

AD _____

Award Number:

W81XWH-08-1-0614

TITLE: Understanding the Biosynthesis of SF2575: A potent antitumor
Compound with novel modes of action

PRINCIPAL INVESTIGATOR:

Yi Tang, PhD

CONTRACTING ORGANIZATION:

University of California Los Angeles
Los Angeles, California 90095

REPORT DATE:

March 2010

TYPE OF REPORT:

Addendum to Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☐ x Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.				
1. REPORT DATE (DD-MM-YYYY) 31-MAR-2010		2. REPORT TYPE Addendum to Final		3. DATES COVERED (From - To) 1 SEP 2009 - 28 FEB 2010
4. TITLE AND SUBTITLE Understanding the Biosynthesis SF2575: A Potent Antitumor Compound with Novel Modes of Action			5a. CONTRACT NUMBER W81XWH-08-1-0614	
			5b. GRANT NUMBER	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Yi Tang Email: yitang@ucla.edu			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES) Regents of the University of California Office of Contract & Grant Adm Univ. of Calif. Los Angeles 11000 Kinross Avenue, Suite 102 Los Angeles, CA 90095			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) DEPARTMENT OF THE ARMY US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND 504 SCOTT STREET FORT DETRICK, MD 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)	
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Distribution is unlimited.				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT: SF2575 is a tetracycline polyketide produced by <i>Streptomyces</i> sp. SF2575 and displays exceptionally potent anticancer activity. The structure is chemically complex and contains unusual angelate and salicylate tailoring groups. In this study, we identified, sequenced and functionally analyzed the <i>ssf</i> biosynthetic gene cluster. Intermediates were isolated from the SF2575 culture extract to suggest the order of pendant groups addition is C-9 glycosylation, C-4 salicylation and C-4' angelacylation. Cytotoxicity studies demonstrated loss of activity following removal of angelate and further inactivity following loss of salicylate indicating that both acyl groups are critical to the potent bioactivity. Two enzymes responsible for C-4 acylation of salicylate were identified and characterized in vitro, an ATP-dependent salicylyl-CoA ligase SsfL1 and acyltransferase SsfX3. Understanding the biosynthesis of SF2575 can therefore expand the repertoire of enzymes that can modify tetracyclines, and facilitate engineered biosynthesis of SF2575 analogs.				
15. SUBJECT TERMS Anticancer, chemotherapy, Natural product, topoisomerase				
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 7
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U		
			19a. NAME OF RESPONSIBLE PERSON USAMRMC	
			19b. TELEPHONE NUMBER (include area code)	

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	5
Task 1. Elucidate the biosynthetic pathway of SF2575 from the native producer	5
Task 2. Probe the mechanism of key enzymes involved in the biosynthesis of SF2575	6
Reportable Outcomes.....	7
Conclusion.....	7
References.....	7

Introduction

Natural products produced by bacteria and fungi encompass a broad range of bioactivity and are an important source of therapeutics in use today. Cancer therapy is no exception to this. One prominent example is doxorubicin, a compound naturally produced by a soil-dwelling bacterium, and is a common component in chemotherapy regimens for many types of cancers including breast cancer. Due to the chemical complexity of these molecules which often prohibits de novo chemical synthesis, biosynthesis is an attractive approach to produce and generate analogs of these bioactive compounds. SF2575, studied here, is a tetracycline family natural product which was found to have exceptionally potent anticancer activity both through an in vitro cytotoxicity assay with P388 leukemia cells, and during an in vivo assay using mouse xenografts¹. More recently, a 60-cell line screening by the National Cancer Institute demonstrated the potent activity of SF2575 against nearly all types of cancer cell lines tested, resulting in an average IC₅₀ value of 11.2 nM. The mechanism of action of several closely related SF2575 analogs, TAN-1518A and TAN-1518B has been identified as inhibition of DNA topoisomerase I². As these structures are nearly identical to that of SF2575, it is likely that this family of compounds shares a common molecular target. As key enzymes during DNA replication, both DNA topoisomerase I and II are known targets for current anticancer therapies in clinical use such as doxorubicin, a topoisomerase II poison³, and camptothecin derivatives which target topoisomerase I⁴.

During this study, we have identified and sequenced the *ssf* gene cluster responsible for the biosynthesis of SF2575 from *Streptomyces* sp. SF2575. This *ssf* gene cluster is only the third tetracycline family gene cluster to be identified and sequenced. The genetic information offers valuable opportunities to further enhance our understanding of tetracycline biosynthesis, and to investigate an entirely new set of tetracycline tailoring modifications that distinguish SF2575 from the previously studied oxytetracycline and chlorotetracycline (Figure 1). SF2575 has a much more complex and heavily decorated structure than previously studied tetracyclines which is reflected in the size of the sequenced gene cluster which was nearly double that of oxytetracycline. Bioinformatic analysis of the gene cluster along with identification of the possible biosynthetic intermediates from *S. sp.* SF2575 fermentation extract led to the assembly of a putative biosynthetic pathway for synthesis of SF2575. We have reconstituted the early portions of the *ssf* pathway in a heterologous host and demonstrated the biosynthesis of the tetracycline core parallels the previously published biosynthetic pathway of oxytetracycline⁵⁻⁷. Additionally, we have identified key tailoring enzymes involved in the attachment of salicylate to the tetracycline aglycon and verified their function through in vitro analysis. Elucidation of the SF2575 biosynthetic pathway and characterization of these key tailoring enzymes is an important first step toward structure activity relationship studies and engineered biosynthesis of new anticancer compounds.

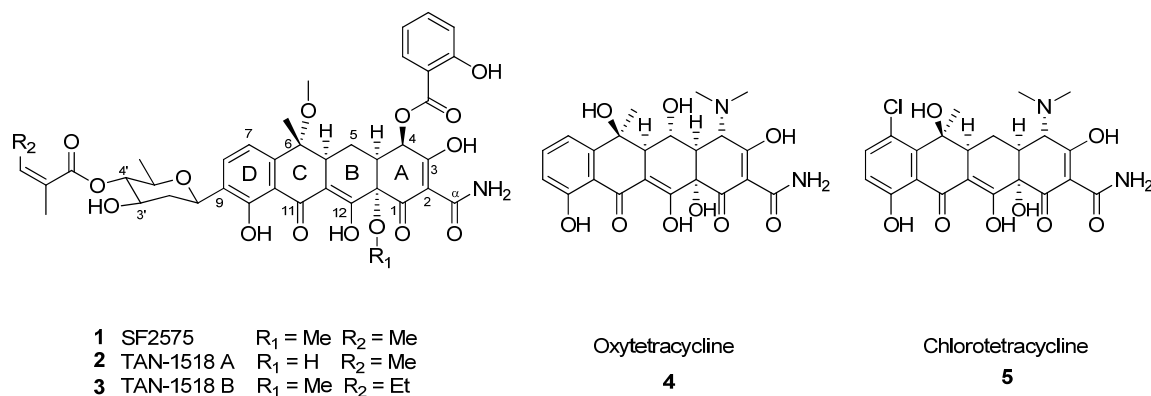


Figure 1: Natural product tetracyclines: SF2575 **1** produced by *S. sp.* SF2575; TAN-1518A **2** and TAN-1518B **3** produced by *Streptomyces* sp. AL-16012; oxytetracycline **4** produced by *S. rimosus*; and chlorotetracycline **5** produced by *S. aureofaciens*.

During the addendum period detailed in this report we have additionally obtained several genetic knockouts using host strain *S. sp.* SF2575 which have further aided our understanding of the biosynthesis and set the stage for production of SF2575 analogs. We have also nearly completed efforts to solve the structure of SsfX3 which will illuminate the mechanism behind the broad substrate specificity and lead into further research to modify this specificity to obtain desired analogs.

Body

Research during the addendum period further develops two main tasks, 1.) Elucidating the biosynthetic pathway of SF2575 from the native producer and 2.) Probing the key mechanisms of enzymes involved in SF2575 biosynthesis.

Task 1. Elucidate the biosynthetic pathway of SF2575 from the native producer.

During the course of the initial research a genetic knockout strategy was developed and used to knockout the SF2575 ketosynthase gene, thus confirming that the gene cluster identification was correct. This knockout scheme, shown in Figure 2, was then used to knock out additional genes to probe the pathway. The first of these was *ssfH*. As shown in the tailoring scheme in Figure 2, SsfH is a putative salicylate synthase which catalyzes the last step in salicylic acid biosynthesis, the conversion of chorismate into salicylate. This gene was targeted in order to produce a strain lacking salicylic acid production which may be then used for mutasynthesis experiments, in which analogs of salicylic acid are fed to this mutant strain. If the scheme proposed below is correct, we expected to see loss of production of **1** and accumulation of **7**. Indeed, LCMS analysis of extract from the mutant strain *S. sp.* SF2575Δ*ssfH* eliminated production of **1** and accumulated **7** as shown in Figure 3.

Additional knockouts have been produced and the results have are shown in table 1. As shown, knockout of genes encoding putative acyl CoA ligase SsfL2, ketoreductase SsfF, and ketosynthase III SsfG have all resulted in the loss of production of SF2575 indicating that they are vital to the biosynthetic pathway. However, intermediates have not been identified in the culture extract that indicate at which point in the pathway these mutants are blocked. This could be due to the instability of the pathway intermediates at these positions. Further investigation will therefore be necessary to characterize these enzymes.

Gene knocked out	Produces SF2575	Intermediate identified
ssfH	No	7
ssfL2	No	none
ssfN	Yes	n/a
ssfF	No	none
ssfG	no	none

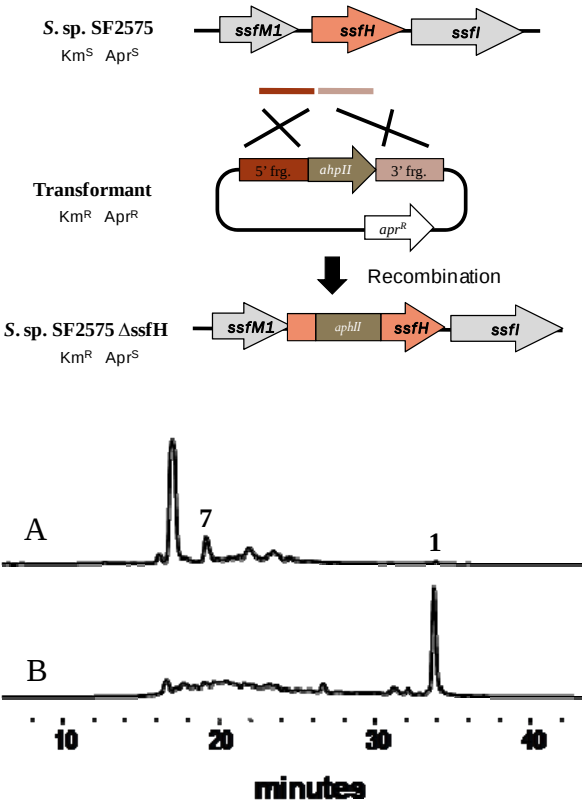


Figure 2: Top: Genetic knockout scheme. Bottom: A. LCMS analysis of *S. sp.* SF2575Δ*ssfH* extract B. LCMS analysis of *S. sp.* SF2575 wild type extract.

Interestingly, SsfN, which was putatively involved in the biosynthesis of the angelic acid substituent was not shown to be necessary for SF2575 biosynthesis.

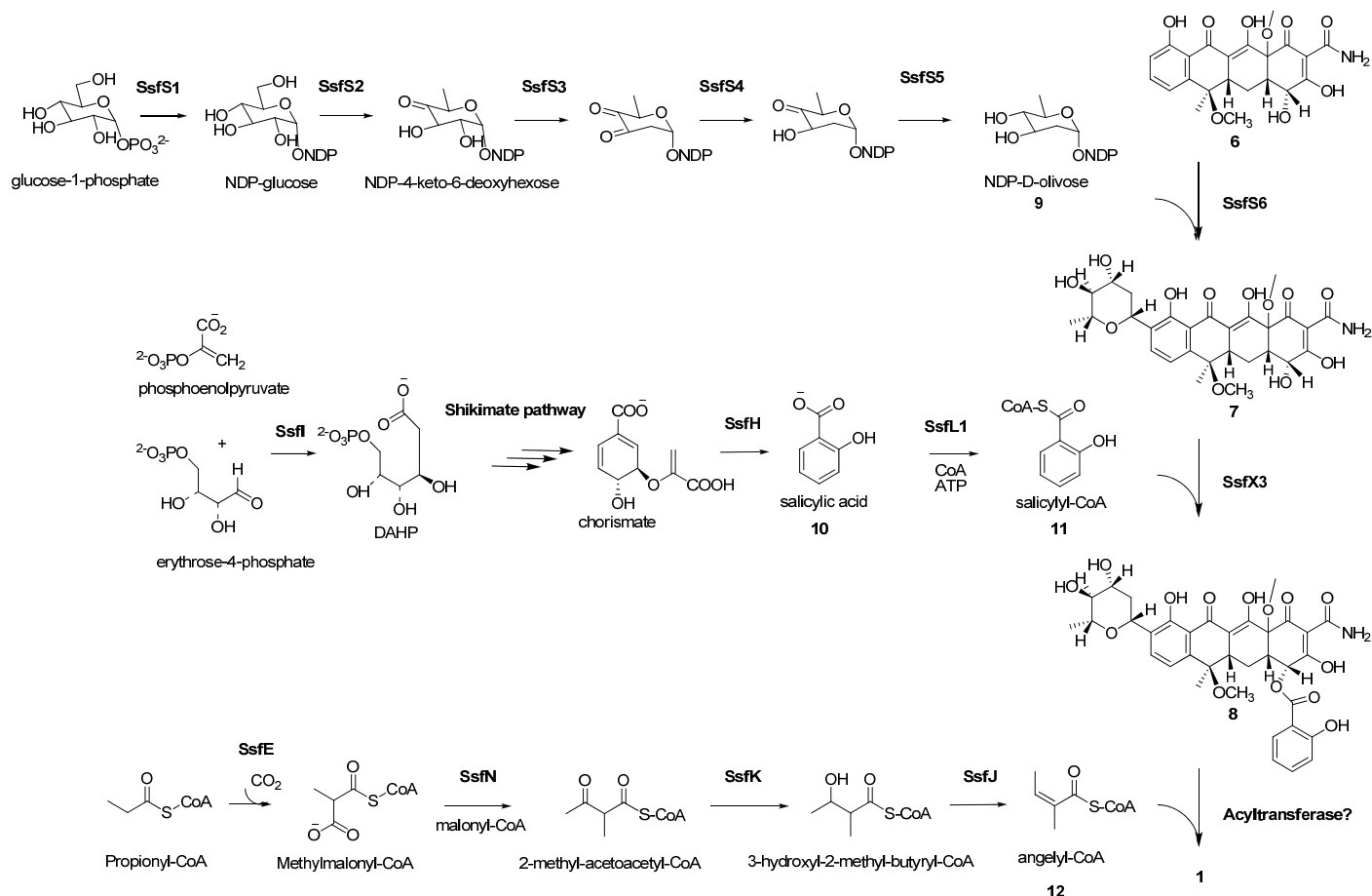


Figure 4: Proposed biosynthetic pathways for the pendants **9**, **11** and **12**, and the conversion of **6** to **1**. The order of these modifications is proposed to be glycosylation of **6** to form **7**, sialylation of the C-4 hydroxyl of **7** to form **8**, and angelicylation of D-olivose to form **1**. The proposed intermediates **7** and **8** have been detected in the extract of *S. sp.* SF2575 culture by LCMS.

Task 2: Probing the key mechanisms of enzymes involved in SF2575 biosynthesis

Following the initial biochemical characterization detailed previously⁸, we aim to gain further understanding of the mechanism and substrate specificity of acyltransferase SsfX3 through crystallization and structure determination. To this end, we have crystallized SsfX3 and obtained diffraction data with resolution as low as 3.1 Å for the selenomethionine and 2.8 Å for the native protein. As there are no close homologs of SsfX3 with known crystal structures, the selenomethionine derivative is necessary to solve the phase. The crystallization has since been further optimized and higher quality crystals have been obtained and are awaiting synchrotron analysis. We expect these crystals to diffract to a resolution below 3 Å at the more powerful x-ray source, which will give data to enable structure determination. Once the structure is known we aim to utilize this information to make a series of active site mutants to probe the effect of these mutations on the substrate specificity of SsfX3.

Key Research Accomplishments for September 2009-February 2010

- Five mutant strains have been produced through knocking out individual genes using double crossover recombination
- Mutant strain *S. sp.* SF2575 Δ *ssfH* may now be used for mutasynthesis studies to obtain analogs with variation at the C4 position
- SsfX3 crystallization trials have resulted in promising crystals from which we expect to shortly obtain structure information.

Reportable Outcomes for September 2009-February 2010

- Publication:

Pickens, L. B., Kim, W., Wang, P., Zhou, H., Watanabe, K., Gomi, S., Tang, Y. "Biochemical Analysis of the Biosynthetic Pathway of an Anticancer Tetracycline SF2575." *J. Am. Chem. Soc.* **2009**, 131, 17677–17689.

- Presentations:

Pickens, L. B., Tang, Y., "Characterization of tetracycline tailoring enzymes toward metabolic engineering of novel derivatives". AICHE annual meeting, Nov 8-12, 2009, Nashville, TN (presentation)

Conclusions

Results from the addendum period have further enhanced our understanding of the biosynthetic pathway of SF2575 through the creation of several genetic knockouts and the successful crystallization of key enzyme SsfX3. In particular the *AssfH* mutant strain and the illumination of the active site of SsfX3 will be important developments to aid the production of novel tetracycline analogs.

References

- (1.) Hatsu, M.; Sasaki, T.; Watabe, H.; Miyadoh, S.; Nagasawa, M.; Shomura, T.; Sezki, M.; Inouye, S.; Kondo, S., *J. Antibiot.* **1992**, 45, 320-324.
- (2.) Horiguchi, T.; Hayashi, K.; Tsubotani, S.; Inuma, S.; Harada, S.; Tanida, S., *J. Antibiot.* **1994**, 47, 545-556.
- (3.) Kellner, U.; Sehested, M.; Jensen, P. B.; Gieseler, F.; Rudolph, P., *Lancet Oncol.* **2002**, 3, 235-243.
- (4.) Pommier, Y., *Nat. Rev. Cancer* **2006**, 6, 789-802.
- (5.) Zhang, W.; Ames, B. D.; Tsai, S. C.; Tang, Y., *Appl. Environ. Microbiol.* **2006**, 72, 2573-2580.
- (6.) Zhang, W.; Watanabe, K.; Cai, X.; Jung, M. E.; Tang, Y.; Zhan, J., *J. Am. Chem. Soc.* **2008**, 130, 6068-6069.
- (7.) Zhang, W.; Watanabe, K.; Wang, C. C. C.; Tang, Y., *J. Biol. Chem.* **2007**, 282, 25717-25725.
- (8.) Pickens, L. B.; Kim, W.; Wang, P.; Zhou, H.; Watanabe, K.; Gomi, S.; Tang, Y., *Journal of the American Chemical Society* **2009**, 131, 17677.