CLICK CHEMISTRY IN GENERATION OF MAMMALIAN ACETYLOME TO STUDY SHWACHMAN-DIAMOND SYNDROME

An Honors Fellows Thesis

by

ROBERT CARSON SIBLEY III

Submitted to the Honors Programs Office
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

April 2011

Major: Biology

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ABSTRACT

Click Chemistry in Generation of Mammalian Acetylome to Study Shwachman-Diamond Syndrome. (April 2011)

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Shwachman Diamond syndrome is an autosomal recessive disease associated with pancreatic insufficiency, skeletal abnormalities, and bone marrow failure. The de Figueiredo lab has recently shown physical and genetic interactions between Sdo1p/SBDS proteins and class I and II histone deacetylases (HDACs) in yeast and mammalian cells, respectively. This study seeks to implement new methods to generate an acetylome to further elucidate the role of acetylation in the function of SBDS, hoping that more information will lead to the development of more effective treatments for those with SDS. Metabolic labeling with click chemistry is a new method that can be used for this purpose. To study if this chemistry is suitable K-562, an erythroleukemia type of mammalian cells, were used as the model organism. The cells were treated with Trichostatin A (TSA), an HDAC inhibitor, to simulate the interaction between SBDS and HDACs. The proteins of these cells were metabolically labeled with sodium 4-pentynoate. These proteins were then isolated and visualized using the integration of

click reaction into a horseradish peroxidase (HRP) labeling system. Since no bands were visualized in the 17 kDa range (the size of HDACs), this data does not support the use of click chemistry in creating an acetylome to further elucidate the cellular mechanism of SDS. Though the concentrations recommended in a previous study did not work for this system, generating 4-pentynoate dosage response data may allow for this strategy to work.

DEDICATION

I would like to dedicate this thesis to my family for always believing in me and to those who suffer from Shwachman Diamond syndrome.

ACKNOWLEDGMENTS

First of all I would like to acknowledge my research advisor, Dr. Paul de Figueiredo, for giving me the chance to enter into the world of research. Dr. de Figueiredo has shown me a vivid view of the wide variety of projects being conducted in his laboratory. This experience has expanded my understanding of cellular biology exponentially. He has always encouraged me to achieve my utmost potential in all that I do.

I would also like to thank Dr. Lei Li. She has spent countless hours explaining theories and demonstrating a wide variety of laboratory techniques. She has encouraged me to pursue research without compromise. She has always encouraged me to never settle for anything less than 100% certainty in my work.

Dr. Qingming Qin has also made a large contribution to my research. He has tutored me in the ways of mammalian cell culture, and has shown me a glimpse into the realities of an endless world of research. I would also like to thank Marshall, Veronica, Cassie, and Katie for listening to my woes and for celebrating with me in my successes.

Most importantly I would like to thank my family for their constant support.

NOMENCLATURE

DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
RPMI	Roswell Park Memorial Institute medium
SBDS	Shwachman-Bodian-Diamond syndrome
SDS	Shwachman Diamond syndrome
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TSA	Trichostatin A

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CHAPTER I

INTRODUCTION

Shwachman Diamond syndrome (SDS) is a congenital disorder, first described in 1964 in five patients showing diarrhea, pancreatic insufficiency, neutropenia associated with bone marrow hypoplasia, elevation in fetal hemoglobin, inconstant galactosuria, and growth retardation. These patients were originally thought to have cystic fibrosis, but normal sweat electrolytes and an absence of pulmonary disease suggested a different diagnosis (1).

Years of research have followed, but medical professionals need a more complete understanding of how Shwachman Diamond syndrome affects patients on the cellular level before further treatment options can be developed. The gene *sbds* is mutated in nearly all cases of SDS (2). These mutations consistently result in at least one of two pseudogene-like sequence changes that result in protein truncation. The protein SBDS is a member of a highly conserved family of proteins of unknown function with putative orthologs found in diverse species allowing for various model systems to be used for research(2).

This thesis follows the style of *Journal of the Biological Chemistry*.

Recent studies suggest that SBDS is implicated in ribosome maturation and mitotic abnormalities (3-6). The process of ribosomal maturation includes RNA polymerase I transcription, pre-rRNA processing, and ribosome subunit trafficking and maturation. While the human SBDS protein has been shown to localize to the nucleolus (3) and associate with 28S rRNA (7), depletion of the human protein has not been associated with specific blocks in pre-rRNA processing or decreased amounts of 60S ribosome subunits(5,6). However, a recent report showed that depleting mouse cells of the SBDS protein decreases 60S subunit levels, suggesting that SBDS is involved in ribosome maturation (8).

Additionally, cells from SDS patients exhibit mitotic abnormalities including centrosome amplifications and multipolar spindles when passaged(9). SBDS has also been localized to the mitotic spindle(10). The observation that SBDS can bind microtubules *in vitro* suggests that SBDS may be multifunctional and have a direct role in spindle maintenance and function. Furthermore, a role for SBDS in chemotaxis has been suggested (11-13).

Defects in ribosome synthesis contribute to disease pathology (4,14) in a number of human diseases including Diamond Blackfan anemia (15,16), Treacher Collins syndrome (17), X-linked dyskeratosis congenital (18), and cartilage hair hypoplasia (19). How the distinct clinical presentations of these disorders are linked to defects in specific stages of ribosome synthesis is unclear. Notably, aside from defects in ribosome

synthesis, most of these disorders also share features such as bone marrow dysfunction, developmental defects, and cancer predisposition, suggesting that the ribosome plays a crucial role in hematopoiesis, development, and oncogenesis (4,14). However, in some instances the genes affected have been shown to have other cellular targets in addition to the ribosome, such as telomerase in dyskeratosis congenita (20) and mRNA turnover in cartilage hair hypoplasia (21). This raises the possibility of extraribosomal targets for these genes, which may account for some aspects of their phenotypes.

The de Figueiredo lab has recently shown physical and genetic interactions between Sdo1p/SBDS and class I and II HDACs in yeast and mammalian cells, respectively. These unexpected findings suggested that Sdo1p/SBDS functions as a natural HDAC inhibitor influencing ribosome synthesis and potentially other processes within cells. By revealing a novel role for acetylation in controlling ribosome synthesis, therapeutics could be developed to treat SDS and related disorders (22).

Posttranslational modification performs integral roles in diverse biological activities in eukaryotes. Protein acetylation is a process by which an acetyl group is added to a lysine residue. Acetylated proteins include cytosolic proteins, mitochondrial enzymes, and plasma membrane-associated receptors (23). A recent study identified over 1,300 acetylated peptides in mitochondrial and cytosolic fractions (24).

Protein acetylation is regulated by lysine acetyltransferases and lysine deacetylases and plays a key role in gene expression (23). Traditionally, acetylated proteins are visualized by employing radiolabeled acetate or acetyl-CoA (25). However, this procedure generates radioactive wastes and lacks the capability for high sensitivity. Using click-chemistry (26), an azide-alkyne reaction, allows analysis of protein acetylation with a higher sensitivity when compared to autoradiography. Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (27) has been demonstrated to rapidly detect acetylated proteins in mammalian cells through the metabolic labeling of alkynyl-acetate analogs (28).

Studies have shown the yeast ortholog of SBDS, Sdo1p, to be an endogenous inhibitor of class I and II histrone deacetylase (HDAC) activities (22). Again the treatment options for those who suffer from Shwachman Diamond Syndrome are limited by our understanding of the cellular mechanisms of this genetic disorder. Given the link of this disorder to protein acetylation, the purpose of this project is to use the CuAAC reaction to label acetylated proteins in mammalian cells in the hopes of further describing the role of acetylation in SBDS function.

CHAPTER II

METHODS

Cell culture

K-562 cells were cultured in 10 mL of RPMI medium 1640 supplemented with 10% FBS, 1% penicillin and streptomycin. Cells were incubated in a 5% CO_2 humidified incubator at 37 ºC.

Protein isolation

The cells were harvested and washed twice with PBS. The proteins were centrifuged at 1200 rpm for 5 minutes at room temperature, and the supernatant was removed. 50 μL of pre-lysis buffer (7mM PMSF, 10X EDTA free protease inhibitors, 8 μM Mg Cl_2, 0.05% SDS, 10 mM Tris-base, pH 7.4) were added to the cell pellet, followed by co-incubating with 0.7 μL of benzonase nuclease (Sigma, St. Louis, MO) for 20 min. The cell suspensions were then lysed by adding 150 μL of lysis buffer (1% SDS, 150 mM NaCl, 50 mM tris-base, pH 7.8) with subsequent vigorous vortexing. Insoluble cell debris was removed by centrifuging at 14,000 rpm for 10 min. The supernatant was collected to yield a total cell lysate. The concentration of protein was calculated using a BCA kit (Pierce, Rockford, IL).

SDS-PAGE

The protein pellets were resuspended in loading buffer (50 mM Tris-HCl; pH 6.8, 2% SDS, 10% Glycerol, 1% b-Mercaptoethanol, 12.5 mM EDTA, 0.02 % Bromophenol Blue) and the proteins were separated running the gels in 12% acrylamide gels at 80V for 4 hours.

Coomassie blue staining

Gels from SDS-PAGE were stained with Coomassie Brilliant Blue R-250 Staining Solution (BIO RAD, Hercules, CA) for 2-3 hours. The gels were then destained Coomassie destaining solution (20% methanol, 5% glacial acetic acid) for 6-8 hours.

Anti-actin western blot

To investigate whether the protein isolation protocol succeeded in recovering the expected proteins and to ensure that the recovered proteins would withstand the conditions of the click reaction, a mock click reaction was performed. In this mock reaction click reagents were not used, but instead, the proteins were subjected to all the manipulations required for the click reaction to occur. Four samples were collected in between steps of the click reaction: (1) Sample P1 was collected after the protein isolation was obtained samples were collected after protein isolation, (2) Sample P2 was collected after freezing and thawing the sample, (3) Sample P3 was collected after allowing the sample to remain at room temperature for 30 minutes, and (4) Sample P4

was collected after the methanol chloroform protein cleanup step described in Click-iT® Protein Reaction Buffer Kit (Invitrogen, Carlsbad, CA).

Preparation of sodium 4-pentynoate

2 g of 4-pentynoic acid were dissolved in 20 mL of ddH_2O, and .82 g of NaOH was dissolved in 10 mL of ddH_2O. This NaOH solution was then added drop wise to 4-pentynoic acid solution. The reaction mixture was placed at -80 ºC for 10-12 hours. After freezing overnight, the mixture was lypholized to dryness leaving a white powder. 2.4 g of powder were collected and were dissolved in 39.2 mL of ddH_2O.

TSA treatment

10mL of K-562 culture were collected and divided equally into four parts. For simplicity these cell samples will be referred to as T1, T2, D3, and D4. These cells were then centrifuged for 5 minutes at room temperature at 1200 rpm and resuspended in 4 mL of RPMI medium 1640 supplemented with 2% FBS. 4μL of 10 μM TSA were added to T1 and T2, and 4 μL of DMSO were added to D3 and D4. These cells were then incubated for 18 hours at 37° C.

Sodium 4-pentynoate treatment

The four samples were centrifuged for 5 minutes at 1200 rpm at room temperature. Then they were washed twice with PBS. Each sample was then resuspended in 4 mL of RPMI medium 1640 supplemented with 2% FBS. 40 μL of 500 mM sodium 4-

pentynoate were then added to samples T1, T2, and D3. 4 μL of TSA were again added to T1. D4 received no Sodium 4-Pentynoate treatment. The cells were incubated for 7 hours before the proteins were isolated.

Click reaction

The Click-iT® Protein Reaction Buffer Kit (Invitrogen, Carlsbad, CA) was used to perform the click reaction (27). The protocol for this kit was followed beginning with a 2 mg/mL protein sample, biotin-azide was used as the detection reagent. The final product was a protein pellet.

CHAPTER III

RESULTS

Confirmation of protein isolation

To confirm that proteins could be isolated from K562 cells and that they would not deteriorate throughout the click reaction, two experiments were performed. The mock click reaction formerly described in Chapter II was performed. The first was a Coomassie blue stain to analyze the protein sample. Second, a western blot was generated to test for the presence of actin.

Coomassie blue stain

I separated proteins in cellular lysates by SDS-PAGE, and then visualized the separated proteins using Coomassie blue staining (Figure 1a). I visualized proteins in each of the lanes, and concluded that the protein isolation protocol successfully isolated proteins from K562 cells.

Actin western blot

Western blot analysis using polyclonal antibodies directed against human actin proteins was performed to test for the presence of actin (Figure 1b). I found that in each sample actin was visualized. I conclude that actin was successfully isolated from K562 cells, and the general protocol used for western blot analysis worked successfully.

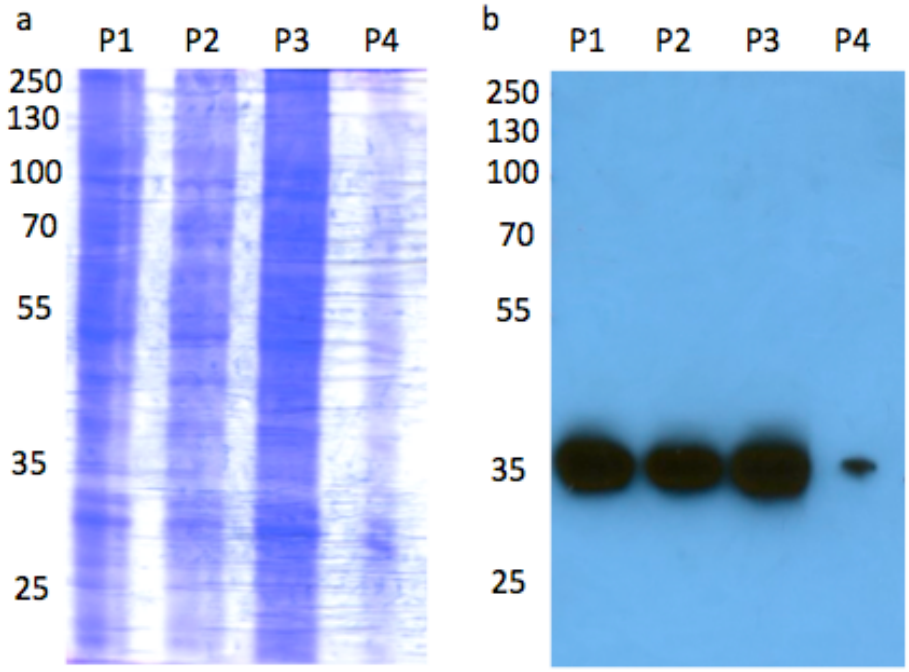

Figure 1. Protein isolation without click reaction. (a) Coomassie blue stain (b) Western blot with anti-actin

Click acetylome generation

The purpose of this aspect of the project was to generate an acetylome via western blot. Samples T1,T2, D3, and D4, described in Chapter II, were used to map an acetylome. These samples were treated with Na 4-pentynoate. The Click-iT® Protein Reaction Buffer Kit (Invitrogen, Carlsbad, CA) was used to perform the click reaction, attaching a biotin tag to those proteins labeled with 4-pentynoate. These samples were then separated using SDS-PAGE. The gels were then visualized using Coomassie blue staining and a click western blot.

Coomassie blue stain

I separated proteins in cellular lysates from samples T1, T2, D3, and D4 by SDS-PAGE, and then visualized the separated proteins using Coomassie blue staining (Figure 2a). I visualized proteins in each of the lanes, and concluded that the proteins were successfully isolated in each of the four samples.

Click chemistry western blot

After protein isolations from samples T1, T2, T3, and T4 were separated by SDS-PAGE, I transferred these proteins to a PVDF membrane. HRP-streptavidin was then used to visualize the biotin labeled proteins (Figure 2b). In this experiment histones can be viewed as a positive control. Histones are acetylated proteins that package DNA into nucleosomes found in the chromosomes of cells. These proteins are approximately 17 kDa. If acetylated proteins were successfully labeled with the click chemical, then a band would be expected to be seen around the 17 kDa range. However, as seen in Figure 2b, no band is present in this region. Since biotin was used to tag the acetylated proteins, any proteins that are naturally biotinylated will give a false positive. Since bands are seen in D4, proteins not labeled with the click chemical were being visualized explaining the presence of bands in T1, T2, and T3. In conclusion, this click labeling system did not work under the conditions present throughout this experiment.

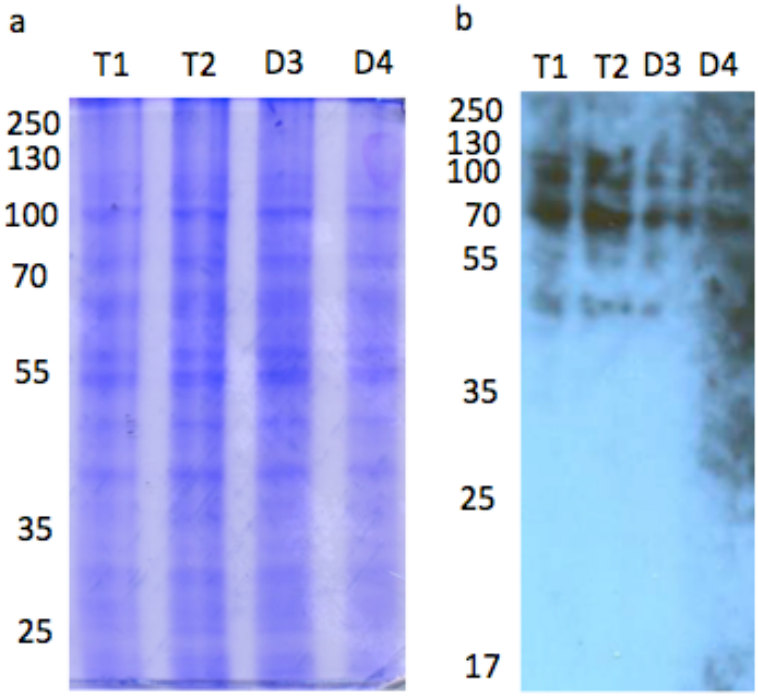

Figure 2. Click chemistry sample analysis. (a) coomassie blue stain (b) western blot using click strategy

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Scientists need more data on the function of SBDS, the mutated protein of Shwachman Diamond Syndrome, to develop effective therapeutic methods to cure the disease. With the recently discovered interaction between SBDS and HDACs, the function of SBDS is one step closer to being fully understood. Now the next piece of the puzzle is needed. A map of the acetylome of a cell with inhibited SBDS function will lead to a more complete understanding of Shwachman Diamond syndrome, and the purpose of this experiment is to demonstrate a method that would effectively generate this acetylome.

A previous study has shown that click chemicals can be metabolically labeled onto acetylated proteins (28). There are many benefits to using the click chemistry suggested in this paper. However, the use of metabolic labeling with click chemicals has yet to become a mainstream strategy. Currently proteomic strategies, which use mass spectrometry to identify the chemical nature of proteins, can be used to generate an acetylome. However proteomic strategies are time consuming and are not immediately available to many labs that work with genetic manipulation. This situation creates an environment that would greatly benefit from an effective system of acetylome mapping.

According to the data collected to confirm the presence of proteins, I found that the proteins were indeed isolated and were able to withstand the conditions for the click

chemistry. As seen Figure 1b, the test for actin is positive in all four samples. This confirms that the proteins were not degraded throughout the mock click-chemistry reaction. Also these results confirmed that the equipment and procedures used for the western blot worked. The results of Figure 1a also support these conclusions; the Coomassie blue stain revealed the presence of proteins and with various sizes. The perceived decrease in signal of P4 seen in both Figures 1a and 1b arise from residual methanol used in the protein clean up step that this sample went through. The density of the protein sample was then decreased and sample did not load completely.

The data of the click chemistry section of this experiment fail to demonstrate that the metabolic labeling of the click chemistry worked. Figure 2a shows that proteins were present in the sample and did not degrade. This shows that the addition of the chemical reagents used to perform the click reaction did not degrade the sample. The presence of proteins visualized by Coomassie blue stain demonstrates that the addition of the chemical reagents used to perform the click reaction did not degrade the sample.

Though the results suggest that the click-chemistry labeling system does not work under condition described in this experiment, there is still hope that a click chemistry strategy can be used. For instance, the dosage of Na 4-pentynoate used was that suggested by the Hang paper. However due to a slightly different mammalian cell (Jurkat vs. K562) line new optimization data of Na 4-pentynoate dosage might allow for this acetylated protein labeling strategy to be used. This new experiment may allow for a quick generation of

an acetylome, bringing scientists one step closer to understanding the cellular mechanisms of SBDS, leading to the development of new strategies for treatment of this disease.

REFERENCES

1. Shwachman, H., Diamond, L. K., Oski, F. A., and Khaw, K.-T. (1964) *The Journal of Pediatrics* **65**, 645-663

2. Boocock, G. R. B. (2003) *Nature Genetics* **33**, 97-101

3. Austin, K. M. (2005) *Blood* **106**, 1253-1258

4. Ganapathi, K. A. (2008) *British journal of haematology* **141**, 376-387

5. Luz, J. S. (1990) *Yeast* **26**, 287-298

6. Moore, J. B., and Moore. (2010) *Haematologica* **95**, 57-64

7. Ganapathi, K. A. (2007) *Blood* **110**, 1458-1465

8. Ball, H. L., Zhang, B., Riches, J. J., Gandhi, R., Li, J., Rommens, J. M., and Myers, J. S. (2009) *Human Molecular Genetics* **18**, 3684-3695

9. Austin, K. M. (2008) *The Journal of Clinical Investigation* **118**, 1511-1518

10. Orelio, C. (2009) *PloS One* **4**, e7084

11. Orelio, C. (2009) *Haematologica* **94**, 409-413

12. Stepanovic, V. (2004) *Cell Motility and the Cytoskeleton* **57**, 158-174

13. Wessels, D., Srikantha, T., Yi, S., Kuhl, S., Aravind, L., and Soll, D. (2006) *Journal of Cell Science* **119**, 17-17

14. Sakamoto, K. M., Shimamura, A., and Davies, S. M. (2010) *Biology of Blood and Marrow Transplantation* **16**, S12-S17

15. Ellis, S. R., and Lipton, J. M. (2008) *Current Topics in Developmental Biology* (James, J. B. ed.) Vol. 82, pp 217-241, Academic Press, Maryland Heights, MO

16. Lipton, J. M., and Ellis, S. R. (2009) *Hematology/Oncology Clinics of North America* **23**, 261-282

17. Dixon, J. (2007) *Orthodontics and Craniofacial Research* **10**, 88-95

18. Walne, A. J. (2009) *British Journal of Haematology* **145**, 164-172

19. Notarangelo, L. D. (2008) *Current Opinion in Allergy and Clinical Immunology* **8**, 534-539

20. Mitchell, J. R., and Collins. (1999) *Nature* **402**, 551-555

21. Ridanpää, M., van Eenennaam, H., Pelin, K., Chadwick, R., Johnson, C., Yuan, B., vanVenrooij, W., Pruijn, G., Salmela, R., Rockas, S., Mäkitie, O., Kaitila, I., and de la Chapelle, A. (2001) *Cell* **104**, 195-203

22. Guo, J. d. F., Paul. (2009) The Shwachman Diamond syndrome protein is an inhibitor of histone deactylase activities. (Unpublished Work) Texas A&M University, College Station

23. Yang, X.-J., and Seto, E. (2008) *Molecular Cell* **31**, 449-461

24. Shimin, Z., Wei, X., Wenqing, J., Wei, Y., Yan, L., Tengfei, Z., Jun, Y., Li, Z., Yaxue, Z., Hong, L., Yixue, L., Jiong, S., Wenlin, A., Hancock, S., Fuchu, H., Lunxiu, Q., Chin, J., Pengyuan, Y., Xian, C., and Qunying, L. (2010) *Science* **327**, 1000-1004

25. Brownell, J. E., and Allis, C. D. (1995) *Proceedings of the National Academy of Sciences of the United States of America* **92**, 6364-6368

26. Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) *Angewandte Chemie (International ed. in English)* **40**, 2004-2021

27. Meldal, M., and Tornøe, C. W. (2008) *Chemical Reviews* **108**, 2952-3015

28. Yang, Y. Y. (2010) *Journal of the American Chemical Society* **132**, 3640-3641

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