

2006 GSCSSA Progress Report

TITLE OF PROJECT: Control of Grassy Weeds. Structural Characterization of a novel, highly-specific herbicide of natural origin.

OBJECTIVES:

- A. Completion of mass spectral studies of GAF and its derivatives.**
- B. Generation of nuclear magnetic resonance (NMR) data for GAF and/or its derivatives.**
- C. Development of a putative model of the structure of GAF and confirmation of this structure by chemical synthesis.**
- D. Maintenance of patent protection of the herbicide.**

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ABSTRACT OF 2005 PROGRESS: A Germination-Arrest Factor (GAF) produced by certain isolates of rhizosphere bacteria specifically arrests germination of the seeds of a wide-range of grassy weeds. We have characterized the biological, physical, and chemical properties of GAF and identified genetic regulatory elements that control GAF production. In the 2004 and 2005 grant years, we have succeeded in purifying GAF to homogeneity and in obtaining mass spectral and nuclear magnetic resonance data that have enabled us to assign a putative structure to the GAF molecule. Chemical synthesis of GAF and tests of several chemically related compounds are underway.

JUSTIFICATION: Contamination of perennial ryegrass and tall fescue seed lots with the seeds of grassy weeds is a major problem to the grass seed industry in the Pacific Northwest. This problem is made worse by the development of Diuron-resistant strains of these weeds. We have identified a naturally occurring herbicide that selectively arrests germination of the seeds of a wide range of grassy weeds, including Diuron-resistant strains. Commercial development of this herbicide will provide a highly specific and effective alternative to synthetic chemical herbicides and one that is likely to have a minimal impact on the environment.

PROGRESS:

Major Accomplishments in 2005: We have previously demonstrated that GAF specifically and irreversibly arrests germination of the seeds of a wide range of grassy weeds, while exerting little if any effect on the growth of established grass seedlings or the germination of any dicot (broad-leaf) species tested to date. Through mutational analysis of the genetic regulation of GAF production, we were able to establish that GAF activity is associated with a specific ninhydrin-positive compound that is very hydrophilic and restricted to culture filtrates of bacterial strains that produce GAF. (Ninhydrin is a reagent that reacts with amino groups to produce a colored derivative of the original compound.) The hydrophilic character of the GAF molecule made the purification of GAF a substantial challenge, because traditional solvent extraction and trace enrichment procedures, which are based on the hydrophobic properties of many small organic molecules, could not be used for GAF purification. However, we were ultimately successful in obtaining GAF preparations of the purity required for structural analysis. A combination of mass spectral analysis and nuclear magnetic resonance studies of these purified GAF preparations have enabled us to assign a putative structure to the GAF molecule. For confirmation of structure, chemical synthesis of GAF is essential. Exploratory investigations during the past year have identified one of several possible synthetic pathways as the most promising, and the requisite work for chemical synthesis of GAF is underway. While this work is progressing, we have used the putative structure of the GAF molecule to identify a small number of commercially available compounds that bear some structural relationship to GAF, as well as several compounds that might be expected to exhibit various types of biological interactions with the herbicide. Tests of the biological activity of these compounds in our grass seed assay systems are underway.

OBJECTIVE A: Completion of mass spectral studies of GAF and its derivatives. Our initial mass spectral analyses were conducted on partially purified GAF preparations obtained by extraction of dried culture filtrates with 90% ethanol solutions followed by preparative TLC chromatography of the extracts on cellulose plates. The results of these mass spectral studies indicated that the GAF preparations in question still contained high concentrations of phosphate salts (presumably from the culture medium) as a major contaminant. This information enabled us to design chromatographic methods to separate GAF from the contaminating phosphate, and the resulting GAF preparations proved sufficiently pure that we could utilize electrospray techniques to obtain useful mass spectra. From these studies, we were able to deduce the molecular weight of GAF, and we obtained rather complete mass fragmentation patterns that provided some initial information concerning the functional groups likely to be present in the molecule. Attempts were also made to obtain a high resolution mass value for the GAF molecular ion (a value that ideally would be accurate to the fourth decimal place). In the best circumstances, such high resolution values enable one to deduce a probable empirical formula for the compound in question (*i.e.* a formula of the type $C_nH_nO_nN_n$). The high resolution values we obtained for GAF proved more variable than we had hoped. Nevertheless, from a list of possible empirical formulas generated from this data, and assuming GAF contained only carbon, hydrogen, oxygen, and

nitrogen, one particular formula appeared most consistent with all of the other mass spectral information we had obtained for the GAF molecule.

OBJECTIVE B. Generation of nuclear magnetic resonance (NMR) data for GAF and/or its derivatives. NMR studies on rigorously purified GAF preparations were carried out in collaboration with Dr. Kerry McPhail of the OSU College of Pharmacy. NMR analysis is a particularly powerful analytical tool for structural work, but in contrast to mass spectral studies which require very small amounts of material, NMR is quantitatively much less sensitive. Thus, it was necessary to effect a considerable scale-up of our purification protocols to obtain sufficient purified material for NMR studies. Because of the time consuming and laborious nature of preparative Thin-Layer Chromatography, it was both a surprise and relief that our first attempt at such a scale-up yielded a GAF preparation that gave clean NMR signals. From this sample, Dr. McPhail was able to obtain both proton (hydrogen) and carbon-thirteen NMR spectra, including 2-D COSY and HSQC spectra. The NMR data has subsequently been replicated on a second, separately purified, sample of GAF with identical results. The latter sample also yielded optical rotation data that should ultimately be of utility in assigning stereochemical configurations to the GAF molecule.

OBJECTIVE C. Development of a putative model of the structure of GAF and confirmation of this structure by chemical synthesis. From the NMR data obtained in the studies described above, we were able to deduce the number of carbons present in the molecule, the functional groups attached to these carbons, and the probable relationship between these groups. Although some stereochemical questions remained to be resolved, the NMR data were sufficient to generate a putative structure for the GAF molecule. This structure is consistent with the molecular weight and empirical chemical formula postulated on the basis of the mass spectral studies. Confirmation of this putative GAF structure requires chemical synthesis and testing of the properties of the synthetic compound. Preliminary work to test several strategies for synthesis has been completed, and an attempt at synthesis utilizing the most promising of these strategies is underway.

The analytical data supporting our putative GAF structure are sufficiently unambiguous that we have been comfortable in proceeding under the assumption that this will prove to be the correct structure. Therefore, in the interval while the synthetic work is progressing, we have utilized the putative structure of GAF to select and test a number of commercially available compounds that have either some structural similarity to GAF or appear likely to interact with or be related to GAF on a biochemical level. Of approximately 30 such compounds tested, several interacted competitively with GAF to overcome the effects of the latter compound on germination of annual bluegrass seeds, some exhibited little if any effect on the germination process in either the presence or absence of GAF, and a small number appeared to be inhibitory to germination in their own right. The three most active of these latter compounds, including two that share some structural features with our putative GAF structure, are being further tested to determine their effects on germination of annual bluegrass and the growth of established ryegrass seedlings in soil-based systems.

OBJECTIVE D. Maintenance of patent protection of the herbicide. To develop patent protection for GAF and the GAF-producing bacterial strains, we have worked with the OSU Technology Transfer Office, with Margaret Connor (the USDA Patent Advisor), and with attorneys at Klarquist Sparkman who handle patentable inventions. A provisional USA patent application was filed in December, 2002, and patent protection was subsequently extended by filing a Patent Cooperative Treaty International Application in December, 2003. In December, 2004, a decision was reached to limit our filings under the Cooperative Treaty to a provisional US-patent application. (Cost considerations have prohibited the University from exercising the option of additional filings in international jurisdictions.) As soon as confirmation of our putative GAF structure has been achieved, our intent is to file an additional patent application covering the structure of GAF, as many GAF analogs as possible, and the use of GAF and/or GAF analogs for the control of grassy weeds.

INTERACTIONS: Because of patent concerns, our interactions outside the circle of individuals with a direct need to know has to date been limited. However, we have tested, and will continue to test, seeds of weed species that are potential targets of GAF as these are brought to our attention by growers and other interested parties. In addition, there have been occasional requests from representatives of industry for additional information concerning GAF, and we have honored such request while continuing to limit precise structural information to the principals directly involved in the project.

TIMELINE FOR ACCOMPLISHMENTS REPORTED HERE AND BEYOND:

Objective A: Purified samples of unmodified GAF were obtained in amounts sufficient to initiate mass spectral analysis in May 2005. Mass spectral data, including fragmentation patterns and high resolution mass estimates, were obtained during the summer of 2005. Nuclear magnetic resonance (NMR) studies of purified GAF samples were initiated in September 2005, and a putative structural formula was assigned to the GAF molecule by the first week of October.

Objective B: Initial tests of the efficacy of GAF in spray applications to soil systems in growth chamber and greenhouse environments were completed in June, 2004.

Objective C: Our work is currently protected under a modified provisional U.S. patent protection application filed in December 2004.

PUBLICATIONS, REPORTS, AND PRESENTATIONS FOR CURRENT YEAR:

Journal publications have not been submitted to date because of patent protection concerns, but three manuscripts are in draft form. The first public presentation of results obtained in studies of GAF occurred at the GSCSSA Meeting in Portland, Oregon in December, 2002. Results of our initial efforts to purify GAF were presented at the Portland GSCSSA Meeting in November of 2003, and progress reports were presented at subsequent GSCSSA Meetings in Moscow, Idaho (November, 2004) and Albany, Oregon (November, 2005).