Inhibition of Hsp90 in *Streptomyces coelicolor*

by

Katherine A. Wu

SUBMITTED TO THE DEPARTMENT OF CHEMISTRY
AS A SUPPLEMENT TO THE DEGREE OF

BACHELOR OF SCIENCE IN CHEMISTRY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2005

Signature of Author: _____

Department of Chemistry
April 29, 2005

Certified by: _____

Sarah E. O'Connor
Latham Career Development Assistant Professor of Chemistry
Thesis Supervisor

Accepted by: _____

Rick Lane Danheiser
Arthur C. Cope Professor of Chemistry
Associate Head of the Department

Inhibition of Hsp90 in *Streptomyces coelicolor*

by

Katherine A. Wu

Sumbitted to the Department of Chemistry on
April 29, 2005
as a Supplement to the Degree of
Bachelor of Science in Chemistry

ABSTRACT

Inhibition of the chaperone protein Hsp90 in plants and insects has been found to result in drastic changes in phenotype. We investigated the effect of Hsp90 inhibition on the bacteria *Streptomyces coelicolor*. These changes were studied by growing *S. coelicolor* in the presence of Hsp90 inhibitors, extracting the growth media, and subjecting the extractions to HPLC and MS analysis. It has been found that reproducibility is difficult to achieve. Since the genome of *S. coelicolor* has been sequenced, attention was turned to studying changes in mRNA expression levels, which gave more reproducible results, as well as insight to the specific genes being affected.

Thesis Supervisor: Sarah E. O'Connor

Title: Latham Career Development Assistant Professor of Chemistry

Table of Contents

1. Introduction

Many organisms have the potential to produce various other metabolites that they would not produce under normal laboratory conditions. This potential is often realized upon stress.[1] One stress factor is specific inhibition of the stress response via inhibition of proteins involved in the stress response. An effective target of such protein inhibition is chaperone proteins, which aid in the folding of other proteins. One such chaperone protein is the heat shock protein 90 (Hsp90). Hsp90 chaperones numerous proteins including transcription factors and proteins that regulate growth and development.[2] Inhibiting Hsp90 may therefore cause many changes in growth and development and alter the genes that are transcribed. This may then result in changes in the proteins that are produced. This has indeed been found to be true in both plants and insects.[2, 3]

Most of the protein targets of Hsp90 are cell-cycle and developmental regulators with conformational instability. This instability is crucial in their roles as molecular switches. Hsp90 interacts weakly with these proteins until they can be stabilized by conformation changes coupled to signal transduction. Through such interactions and stabilizing of certain pathways, hidden phenotypes can be exposed to natural selection, suggesting a possible evolutionary role of Hsp90.[3]

Inhibition of Hsp90 is brought about pharmacologically, using drugs such as geldanamycin (GDA) and radicicol. Although structurally unrelated, each inhibits the ATP-dependent Hsp90 by interacting with residues in its unique ATP-binding pocket.[2]

Streptomyces is a family of soil bacteria that produces two-thirds of the naturally derived antibiotics used in medicine via secondary metabolic pathways. The genome of *Strepotmyces coelicolor* has been completely sequenced, revealing 20 gene clusters coding for secondary metabolites, including the blue-colored actinorhodin and red-colored prodiginines (Figure 1).[4]

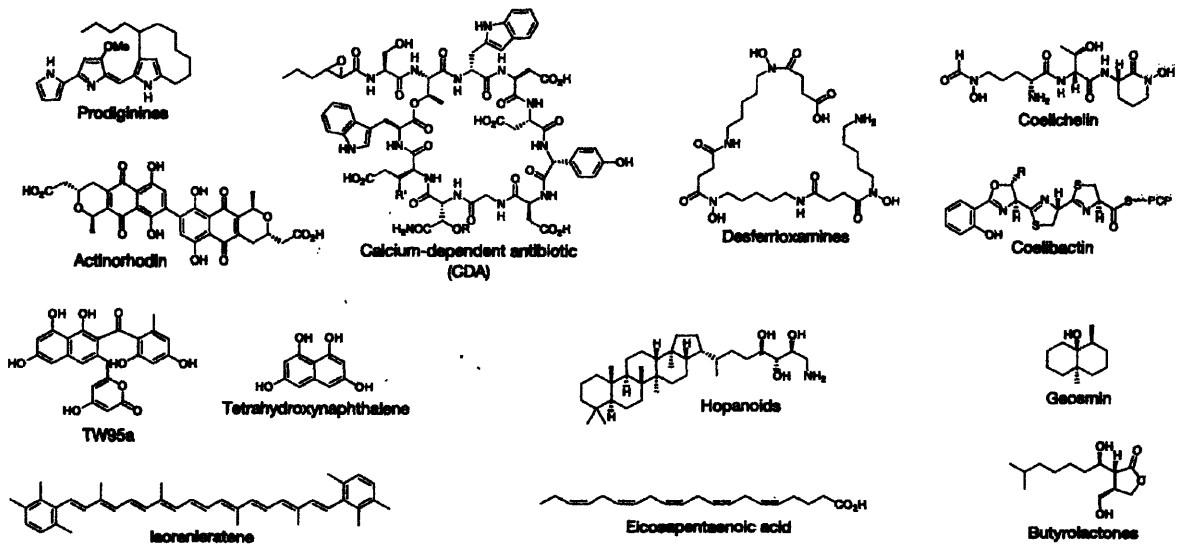

Figure 1. Secondary metabolites known or predicted to be made by *S. coelicolor*.[4]

Hidden gene clusters of putative natural products have also been revealed, suggesting the capability of *S. coelicolor* to produce novel metabolites in unnatural environments. Wild type *S. coelicolor* phenotype includes a purple color because of these colored products. If inhibition of Hsp90 in *S. coelicolor* results in changes in production of these known natural products, changes in bacteria colony color may be visualized. Changes in mRNA expression levels of proteins responsible for the production of these natural products should also be observed. In the present report, we attempt to monitor these changes to make an overall assessment on the effect of Hsp90 inhibition on secondary metabolism.

2. Methods

To study changes in natural product production brought about by Hsp90 inhibition, the natural products and total RNA were extracted and analyzed.

2.1 Analysis of natural products. S. coelicolor were grown in solid SMMS media with and without the Hsp90 inhibitors, radicicol or GDA (Sigma) and incubated at 30°C according to standard protocol.[5] Varying concentrations of radicicol dissolved in a 50:50 mixture of water and ethanol (15nM, 17nM, 50μM) or geldanamycin (10nM in DMSO, 1μM in water/ethanol, 2μM in DMSO, 50μM in water:ethanol or DMSO) to the media. The appropriate controls (equivalent volume of corresponding solvent without inhibitor) were also made. Observation of colony color and density were made after 2-4 days, after which the observations were stable for at least 2 days.

After about 5 days of growth, the media was scraped into a beaker. The appropriate solvent (either DMSO or a 50:50 mixture of water and ethanol) was added and the media crushed to fine pieces with a spatula. The mixture was then filtered through cheesecloth and dried down in a rotavap or a Speedivac in 1mL aliquots. The aliquots were then combined and methanol added to the dried product. The mixture was spun down for 2min at 13,000 rpm to remove particulates and analyzed by HPLC on a gradient of water (0.1% TFA) and acetonitrile and monitored by UV at 280nm. Any peaks unique to Hsp90 inhibition were collected and analyzed with electrospray mass spectrometry.

2.2 Analysis of mRNA. Total RNA of *S. coelicolor* was extracted and analyzed by modified Northern blotting. *S. coelicolor* were grown in solid SMMS media either in 50μM radicicol dissolved in DMSO or in the corresponding volume of DMSO and incubated at 30°C. After 4 days of growth, total RNA was extracted.

All solutions involved in RNA extraction and analysis were treated with 0.01% DEPC. Several protocols were used including various kits and homemade recipes. The final protocol used was described by Kormanec.[6] Briefly, cells were scraped from plates, washed in cold water, and lysed with glass beads in a solution containing guanidine isothiocyanate, sodium acetate,

phenol, and chloroform. Several phenol/chloroform extractions were performed to isolate total RNA from proteins and DNA. Total RNA was then precipitated with isopropanol and redissolved in water. Total RNA was then analyzed by a denaturing formaldehyde-agarose gel[7] and relative concentrations determined with UV_{260} spectroscopy.

Northern blotting was performed as described by Ausubel, et. al.[7] Modified Northern blotting was performed as described with the following changes. Equal amounts of total RNA, as determined by UV spectroscopy, from each of the inhibited and control groups was dried down in a Speedivac and resuspended in 2μL DEPC-treated water. Instead of transferring total RNA from a denaturing gel, total RNA was spotted directly onto positively charged nitrocellulose membrane strips (Roche) as described by Ausubel, et. al. Control DNA of the same sequence as probe was also spotted directly onto the membrane. Detection was then performed using a DIG Luminescent Detection Kit (Roche). Each experiment was repeated.

Detection involved hybridizing DIG-labeled probes (short complementary DNA fragments labeled on uracil bases with DIG-dUTP) to the membranes. Anti-DIG antibodies conjugated to an alkaline phosphatase were then hybridized to the DIG-labeled probes. The membrane was then washed in the chemiluminescent substrate CSPD which dephosphorylates into CSD, emitting at 477nm. This emission was then recorded on chemiluminescent X-ray film (Kodak).

Genomic DNA extraction for probe and DNA control synthesis was performed as described using a Bactozol Kit (Molecular Research Center, Inc.). DIG-labeled probes were synthesized using a PCR DIG Probe Synthesis Kit (Roche). Six genes were chosen for analysis, five involved in natural product production and one involved in DNA repair. Their functions and

Table 1. Genes, functions, and sequences analyzed.		
Gene	**Function**	**Primer sequences (5' ◊ 3')**
Actinorhodin (actII)	production of actinorhodin	Forward: ACG GGG GCG CAG ATG AGA TTC AAC TTA TT Reverse: GTG CTA CAC GAG CAC CTT CTC ACC GTT GAG A
Calcium-dependent antibiotic (cda)	production of calcium-dependent antibiotic	Forward: ATG AGT ACG GAC CCC AAG TCG GTT GT Reverse: TCA CGC CGC TTC CAG ACC CGA GCC GG
Prodiginine (redD)	production of prodiginines	Forward: GTT CCG TTG AAC CGG AGT GCG GTA CG Reverse: GCT GTG CGA CGT GTC GAT CGA TAC GG
Coelichelin	production of coelichelins	Forward: GCC GAG CGC ACG GCA CGG GT Reverse: TTT GAC GGT GAG CCG GTC GCC GGG GG
Chalcone	production of chalcone	Forward: TGC TCG CCG ACT GCC CGT TCC TGC CT Reverse: AAA ACG GCC GCG GTC AGC CAC GCC GA
RecA	DNA repair	Forward: ACC TCA TCG TCA TCG ACT CC Reverse: AAC ATC ACG CCG ATC TTC TC

PCR primer sequences are given in Table 1. DNA controls were also synthesized with the same sequence as the probes using the polymerase chain reaction.

3. Results

Changes in natural product production caused by Hsp90 inhibition in *S. coelicolor* were studied by growing *S. coelicolor* in the presence of Hsp90 inhibitors. The natural products as well as mRNA were then extracted and analyzed.

3.1 Analysis of natural products. S. coelicolor were initially grown in plain (without additional additives) SMMS solid media, in 50uM GDA or 15nM radicicol. A control was performed with the appropriate volume of co-solvent (a 50:50 mixture of water:ethanol corresponding to the volume of GDA used) added to the media since the co-solvent itself may affect natural product production. Solid media was chosen because of shown enhancement of natural product expression. The plates were extracted with either ethyl acetate or methanol. The extracts were analyzed by HPLC. Representative HPLC chromatograms are shown in Figures 2-9. The experiment was repeated and resulted in colonies of a dark purple color on plain media, a

medium purple on the GDA and radicicol plates, and a light purple on the GDA control. This experiment was repeated again but resulted in no differences in color. Methanol extracts of these plates resulted in HPLC traces that were drastically different from the previous traces, with significantly fewer peaks. These initial results indicated that they were not reproducible.

3.1.1 Optimization of extraction protocol. To optimize the extraction solvent, a plate of *S. coelicolor* was divided and extracted in methanol at pH 8, by drying the media down and then dissolving in methanol, pure methanol, methanol with sonication, chloroform, methanol at pH 5, and with DMSO. These extracts were then subjected to HPLC. The most peaks appeared in the trace corresponding to methanol at pH 8.

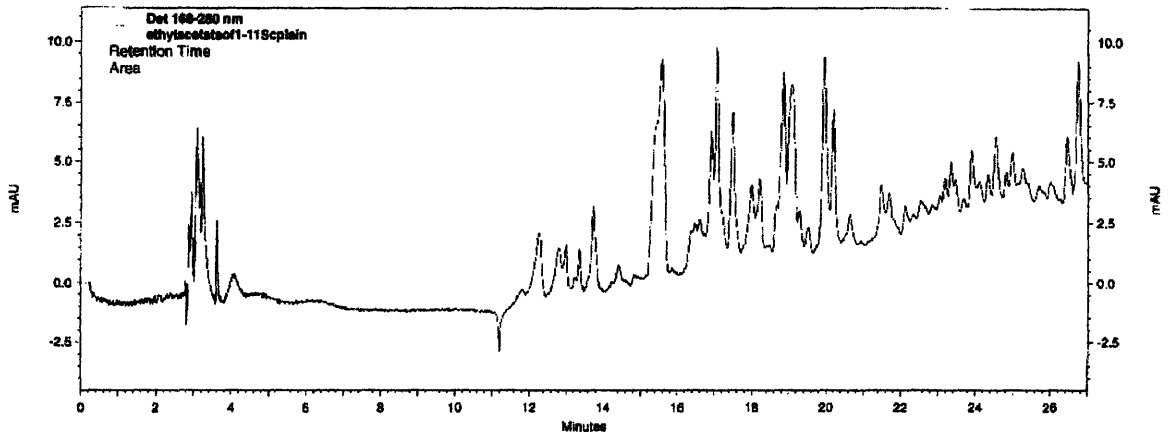

Figure 2. HPLC trace of the ethyl acetate extraction of *S. coelicolor* grown on plain media.

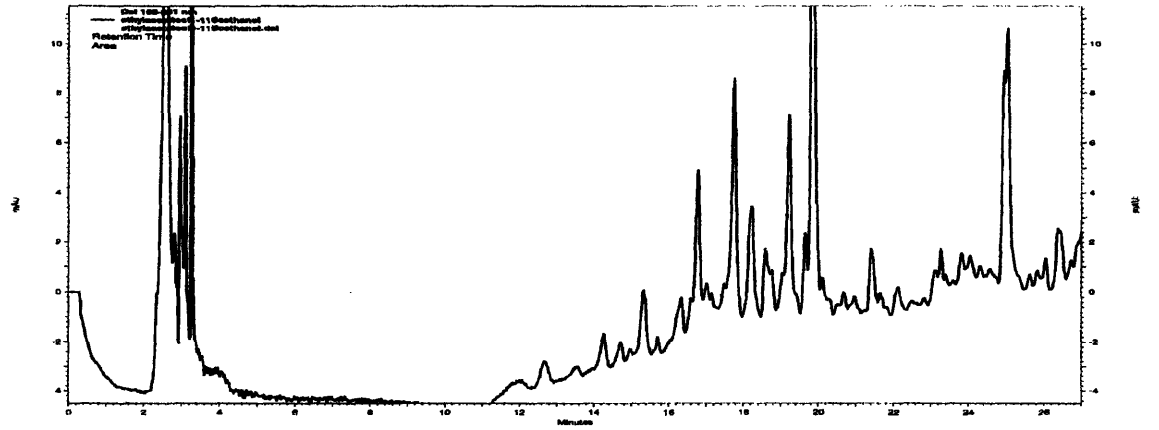

Figure 3. HPLC trace of the ethyl acetate extraction of *S. coelicolor* grown on media with water/ethanol.

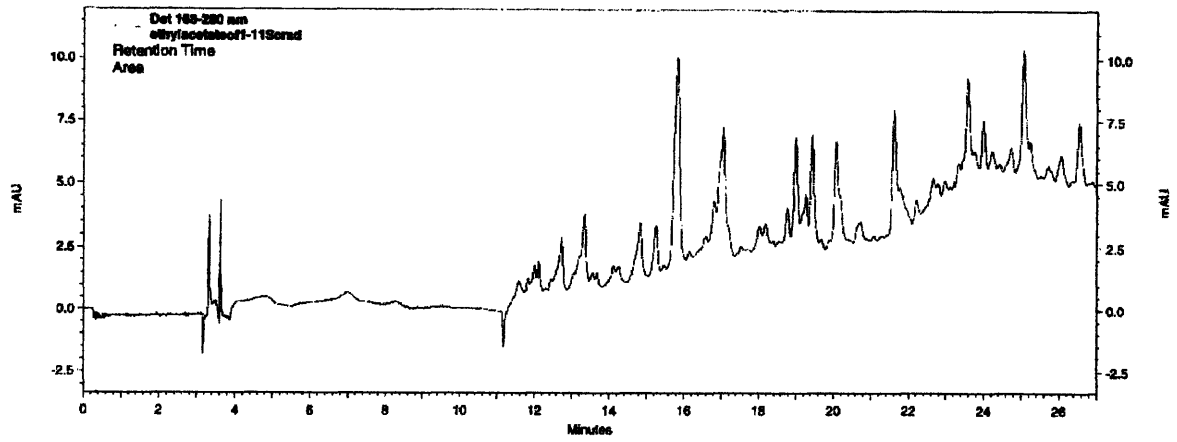

Figure 4. HPLC trace of the ethyl acetate extraction of *S. coelicolor* grown on media with radicicol.

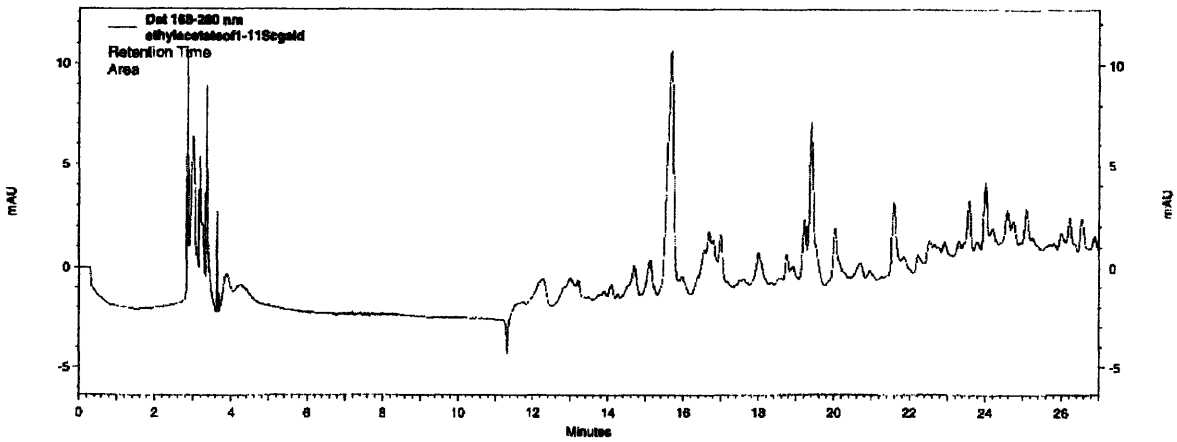

Figure 5. HPLC trace of the ethyl acetate extraction of *S. coelicolor* grown on media with geldanamycin.

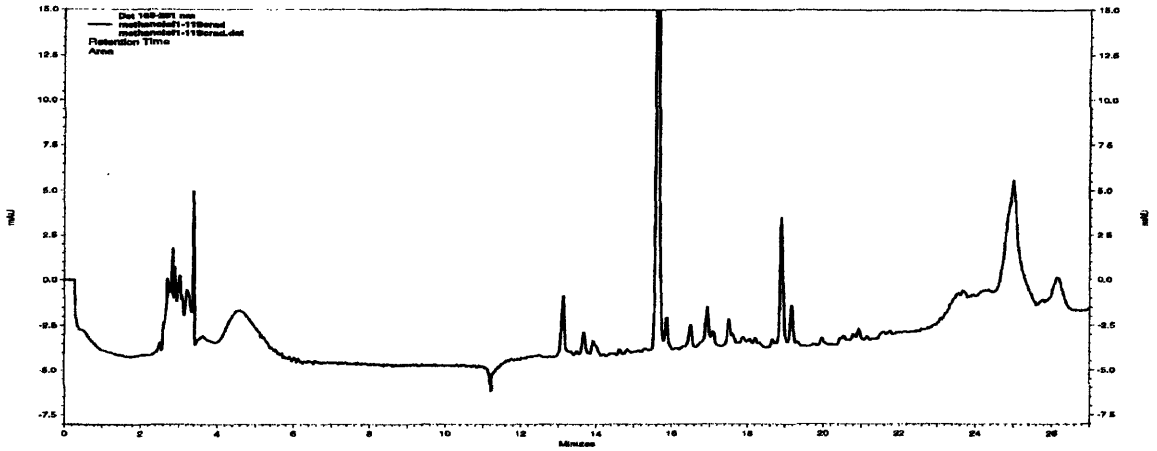

Figure 6. HPLC trace of the methanol extraction of *S. coelicolor* grown on media with radicicol.

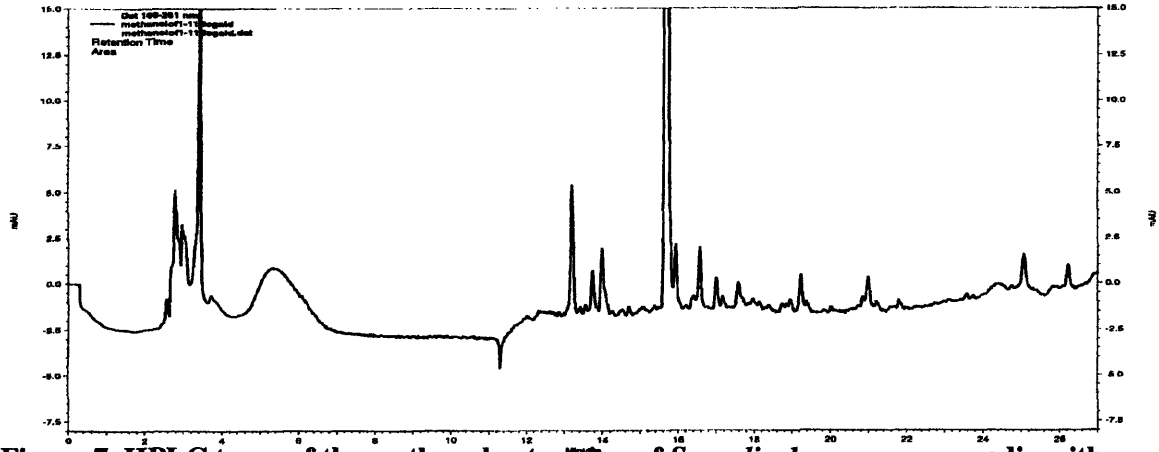

Figure 7. HPLC trace of the methanol extraction of *S. coelicolor* grown on media with geldanamycin.

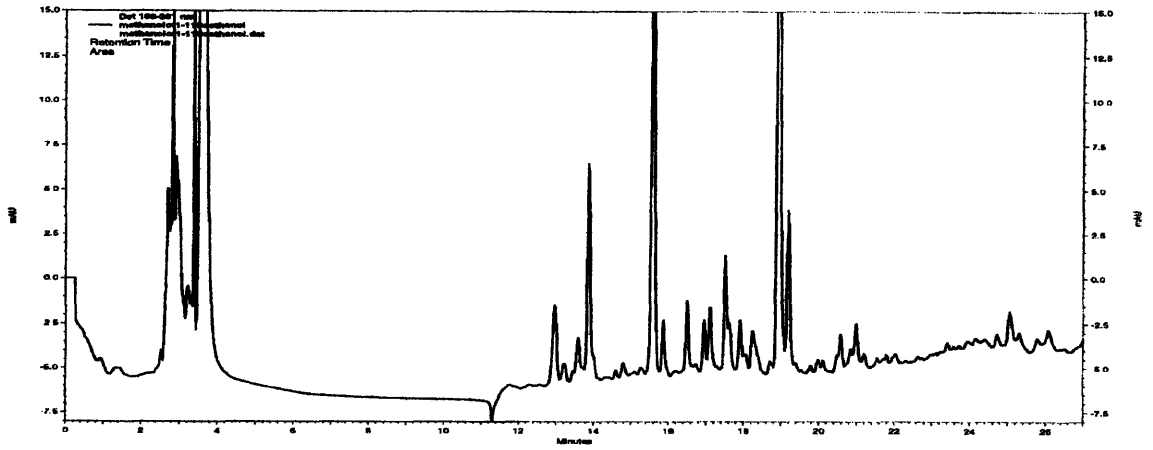

Figure 8. HPLC trace of the methanol extraction of *S. coelicolor* grown on media with water/ethanol.

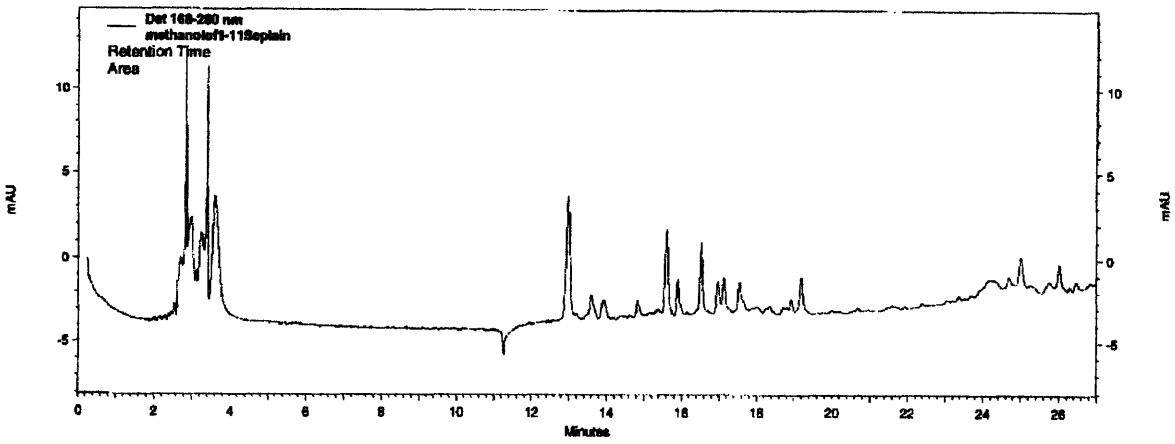

Figure 9. HPLC trace of the methanol extraction of *S. coelicolor* grown on plain media.

Optimization of extraction solvent was once again performed by extracting the plate with methylene chloride, methanol, and chloroform and analyzing the extracts by HPLC. Methylene chloride appeared to solubilize the greatest number of metabolites.

3.1.2 Optimization of co-solvent for inhibitors. It is possible that the co-solvent used to dissolve the chemical inhibitors may itself have an effect on natural product production. To examine DMSO as a possible solvent of the Hsp90 inhibitors, *S. coelicolor* were grown in media with and without DMSO corresponding to a 50uM GDA concentration. Methanol extracts produced identical traces indicating DMSO did not significantly affect secondary metabolism. The experiment was repeated with identical results.

3.1.3 Analysis using radicicol. S. coelicolor were grown on media with 17nM radicicol with the corresponding control of 50:50 water:ethanol but the differences in color were only slight, with bacteria grown in radicicol slightly darker pink than in the control (Figure 10). Methanol extracts

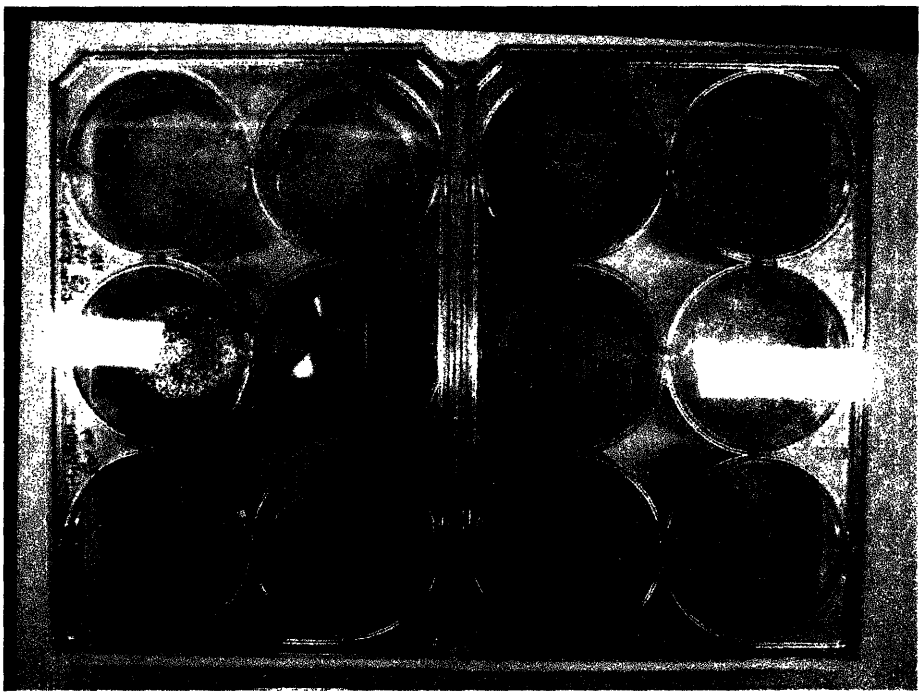

Figure 10. *S. coelicolor* in 17nM radicicol (right) with control (left). Bacteria grown in radicicol are slightly darker pink than in the control.

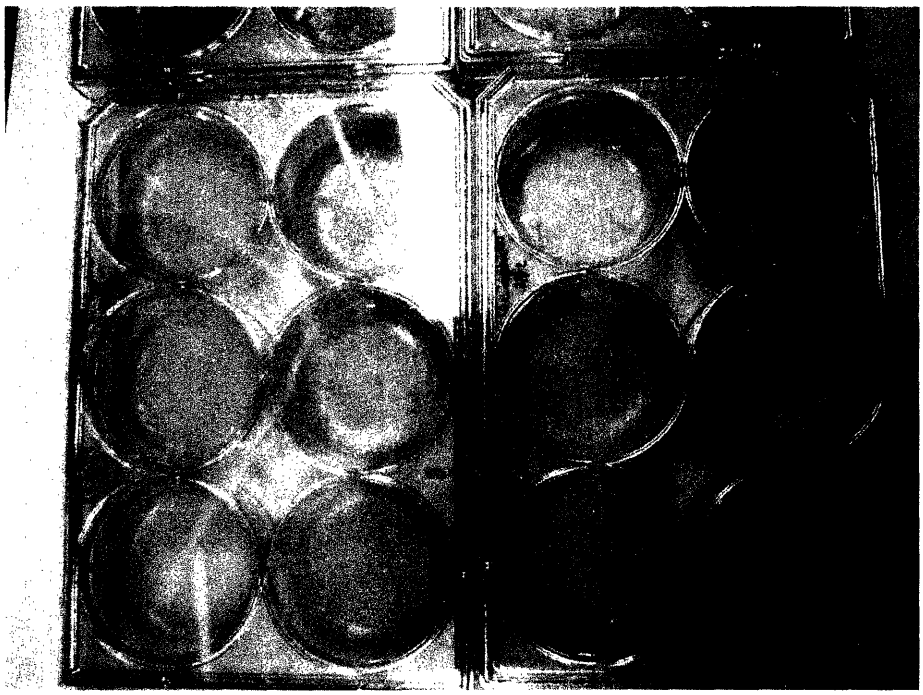

Figure 11. Repeated experiment of *S. coelicolor* in 17nM radicicol (left) with control (right). No apparent color difference exists between the two plates.

redissolved in DMSO were subjected to HPLC and resulted in traces similar to each other. This was repeated with another stock of *S. coelicolor* which resulted in almost no color difference (Figure 11). The experiment was repeated again with the first stock and resulted again in only slight differences in color. Methanol extracts redissolved in DMSO gave HPLC traces that were very different, however. The experiment was repeated again with the first stock which again resulted in only slight differences in color. Methanol extracts redissolved in DMSO gave HPLC traces that were inconsistent with the previous traces.

The experiment was repeated again with the first stock of bacteria and resulted again in very slight differences in color. A 50:50 methanol:chloroform extraction produced an HPLC trace with 3 distinct peaks. These peaks were analyzed by MS but the compounds could not be identified. The experiment was repeated again with the first stock of bacteria with the same color

differences. HPLC traces were different from the previous traces, with more peaks. These peaks were analyzed by MS as well (data not shown) but the compounds could not be identified. It was repeated again with the second stock once more, which gave the same result.

Two different stocks of bacteria were grown on media with 50uM radicicol and the corresponding water:ethanol control. This resulted in white colonies on the radicicol plates and red colonies on the control plates. The experiment was repeated with DMSO instead of water/ethanol. This resulted in pale blue colonies on the radicicol plates and darker blue colonies on the control plates. This indicated that ethanol might have a significant effect on secondary metabolism. These experiments also indicated that a high concentration of radicicol (50μM versus 17nM) is required to observe a reproducible effect..

Overall, these results indicated that a reliable extraction protocol was not achieved. Additionally, although initial results gave only slightly darker colors with the radicicol-inhibited bacteria, ultimately, radicicol-inhibted bacteria reproducibly resulted in lighter purple colors which were later consistently reproduced in the mRNA analysis.

3.1.4 Analysis using GDA. Bacteria were grown in 50uM GDA with the appropriate control of water:ethanol which resulted in light pink colonies with GDA and purple colonies with the control. The experiment was repeated and resulted in orange colonies with GDA and red colonies with the control. The plates were extracted with a 50:50 mixture of methanol and methylene chloride and analyzed by HPLC which resulted in dissimilar traces. The unique peaks were analyzed by MS as well (data not shown) but the compounds could not be identified.

The experiment was repeated and resulted in orange colonies with GDA and purple colonies with the control. This was repeated with GDA dissolved in DMSO which resulted in bright pink colonies on the control plates and light pink colonies on the GDA plates. This was

repeated with two other stocks of bacteria. One stock resulted in sparse, pinkish-purple colonies on the GDA plates and dense purple colonies on the control plates.

Overall, these results indicated that GDA-inhibited bacteria were less purple, although a reliable extraction protocol was not achieved.

3.2 Analysis of mRNA. mRNA from bacteria grown with and without the inhibitor radicicol was analyzed. The phenotype of the bacteria was consistent and reproducible. Inhibited bacteria were white whereas control bacteria were purple after four days (Figure 12).

Several total RNA extraction procedures were used including kits and homemade recipes. Each gave no RNA as visualized on denaturing formaldehyde-agarose gels (data not shown). The final protocol as described by Kormanec[6] gave reasonable total RNA concentrations (Figure 13).

Modified Northern blotting (direct spotting of total RNA onto the membrane as opposed to transfer to the membrane from a gel) was first performed on the DNA control for calcium-dependent antibiotic (CDA) and consistently resulted in a strong signal (data not shown). Modified Northern blotting was then performed on total RNA samples from control plates with CDA DIG-labeled probes and consistently resulted in strong signals (data not shown). Northern blotting (transfer of total RNA from a gel onto the membrane as opposed to direct spotting of total RNA) was then performed on total RNA samples but consistently resulted in no signal, perhaps because of the difficulty to isolate mRNA of substantial concentrations and the lower sensitivity of the method. Thus, all further analysis was performed using modified Northern blotting. Analysis of two of the six genes gave reproducible results as shown in Figure 14 and summarized in Table 2. The remaining 4 genes are continuing to be analyzed.

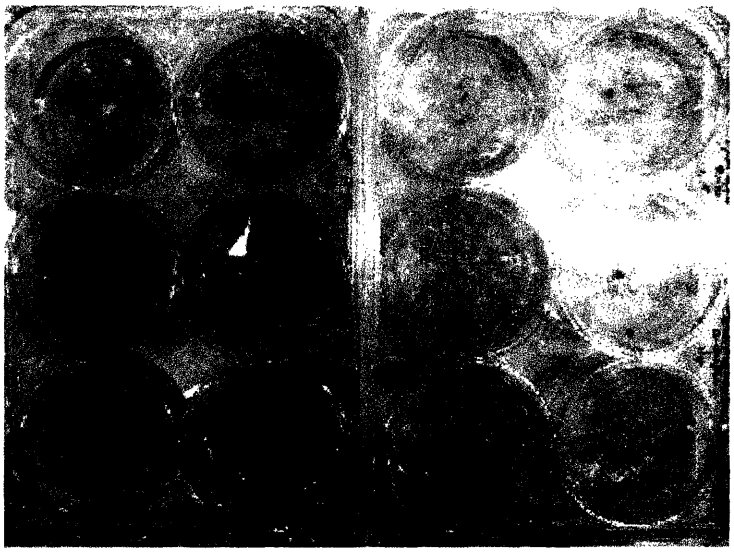

Figure 12. Control (left) and inhibited (right) *S. coelicolor* after 4 days of growth. Control bacteria are bluish-purple while inhibited bacteria are white.

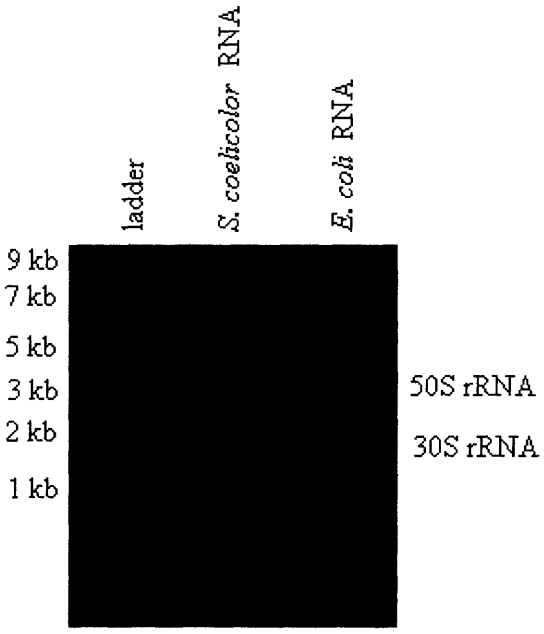

Figure 13. Total RNA isolated from *S. coelicolor* and *E. coli* (positive control) as visualized on a denaturing formaldehyde-agarose gel. The band belonging to the 50S and 30S rRNA, the most abundant RNA species in cells, can be clearly seen, indicating successful RNA isolation.

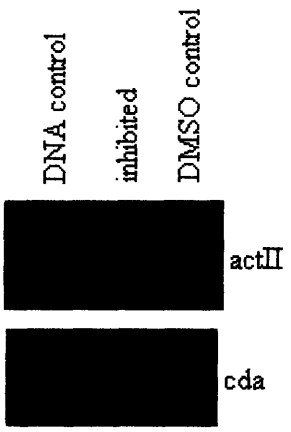

Figure 11. Modified Northern blots of inhibited and control *S. coelicolor* probed for actII and cda mRNA.

Table 2. Summary of modified Northern blotting analysis.	
Gene	**mRNA Expression in Inhibited Bacteria Relative to DMSO Control**
actII	Upregulated
Cda	Upregulated
recA	work in progress
redD	Upregulated (work in progress)
Coelichelin	work in progress
Chalcone	work in progress

4. Discussion

4.1 Analysis of natural products. The difference in HPLC traces between *S. coelicolor* grown with and without Hsp90 inhibition with both radicicol and GDA indicate irreproducibility and inconsistency. Any MS data taken could not be identified with the known products of *S. coelicolor.*

The reason for such inconsistency is unknown, It is possible that radicicol behaves differently from GDA and thus would produce different effects, such as different changes in color. It is also possible that the solvent system used to extract the natural products is not completely optimized and another solvent system would be better at giving more reproducible HPLC traces. It is also possible that the source of the GDA could cause such inconsistency.

Nevertheless, overall differences in color seen with both radicicol and GDA were more consistent, with inhibited bacteria giving an overall less purple color. The results thus indicate

that there is indeed some kind of effect in natural product production resulting from Hsp90 inhibition.

4.2 Analysis of mRNA. Another method of monitoring changes brought about by Hsp90 inhibition is to monitor levels of mRNA expression, such as by Northern blotting. Such analysis would show which genes are being turned on and off because of Hsp90 inhibition. In bacteria, the genes for a particular metabolic pathway are clustered together within a single open reading frame, so that the expression of one gene in the pathway would imply that other genes in the pathway are likewise expressed. Analysis of one particular gene would therefore be representative of the entire pathway.

Results from such work have proved to be more intriguing. Inhibited colonies were consistently white in color; indicating a down-regulation in actinorhodin production (Figure 1). We thus expected the genes involved in actinorhodin production to also be down-regulated. However, the opposite was observed. mRNA levels of one of the genes involved in actinorhodin production, actII, were consistently observed to be expressed more than in the controls. Since the genes of a prokaryotic gene cluster are typically expressed simultaneously, this implies that all proteins in the entire actinorhodin metabolic pathway are being upregulated. Reasons for this are currently unknown, although post-transcriptional regulation seems to be in effect. What is clear, however, is that Hsp90 inhibition by radicicol consistently affects gene regulation.

Likewise, upregulation of calcium-dependent antibiotic mRNA expression (and prodiginine mRNA expression, although these results are still being confirmed) is observed, although translation of the mRNA may or may not be occurring. Since some natural products that are produced might not be transported outside the cells and be extracted, Northern blotting in

combination with HPLC analysis might give a more complete picture of the effects of Hsp90 inhibition, as might be the case with actinorhodin.

A more perfect analysis of these transcripts would involve unmodified Northern blotting. Modified Northern blotting involves spotting total RNA onto a small area without separating different RNA species by size. This can create a mesh of RNA into which probes may get trapped and thus result in unspecific binding. However, the observation of consistent results from unmodified Northern blotting seems promising. The reason unmodified Northern blotting did not work initially is currently unknown, although performing blotting with DNA species as well as with RNA may pinpoint the problems.

Future work might include all of these issues. Additionally, the analysis of gene expression in the presence of an Hsp90 "trap" might prove intriguing.[8] Such a trap would involve site-directed mutagenesis of the single residue required for ATP hydrolysis in the protein, disabling it, much as GDA or radicicol does. Results with this mutated protein might be expected to be identical to those observed with chemical inhibition. Furthermore, analysis of mRNA expression on a time scale, measuring mRNA expressed in a certain time period, for example before and after inhibitor is added to the media may also give insight into the mechanism of inhibition. Expression analysis of other genes such as the gene encoding Hsp90 itself, may give insight into the response of Hsp90 itself upon its inhibition.

5. References

1. Bode, H. B., et. al. Big effects from small changes: possible ways to explore Nature's chemical diversity. *ChemBioChem* **3**, 619-627 (2002).

2. Queitsch C., et. al. Hsp90 as a capacitor of phenotypic variation. *Nature* **417**, 618-624 (2002).

3. Rutherford, S. L., et. al. Hsp90 as a capacitor for morphological evolution. *Nature* **396**, 336-342 (1998).

4. Bentley, S. D., et. al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**, 141-147 (2002).

5. Keiser, T., et. al. Practical *Streptomyces* genetics. John Innes. Norwich, England (2000).

6. Kormanec, J. Analyzing the developmental expression of sigma factors with S1-nuclease mapping. In: Schein, C. H. (ed). Nuclease methods and protocols. Methods in molecular biology. Humana Press Inc. Totowa, NJ. **160**, 481-494 (2001).

7. Ausubel, F. M., et. al. (ed). Current protocols in molecular biology. John Wiley and Sons Inc. **1** (1994-2003).

8. Nathan, D. F., et. al. Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. *Mol Cell Bio* **15**, 3917-3925 (1995).