

# Stress and Release: Chemical Modulation of Secondary Metabolite Production in *Aspergillus* sp.

By

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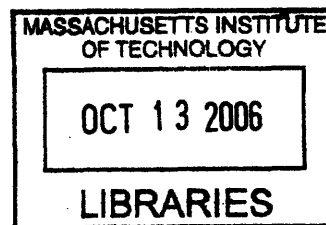
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# **Stress and Release: Chemical Modulation of Secondary Metabolite Production in *Aspergillus* sp.**

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## **ABSTRACT**

**Cyclosporin A induced biosynthesis of colored compounds in three species of *Aspergillus*. Diode array HPLC MS analysis of culture extracts revealed *Aspergillus terreus* demonstrated the most profound response, with upregulation of more than twelve compounds from three distinct chemical families; butyrolactones, aspulvinones, and asterriquinones. Compounds from these three families are prenylated, and biosynthetically derived from homodimers of amino acids. The majority of the upregulated compounds were the aspulvinones, a class of butenolides. Structural elucidation of four isolated aspulvinones revealed both known and novel structures. Inducer concentration had a significant impact on aspulvinone profile. Bioassay revealed previously unreported antibacterial activity for the aspulvinones.**

**A high-throughput colorimetric screen was designed to probe the response. The screen of 2480 known bioactives revealed multiple compounds capable of inducing aspulvinone production, and one compound, rapamycin, capable of inhibiting the response. Additionally, the increase in aspulvinone production was correlated with a decrease in culture density, indicating aspulvinone production is a general stress response.**

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**Introduction** *Aspergillus*, a genus of ubiquitous filamentous fungi, is renowned for production of diverse bioactive secondary metabolites, such as lovastatin, a potent hydroxymethylglutaryl coenzyme A reductase inhibitor,<sup>1</sup> used clinically to reduce cholesterol levels, and the aflatoxins<sup>2</sup>, potent mycotoxins which cause both short-term and long-term adverse health effects, ranging from immediate toxic response and immune suppression to the potential for long-term carcinogenic effect. These secondary metabolites have evolved to confer a selective advantage to the producing organism, with biosynthesis generally triggered by specific environmental conditions. For this reason, although an organism may possess the gene clusters encoding secondary metabolite biosynthetic pathways, in typical culture conditions the corresponding products are either absent or present only in minute quantity.

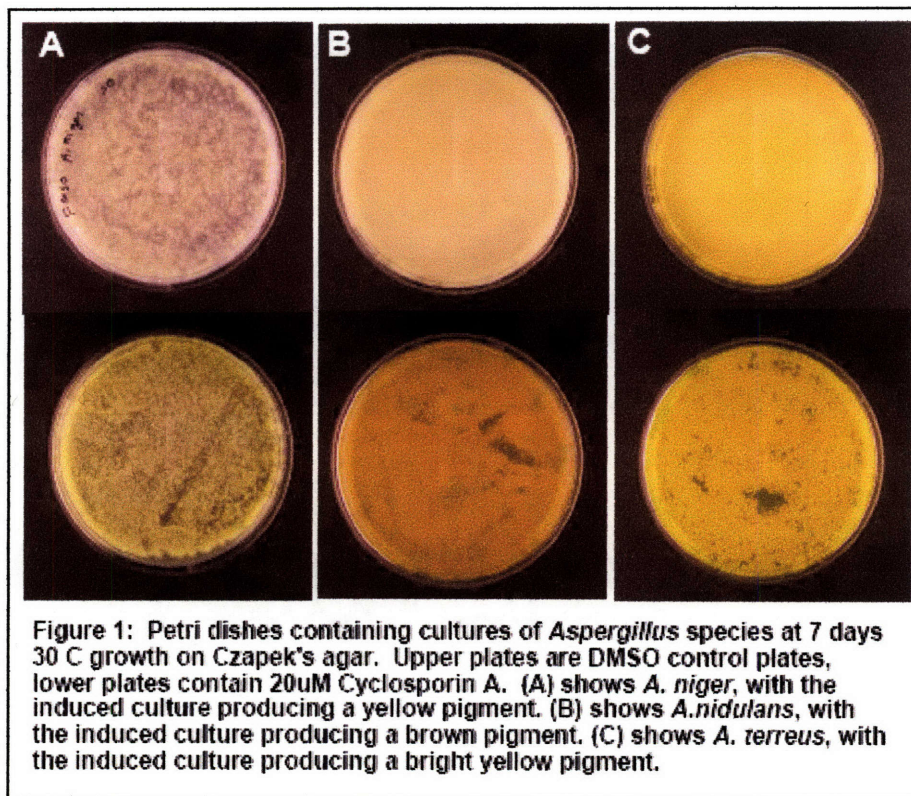
The vast number of silent pathways represents an untapped source of diversity in both chemical structure and potential for biological activity. Methods that release this cryptic variation are essential, not only to access diversity, but also to gain an understanding of the regulation of biosynthesis, particularly for those compounds that are, or will be, commercially fermented for clinical use. As production of secondary metabolites is environmentally mediated, inhibition of central nodes in signal transduction pathways was proposed as a method to induce upregulation of silent pathways. Perturbation of the organism's signaling network could mimic any trigger resulting in the production of secondary metabolites.

High-throughput screening revealed that the induction of secondary metabolism was non-specific to signal transduction inhibitors. Instead, aspulvinone production correlated with a decrease in culture density, suggesting upregulation is a general stress response. Release of secondary metabolites in response to stress is precedented, as fungi of the genus *Fusarium* were recently shown to produce colored naphthoquinone pigments in response to conditions of growth inhibition and arrest.<sup>3</sup> This indication of generality of the fungal "stress and release" response indicates a new approach to discovery of diversity. Although elegant genome-based methods for secondary metabolite discovery have been successfully employed in *Aspergillus*<sup>4</sup>, the vast majority of fungal genomes remain unsequenced. Additionally, there can be considerable disparity of secondary metabolite profiles in the unsequenced strains of the same species. For these reasons, chemical stress may represent a rapid and facile method of releasing cryptic secondary metabolite diversity in unsequenced fungi. Additionally, once the mechanism of upregulation is understood, harnessing this response to upregulate important pharmaceuticals, such a lovastatin, is entirely possible. This could result in less expensive production and lower costs for the millions of consumers of *Aspergillus*-derived pharmaceuticals.

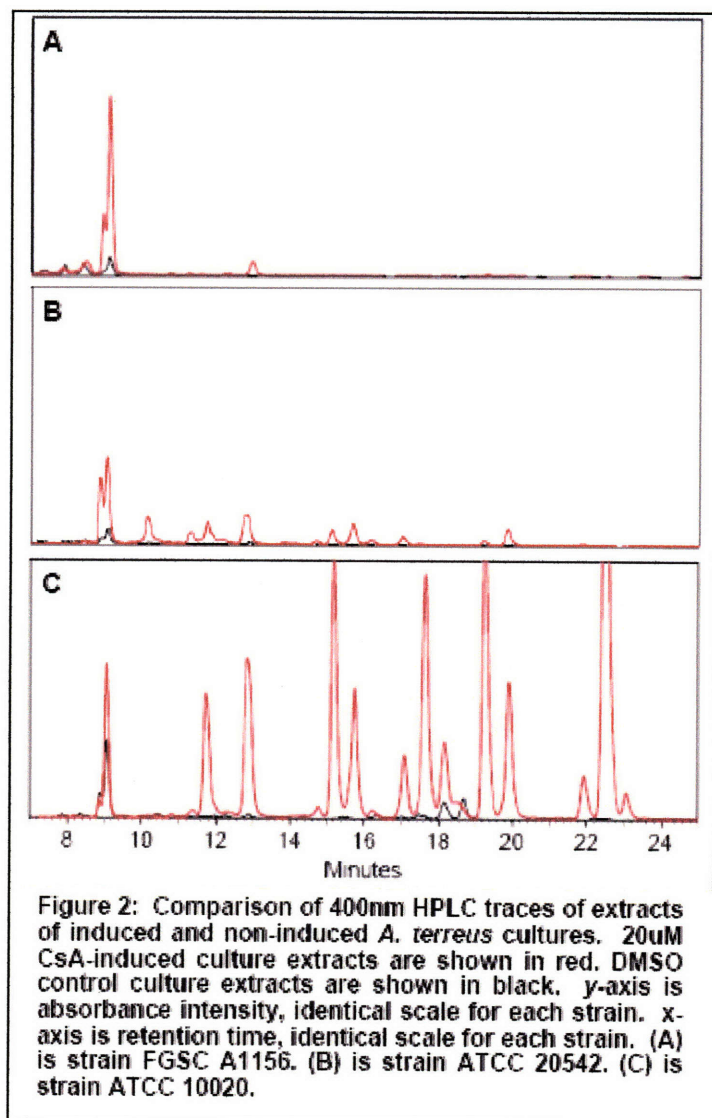
## Results and Discussion

### Experimental Organism Selection

A number of signal transduction inhibitors were tested against the *Aspergillus* species, *A. niger*, *A.nidulans*, and *A. terreus*. Cyclosporin A (CsA), a calcineurin inhibitor<sup>5</sup>, induced upregulation of colored metabolites in all species tested (Fig.1). Please note that poor printer quality may skew color of images. Pdfs of figures are available upon request. Of the three species, *A. terreus* demonstrated the strongest response, showing dramatic upregulation of yellow pigmentation.



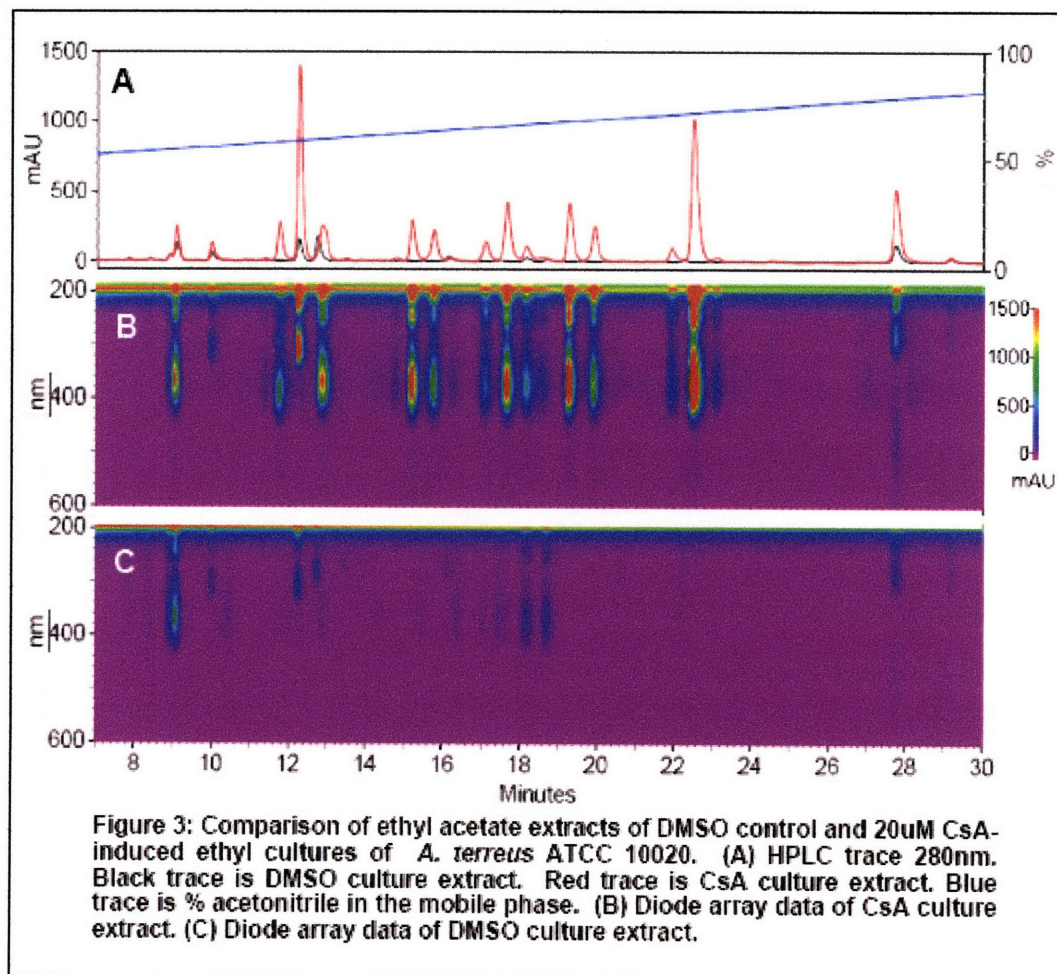
*A. terreus* was therefore selected for further study, and three strains were selected for response comparison; *Aspergillus terreus* American Type Culture Collection (ATCC) 10020, ATCC 20542 and Fungal Genomics Stock Center (FGSC) A1156. All *A. terreus* strains exhibited an increase in colored metabolite biosynthesis. Ethyl acetate extracts of 20uM CsA-induced *A. terreus* cultures were analyzed by UV-Vis HPLC, and all revealed upregulation of colored compounds relative to the uninduced control. However, there were significant differences in both the number of compounds upregulated, and the intensity of the upregulation. ATCC 10020 demonstrated the strongest response in both categories (Fig.2).



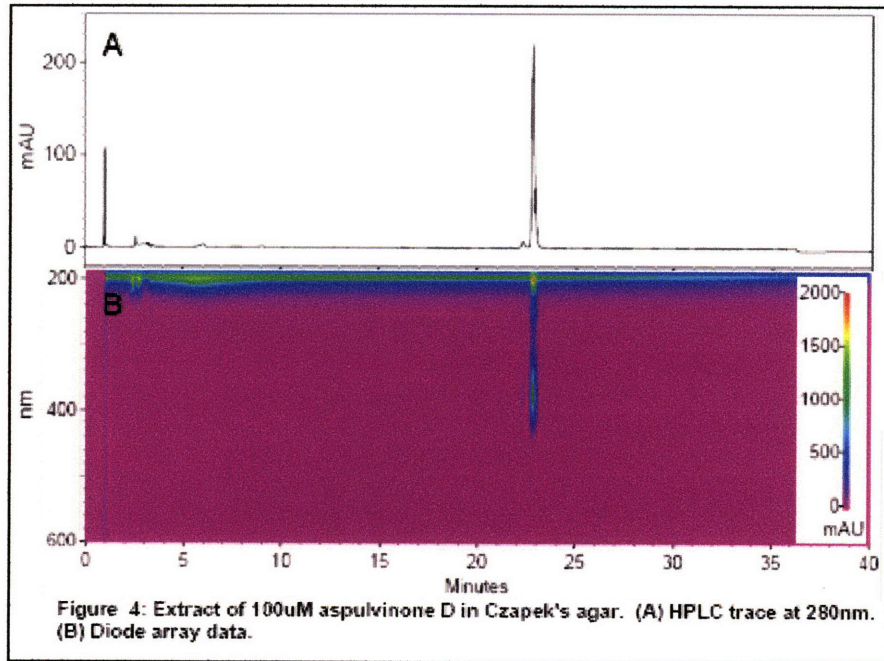
### Identification of Upregulated Compounds

ATCC 10020 was therefore selected for further analysis. Large scale (4X 1L) solid cultures were grown, extracted, and separated by preparatory HPLC to yield mg quantities of 6 compounds. These compounds were selectively subjected to 1D and 2D NMR analysis by  $^1\text{H}$ ,  $^{13}\text{C}$ , DEPT, gCOSY, gHMQC, and HMBC. Diode array hplc ms (Fig.3), and NMR revealed upregulation of three distinct classes of compounds; aspulvinones, butyrolactones, and terriquinones. The diode array data was particularly helpful in identifying compound type, as each family had a characteristic uv-vis spectra (Appendix C). Although structurally distinct, all identified compounds are prenylated homo-dimers, biosynthetically derived from pairs of identical amino acids, with butyrolactones<sup>6</sup> and aspulvinones<sup>7</sup> derived from tyrosine, and asterriquinones derived

from tryptophan<sup>8</sup>. The simultaneous release of three distinct structures, all of the homo-dimer origin, denotes *Aspergillus terreus* as a highly significant candidate for the study of biosynthetic regulation.

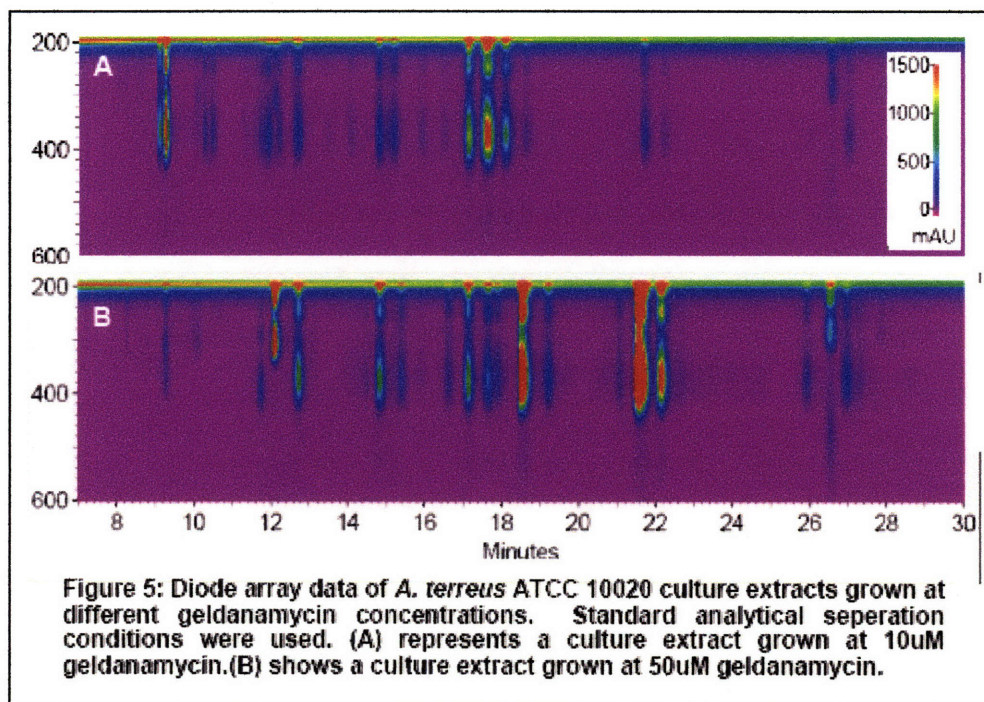


The majority of the upregulated compounds were members of the aspulvinone family. Two of the aspulvinones were previously identified, aspulvinone D and aspulvinone F. All other isolates had spectral differences from the previously characterized aspulvinones A-I.<sup>9</sup> Doping cell-free culture with isolated aspulvinone D, and re-extracting revealed that no compound breakdown occurs during the very mild extraction procedure (Fig.4) This experiment supports that all compounds isolated are compounds produced by the organism, rather than break-down products of a single compound. The determined structures are shown in Appendix D. Name, mass, bioassay results, UV-Vis  $\lambda_{max}$  retention time, bioassay results and unoptimized yield are shown in Appendix A. Correlation of the analytical and preparatory systems, and traces of isolates, are given in Appendix B. Examples of UV spectra are given in Appendix C.



### Change of Upregulated Compound Profile in Response to Inducer Concentration

The concentration, but not the identity, of inducer was determined to have a substantial effect on the profile of the upregulated compounds. Multiple concentrations of CsA, radicicol, and geldanamycin were measured to determine the effect, and a shift to more non-polar aspulvinone production was seen in all cases. The effect was most striking with geldanamycin, as shown in figure 5.



#### Determination of Upregulated Compound Bioactivity

The crude extracts of induced and non-induced *A. terreus* cultures were tested for bioactivity using a simple disc-diffusion bioassay. Activity against three species was tested; *B. subtilis*, a gram positive bacterium, *E. coli*, a gram negative bacterium, and *S. cerevisiae*, a yeast. Only *B. subtilis* registered a response, with the extract of the induced-culture extract resulting in a zone of inhibition substantially larger than that of the uninduced control. Non-identical zones of inhibition indicated that at least one of the induced compounds possessed significant activity against *B. subtilis*. Isolated compounds were then tested individually at 50ug per disc. Photos of bioassays of crude extracts are shown in figure 6. Interestingly, the only isolate not to display bioactivity was aspulvinone 4, the monoprenylated derivative, suggesting that two free prenyl groups may be required for antibacterial activity (Appendix A).



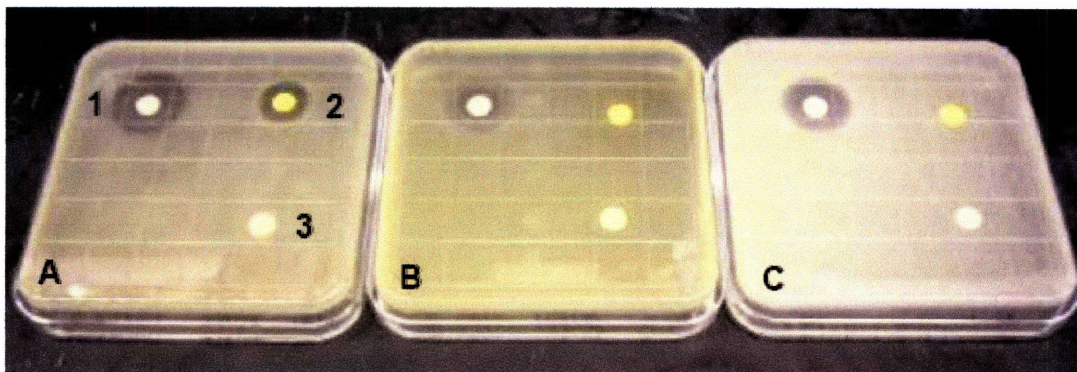
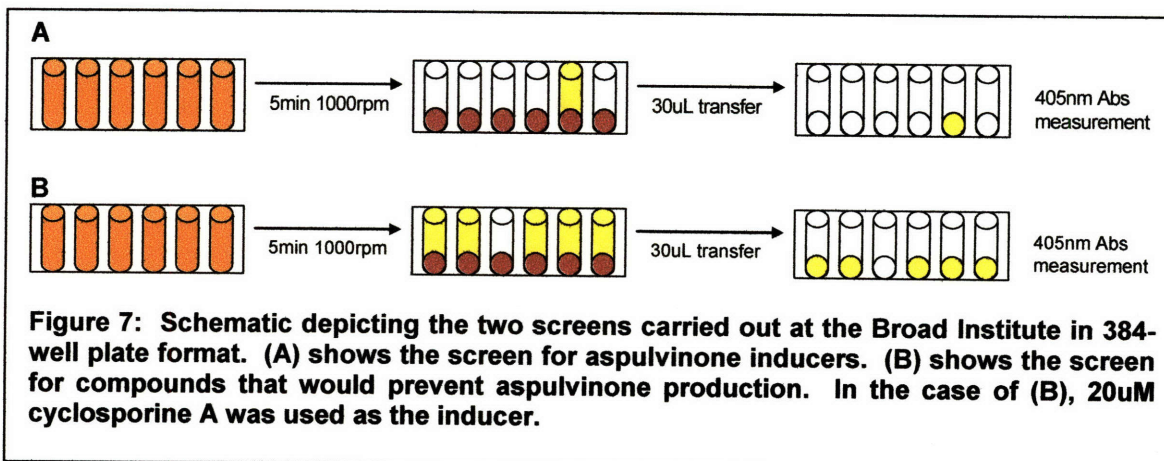


Figure 6: Disc-diffusion bioassays of crude *A. terreus* 10020 induced and non-induced culture extracts. Discs in position (1) are the positive control disc for the specific species. Discs in position (2) are innoculated with 10uL of crude extract from the induced culture. Discs in position (3) are innoculated with 10uL of the non-induced culture extract. (A) is a *B.subtilis* bioassay. (B) is an *E.coli* bioassay. (C) is a *S.cerevisiae* bioassay. Note only *B.subtilis* shows zone of inhibition in response to the upregulated compounds.

## High-Throughput Screen

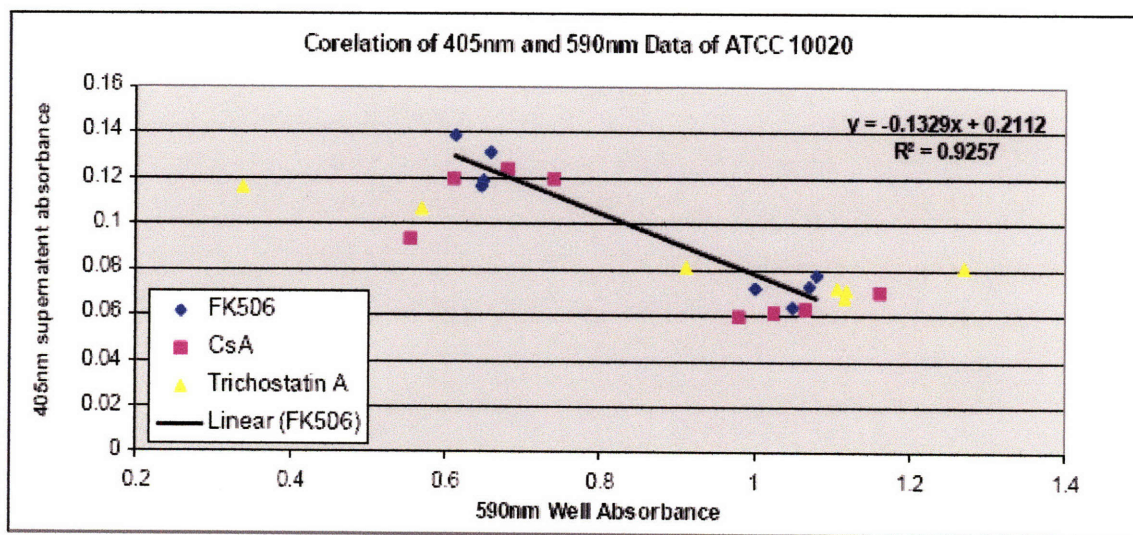
### Investigation of Specificity of Induction

In order to investigate the upregulation of these bioactive compounds, a liquid-based 384-well plate micro-assay was developed for use at the Broad Institute high-throughput screening facility. The diverse bioactive library of 2480 compounds, provided by the Broad Institute at MIT, courtesy of NCI's Initiative for Chemical Genetics (ICG) screening outreach program, was screened. Two goals were set, first to identify additional inducers of aspulvinone upregulation, and second to identify any compound that could prevent aspulvinone upregulation by the previously identified inducer, cyclosporine A. ATCC 10020 microcultures were doped with approximately 10 $\mu$ M of each library compound (Fig.7).



The screen revealed multiple compounds capable of inducing aspulvinone production. The most promising 10 candidates were rescreened from 0.01 $\mu$ M to 50 $\mu$ M. A high false positive rate was expected for this whole-cell assay, and rescreening eliminated many of the tested compounds. However, trichostatin A, a histone deacetylase inhibitor, and FK506, an inhibitor of calcineurin via the FK506 binding protein, were confirmed to be colored-metabolite inducers (data not shown). The Hsp90 inhibitors, geldanamycin and radicicol, were also identified as aspulvinone/terriquinone inducers. As the screen tests a single set of conditions, and the aspulvinone production is dependent on inducer concentration, it is likely that additional compounds from the library have the potential to induce aspulvinones. Initial work with CsA showed that that aspulvinone upregulation is dependent on concentration.

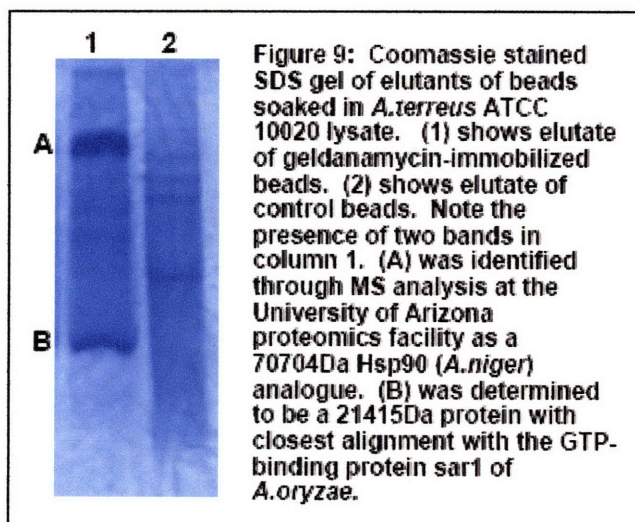
Initial screening data also revealed a rough correlation between decrease in 600nm absorption and increase in 405nm absorption, and this correlation was confirmed for all inducers tested (Fig. 8).



**Figure 8: Correlation of 405nm supernatant absorbance increase with 590nm well absorbance decrease. The data for a concentration series is shown for 0-40 $\mu$ M for each of three inducers: FK-506, diamonds, CsA, squares, and trichostatin A, triangles. The trendline of the FK506 data is shown in black, the equation given in the upper right hand of the figure.**

In the case of geldanamycin, binding of the fungal target protein, Hsp90, was verified using geldanamycin immobilized on beads<sup>10</sup> (Fig. 9). The expected Hsp90-homolog

was obtained from *A. terreus* lysate, which suggested that although the compounds may bind their unique fungal targets, the upregulatory response is not specifically a result of the inhibition of the specific protein target, but rather the result of the non-specific cytotoxic effect of the compound.



### Identification of Inhibitor of Induction

The high-throughput screen identified only one compound which prevented aspulvinone production by cyclosporine A; rapamycin. Rapamycin was then tested in 96-well plate format against all the inducers, and found to prevent the production of aspulvinones, independent of the cellular target of the inducer (Fig. 10).

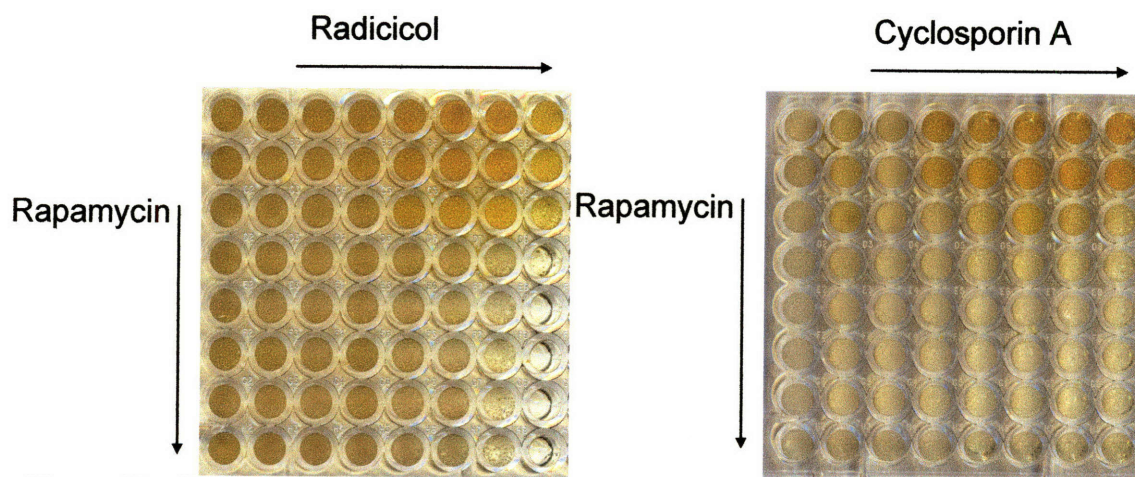


Figure 10: Matrix assays showing aspulvinone production by inducer, and inhibition of aspulvinone production by rapamycin. The compound concentrations increase in the direction of the arrow in the following series; plain media, DMSO control, 0.1uM, 1uM, 5uM, 10uM, 20uM, 50uM inducer. Only 1uM rapamycin is needed to prevent aspulvinone production by both inducers.

The response to rapamycin indicates that the aspulvinone biosynthetic pathway could be under the control of TOR. As fungal biosynthesis regulation is poorly understood, this facile and robust assay could be used to further probe this system to yield crucial information. Further study would yield an understanding of the mechanism of secondary metabolite upregulation, and be applied to another commercially relevant pathway, such as that of lovastatin, to dramatically boost production of this important drug.

## **Experimental**

*General Experimental Procedures.* All 1D and 2D NMR spectra were recorded in d<sub>6</sub>-dimethylsulfoxide on 400MHz and 500MHz instruments, as noted. HPLC was performed on a Beckman Coulter reversed-phase C18 analytical column (4.6 x 25mm, 5µm) on a Beckman-Coulter system employing a photodiode array (PDA). HPLC MS utilized the same column on an Agilent system, with a PDA and an electrospray time-of-flight (ESI-TOF) mass spectrometer. All HPLC solvents were of HPLC grade, while other solvents were of ACS grade. Cyclosporin A was obtained from Toronto Research Chemicals, Trichostatin A was obtained from Biomol, FK506, radicicol, and geldanamycin were obtained from A.G. Scientific.

*Biological material.* *A. terreus* strains ATCC10020, ATCC 20542, and *A. niger* ATCC 6275, *E.coli*, and *B. subtilis* were obtained from the American Type Culture Collection. *A. terreus* FGSC A1156 and *A.nidulans* FGSC A4 were both obtained from the Fungal Genomics Stock center. Fungal spore solutions were made according to the procedure of the National Committee for clinical laboratory standards (NCCLS) M38-A, Vol. 22, No. 16, with the exception of spore solutions containing 10% glycerol to enable storage at -80°C.

*Culture conditions: Solid culture.* All fungal strains were grown on Czapek's agar, in Petri dishes, at 30°C. Solid culture enabled excellent reproducibility in secondary metabolite profiles. Inducers were dissolved in DMSO and added to media prior to solidification. *Liquid culture.* Liquid cultures were grown in minimal media, with 0.01 OD<sub>600</sub> spore density for 384-well plate assays, and 0.001 OD<sub>600</sub> spore density for 96-well plate assays. 100µL cultures were used in 384-well plates, and 250µL cultures were used in 96-well plates. 96-well plate cultures were allowed to develop for 7 days at ambient temperature in a humidity chamber, and 384-well plate cultures were allowed to develop for 11 days in a humidity chamber at 25°C.

*Extraction and Separation- Analytical Scale.* 15mL solid cultures vortexed with 15mL ethyl acetate and 10mL water. Samples were then centrifuged at 5000rpm, 4°C, for 10 minutes. Ethyl acetate was removed, dried under vacuum at 35°C, and the resulting solid was dissolved in methanol and subjected to reverse phase HPLC, (50-100% acetonitrile in 0.1%trifluoroacetic acid-H<sub>2</sub>O), or (50-100% 0.1%trifluoroacetic acid-acetonitrile in 0.1%trifluoroacetic acid-H<sub>2</sub>O) for HPLC MS.

*Extraction and Separation- Preparative Scale.* 4 X1 liter of solid cultures each extracted with one liter ethyl acetate, 300mL H<sub>2</sub>O for one hour at 225rpm in 4L flasks. Ethyl acetate was removed, dried under vacuum at 35°C, and the resulting solid was

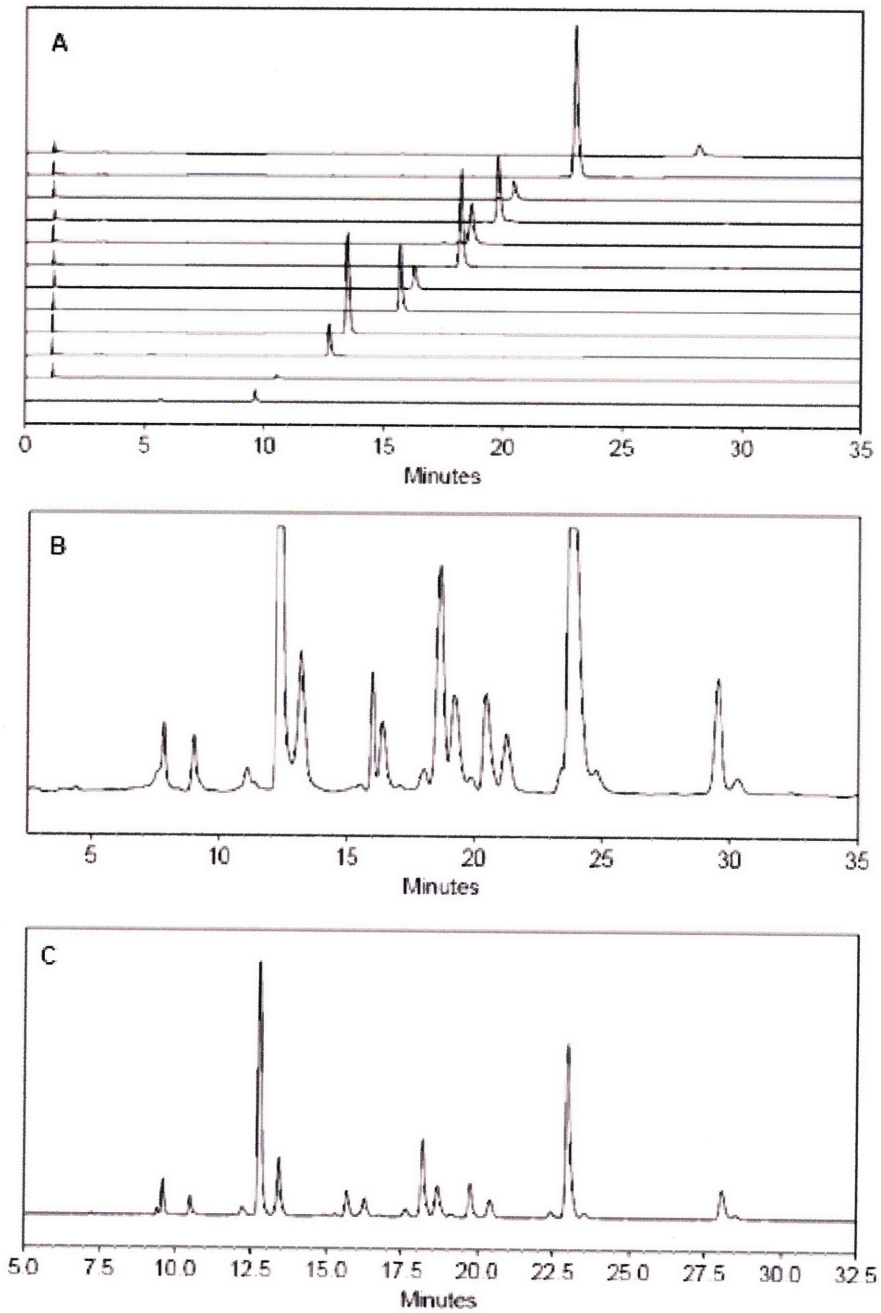
dissolved at 50mg/mL methanol and subjected to preparatory HPLC using a (30-100% acetonitrile in 0.1%trifluoroacetic acid-H<sub>2</sub>O) gradient optimized to match that of the analytical column. Individual peaks were collected for multiple runs, fractions pooled and dried under vacuum at 35°C. Further purification was not required for spectroscopic elucidation.

*Bioassay.* *B. subtilis* were grown to late log phase in Difco Nutrient Broth at 30°C. Culture was then diluted with 10% glycerol Difco Nutrient Broth and stored at -80°C. Aliquots of bacterial suspension were then added to molten Antibiotic Assay Media cooled to 50°C, and allowed to solidify. 50µg of the isolated compounds were applied to sterile antibiotic assay discs. 10µg gentamycin antibiotic assay discs served as a positive control. Discs were placed on the surface of the seeded agar and the compounds were allowed to diffuse for an hour at 4°C, prior to 24 hour incubation at 30°. Existence of a zone of inhibition diameters were then recorded. Extracts of cell-free CsA plates showed no bioactivity, indicating increase in bioactivity of induced cultures is not due to the inducer itself.

*High-Throughput screen.* All large scale screening occurred at the Broad Institute Screening facility, Cambridge, Massachusetts. Seeded media was aliquoted into 384-well plates using a liquid handling robot, and 250nL of library compound stock solutions were transferred from stock plates into assay plates using an automated pin-based transfer robot, to yield compound concentrations of approximately 10µM. Following incubation, the assays were analyzed at 600nm using an automatic plate reader. The plates were then spun at 1000rpm for 5 minutes, and 30µL of culture supernatant was transferred to new 384-well plates using a robotic transfer system. These plates were then analyzed at 405nm using an automatic plate reader. Data was then subjected to statistical processing by the Broad computational team, and the resulting data sets were analyzed with Spotfire software to identify positive hits of the screen. Initial experiments indicated a substantial increase in 405nm absorption of the 20µM CsA cultures relative to the DMSO controls.

*Acknowledgements.* This work was supported by the Beckman foundation. I thank Leah Cowen, Ph.D, postdoctoral fellow, and Susan Lindquist, Ph.D., professor of Biology, both of the Whitehead Institute, for invaluable discussion and advice; John S. Wishnok, Ph.D., Senior Research Scientist, MIT Biological Engineering Mass Spectrometry Lab, for HPLC MS analysis of samples; Peter J. McCarthy, Ph.D., Associate Director and Head of Microbiology, Harbor Branch Oceanographic Institution, Inc. for bioassay protocol and advice; Nicola Tolliday, Ph.D., Head of Screening, and Jason Burbank Screening Room Manager, Paul Clemons, Ph.D., all of the Broad Institute, for invaluable advice, assistance, and preliminary screen data processing.

**Appendix B-Summary of HPLC isolation of Compounds.**

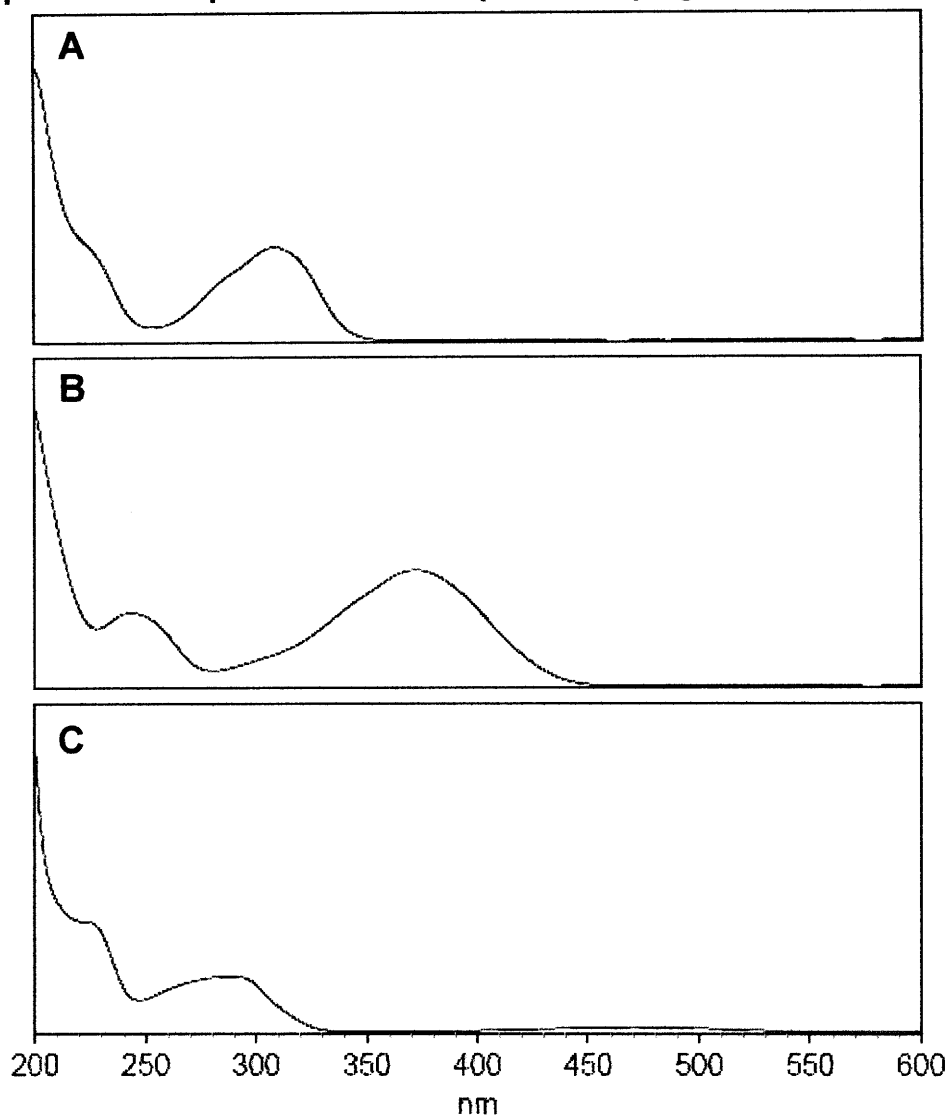


**Appendix B: Comparison of profiles from optimized analytical(C) and preparatory(B) HPLC traces, shown with isolated compounds run on the analytical (A). All traces are shown at 280nm.**

Appendix A- Summary of isolated compound data

Tentative name	<i>B. subtilis</i> bioassay	1H	13C	gCOSY	DEPT	gHMQC	HMBC	M+	UV-VIS HPLC maxima (nm)	50-100X std. config retention time (min)	MASS obtained 4X1L (mg)
<b>3'-(3-Methyl-2-butenyl) butyrolactone II</b>	+	X	X					425.1587	203,308,404	12.74	25
<b>Monoprenylated aspulvinone</b>	-	X	X	X	X	X	X	381.1347	200,242,367	13.5	37
<b>diprenylated aspulvinone D</b>	+	X	X	X	X	X	X	449.1973	199,244,374	15.7	29
aspulvinone	-	X						465.1925	196,241,377	16.33	15
<b>aspulvinone F</b>	-	X	X	X	X	X	X	465.1926	200,244,373	18.24	24
aspulvinone	na	X						465.1953	197,245,376	18.68	19
aspulvinone	+	X						433.253	199,244,372	19.79	12
aspulvinone	+	X		X				449.1972	196,239,375	20.46	12
<b>aspulvinone D</b>	+	X	X	X	X	X		449.1977	204,245,362	23.02	49
<b>asterriquinone</b>	+	X	X					507.2296	193, 226(s), 287,471	28.19	15

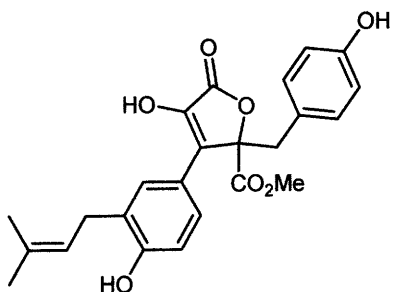
### Appendix C- Comparison of UV-Vis Spectra of Upregulated Compounds



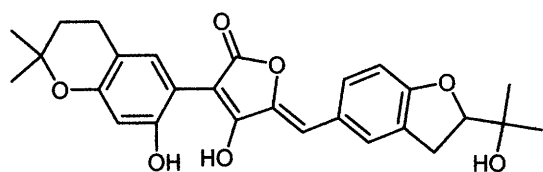
Appendix C: Comparison of typical UV-Visible Spectra of the three different families of upregulated compounds. (A) Butyrolactones (B)Aspulvinones (C)Asterriquinones.



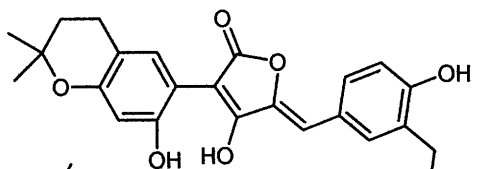
**Appendix D: Structures of Isolated and Structurally-Elucidated Stress-Induced Compounds. Note all are prenylated homodimers. Compounds marked "NOVEL" have not been previously isolated.**



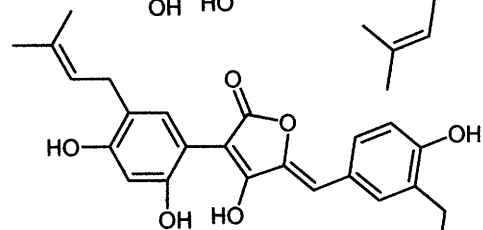
Butyrolactone II



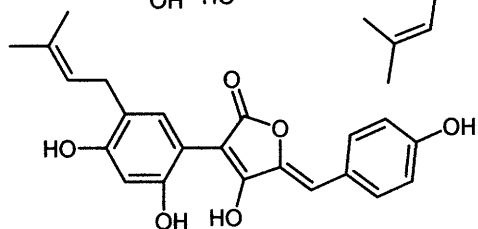
Aspulvinone F



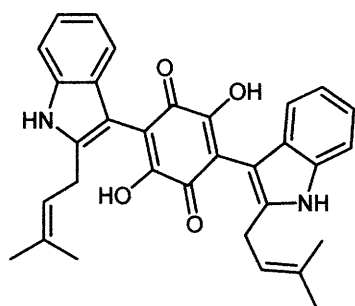
Aspulvinone D



Diprenylated Aspulvinone - NOVEL



Monoprenylated Aspulvinone- NOVEL



Asterriquinone

- 
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