissolved in pyridine (0.5 mL) were added to the amine. The reaction mixture stirred for 12 h, after which it was quenched with acetic acid (1 M, 32 μL). The solvent was removed under vacuum to afford an orange crude product. Purification by preparative TLC (CH_2Cl_2/EtOAc, 4:1, R_f 0.6) yields **5** as a yellow oil (2.9 mg, 68%). The radiolabeled version of photoaffinity probe **5** (1.2 mg, 1.84 mmol, 33.5 mCi/mmol) was prepared in a similar fashion from the corresponding tritiated acylating agent (47.0 mCi/mmol) in 38% yield. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.78 (2H, d, J=8.8 Hz), 7.07 (2H, d, J=8.8 Hz), 6.19 (1H, br s), 6.03 (1H, s), 4.21 (1H, dd, J=9.8, 3.2 Hz), 3.86 (3H, s), 3.69 (2H, dd, J=10.6, 3.7 Hz), 2.86 (2H, br s), 2.81 (1H, d, J=13.2 Hz), 2.53 (1H, d, J=12.6 Hz), 2.33 (2H, t, J=7.3 Hz), 1.80–1.54 (10H, m), 1.46–1.13 (8H, m), 0.85 (3H, s), 0.83 (3H, s), 0.81 (3H, d, J=6.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 183.0, 181.9, 175.8, 170.0, 160.8, 157.4, 146.1, 143.6, 138.1, 137.7, 133.7, 120.2, 110.2, 69.7, 55.8, 53.3, 51.9, 49.1, 43.9, 41.9, 40.8, 39.8, 38.0, 35.1, 33.7, 31.9, 31.2, 29.9, 28.2, 27.8, 26.0, 23.9, 20.2, 19.0, 17.5; IR (thinfilm, NaCl) 3360, 2925, 2867, 2116, 1697, 1681, 1650, 1633, 1602, 1503, 1284 cm^{-1}; UV-Vis (H_2O) 228.4 (6.64), 258.4 (6.63), 291.9 (6.62) nm; $[\alpha]^{25}_D$ +32.0 (*c* 0.12, $CHCl_3$); Anal. calcd for $C_{35}H_{40}ClN_4O_6$: C 64.36, H 6.94. Found: C 64.32, H 6.90.

Photoaffinity labeling study

Protein stability. Alcohol dehydrogenase (127.9 μg, amount of protein found in 66.4 μL of liver supernatant) dissolved in homogenizing buffer (100 μL) was irradiated at 254 nm for different lengths of time. Protein concentrations after irradiation were determined in a Bradford assay.[20]

Decomposition of photoaffinity label. An aqueous solution of photolabel **5** (1 mM) was irradiated at 254 nm for 2 min. The sample was diluted tenfold and placed in a 1 mL quartz cuvette. The absorbance spectra was recorded.

Preparation of liver cytosol. Fresh calf tissue was cut into small cubes (2 cm×2 cm) and homogenized in a Waring blender (homogenizing buffer: 4 M glycerol, 100 mM PIPES, 1 mM DTT, 2 mM EDTA, 5 μg/mL Pepstatin, 75 μg/mL PMSF, 2 μg/mL Aprotinin, 2 μg/mL Leupeptin pH 6.9, 1:1 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 until needed at −78 °C.

Photolabeling of target(s) in cellular supernatant. Experiments were set up in parallel where the photolabel **5** was added to the cytosol in the presence and absence of 100 mM chloroquinone **2**. Specifically, photolabel **5** (0.33 mCi) dissolved in EtOH (3.26 μL) and homogenizing buffer or DMSO (28.2 μL) was added to the desired supernatant (66.4 μL) to yield a total volume of 100 μL. For the competition experiment, chloroquinone **2** (3.74 mg) dissolved in DMSO (28.2 μL) was added to the photolysis tube. Both samples were shaken at 4 °C in 10 mm×120 mm quartz test tubes for 12 h, then they were irradiated for 2 min at 254 nm. Both samples were then diluted with homogenizing buffer to a total volume of 200 μL.

Ion exchange liquid chromatography. A Sephacel column (120 mm×180 mm) was equilibrated with buffer A (2 mM DTT, 25 mM Hepes, pH 7.5). Proteins in each experiment were eluted at 0.2 mL/min with a linear gradient of buffer B (buffer A plus 0.15 M KCl). The eluents were monitored for absorbance and radioactivity. Fractions from the experiment run without chloroquinone **2** that displayed a large difference in radioactivity from the experiment with **2** were pooled and concentrated using a centri-prep-10 to a final volume of 350 μL.

Size exclusion liquid chromatography. A Sepharose column (15 mm×70 mm) was equilibrated with buffer (buffer A plus 0.3 M KCl). Proteins isolated from ion exchange (loading volume 350 μL) were separated at 0.1 mL/min. The eluents were monitored for absorbance and radioactivity. Fractions displaying the largest difference in radioactivity between the two experiments were concentrated (20 μL) and denatured with 2X SDS-PAGE sample buffer (20 μL).

Separation of target proteins. Proteins were separated by SDS–PAGE electrophoresis using a 10% running gel (16 cm×12 cm, 50 mA). The gel was stained with Coomassie Blue to visualize protein bands. The gel was sliced into sections and the individual regions were dissolved in 0.5 mL Solvable/0.5 mL H_2O for 3 h at 60 °C, and then measured for radioactivity. The radioactive bands were washed twice in 0.5 mL 50% CH_3CN for 10 min for sequence analysis. Protein bands displaying the greatest difference in radioactivity were sent out for sequencing.

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References and Notes

1. (a) Luibrand, R. T.; Erdman, T. R.; Vollmer, J. J.; Scheuer, P. J.; Finer, J.; Clardy, J. C. *Tetrahedron* **1979**, *35*, 609. (b) Capon, R. J.; MacLeod, J. K. *J. Org. Chem.* **1987**, *52*, 5060.

2. (a) Loya, S.; Tal, R.; Kashman, Y.; Hizi, A. *Antimicrob. Agents Chemother.* **1990**, *34*, 2009. (b) Kushlan, D. M.; Faulkner, D. J.; Parkanyi, L.; Clardy, J. C. *Tetrahedron* **1989**, *45*, 3307. (c) Bourguet-Kondracki, M.-L.; Longeon, A.; Morel, E.; Guyot, M. *Int. J. Immunopharmacol.* **1991**, *13*, 393. (d) Schröder, H. C.; Wenger, R.; Gerner, H.; Reuter, P.; Kuchino, Y.; Sladic, D.; Müller, W. E. G. *Cancer Res.* **1989**, *49*, 2069. (e) Sarin, P. S.; Sun, D.; Thornton, A.; Müller, W. E. G. *J. Natl. Cancer Inst.* **1987**, *78*, 663. (f) Belisario, M. A.; Maturo, M.; Pecce, R.; De, R. S.; Villani, G. R. D. *Toxicology* **1992**, *72*, 221. (g) Müller, W. E. G.; Dogovic, N.; Zahn, R. K.; Maidhof, A.; Diehl, S. B.; Bedker, C.; Sachsse, W.; Gasic, M. J.; Schroeder, H. C. *Basic Appl. Histochem.* **1985**, *29*, 321.

3. Nambiar, M. P.; Wu, H. C. *Exp. Cell Res.* **1995**, *219*, 671.

4. (a) Talizawa, P. A.; Yucel, J. K.; Veit, B.; Faulkner, D. J.; Deerinck, T.; Soto, G.; Ellisman, M.; Malhotra, V. *Cell* **1993**, *73*, 1079. (b) Veit, B.; Yucel, J. K.; Malhotra, V. *J. Biol. Chem.* **1993**, *122*, 1197.

5. Wang, H.-J.; Benlimane, N.; Nabi, I. R. *J. Cell Sci.* **1997**, *110*, 3043.

6. (a) Vogel, J. P.; Andrews, H. L.; Wong, S. K.; Isberg, R. R. *Science* **1998**, *279*, 873. (b) Greber, U. F.; Willetts, M.; Webster, P.; Helenius, A. *Cell* **1993**, *75*, 477. (c) Nixon, R. A.; Cataldo, A. M.; Paskevich, P. A.; Hamiliton, D. J.; Wheelock, T. R.; Kanaley, A. L. *Ann. N.Y. Acad. Sci.* **1992**, *647*, 65. (d) Kosik, K. S. *Science* **1992**, *256*, 5085, 780. (e) Oda, T.; Wu, H. C. *J. Biol. Chem.* **1993**, *268*, 17, 12596. (f) Liscum L.; Dahl, N. K.; *J. Lipid Res.* **1992**, *33*, 1239. (g) Amigorena, S.; Drake, J.R.; Webster, P.; Mellman, I. *Nature* **1994**, *369*, 113.

7. Jamora, C.; Takizawa, P. A.; Zaarour, R. F.; Denesvre, C.; Faulkner, D. J.; Malhotra, V. *Cell* **1997**, *91*, 617.

8. Bruner, S. D.; Radeke, H. S.; Tallarico, J. A.; Snapper, M. L. *J. Org. Chem.* **1995**, *60*, 1114.

9. Radeke, H. S.; Digits, C. A.; Bruner, S. D.; Snapper, M. L. *J. Org. Chem.* **1997**, *62*, 2823.

10. Mathew, A. E.; Mejillano, M. R.; Nath, J. P.; Himes, R. H.; Stella, V. J. *J. Med. Chem.* **1992**, *35*, 145.

11. Swindell, C. S.; Heerding, J. M.; Krauss, N. E.; Horwitz, S. B.; Rao, S.; Ringel, I. *J. Med. Chem.* **1994**, *37*, 1446.

12. For lead references, see: (a) de la Haba, G.; Cantoni, G. L. *J. Biol. Chem.* **1959**, *234*, 603. (b) Cantoni, G. L. In *Biological Methylation and Drug Design*; Borchardt, R. T.; Creveling, C. R.; Ueland, P. M., Eds.; Humana: Clifton, 1986; pp 227–238. (c) Fujioka, M.; Takata, Y. *J. Biol. Chem.* **1981**, *256*, 1631.

13. Protein sequencing was performed by the Harvard Microchemistry Laboratories, Harvard University, Cambridge, MA. Reported sequences include: VPAINVNDSVTK and

GISEETTTGVHNLYK. Sequence comparisons to known proteins in the SwissProt database were carried out on the Computational Biochemistry server at Eidgenbssische Technische Hochschule Zürich (ETHZ).

14. Palmer, J. L.; Abeles, R. H. *J. Biol. Chem.* **1979**, *254*, 1217.

15. Prestwich, G. D. *Insect Biochem.* **1991**, *21*, 27.

16. *Manuscript in preparation.*

17. (a) Borchardt, R. T. In *The Biochemistry of Adenosylmethionine*; Salvatore, F.; et al., Eds; Columbia University: New York, 1977; pp 151–171. (b) Capdevila, A.; Decha-Umphai, W.; Song, K.-H.; Borchardt, R. T.; Wagner, C. *Arch. Biochem. Biophys.* **1997**, *345*, 47. (c) Chagoya de Sánchez, V.; Hernández-Muñoz, R.; Sánchez, L.; Vidrio, S.; Yáñez, L.; Suárez, J. *Int. J. Biochem.* **1991**, *23*, 1439.

18. (a) Diliberto, Jr., E. J.; Viveros, O.H.; Axelrod, *J. Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 4050. (b) Hirata, F.; Axelrod, *J. Science* **1980**, *209*, 1082. (c) Kim, S.; Galletti, P.; Paik, W. K. *J. Biol. Chem.* **1980**, *255*, 338. (d) Benyon, R. C.; Church, M. K.; Holgate, S. T. *Biochem. Pharmacol.* **1984**, *33*, 2881. (e) Best, L.; Lebrun, P.; Saceda, M.; Garcia-Morales, P.; Hubinont, C.; Juvent, M.; Herchuelz, A.; Malaisse-Lagae, F.; Valverde, I.; Malaisse, W. J. *Biochem. Pharmacol.* **1984**, *33*, 2033. (f) de Groot, P. G.; Gonsalves, M. D.; Loesberg, C.; van Buul-Wortelboer, M. F.; van Aken, W. G.; van Mourik, J. A. *J. Biol. Chem.* **1984**, *259*, 13329.

19. For representative references, see: (a) Wnuk, S. F.; Liu, S.; Yuan, C.-S.; Borchardt, R. T.; Robins, M. J. *J. Med. Chem.* **1996**, *39*, 4162. (b) Siddiqi, S. M.; Chen, X.; Schneller, S. W. *J. Med. Chem.* **1994**, *37*, 551. (c) Wnuk, S. F.; Dalley, N. K.; Robins, M. J. *J. Org. Chem.* **1993**, *58*, 111. (d) Wolfe, M. S.; Lee, Y.; Bartlett, W. J.; Borcherding, D. R.; Borchardt, R. T. *J. Med. Chem.* **1992**, *35*, 1782. (e) Samano, V.; Robins, M. J. *J. Org. Chem.* **1991**, *56*, 7108.

20. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.