4-Formylaminooxyvinylglycine, an Herbicidal Germination-Arrest Factor from *Pseudomonas* Rhizosphere Bacteria

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A new oxyvinylglycine has been identified as a naturally occurring herbicide that irreversibly arrests germination of the seeds of grassy weeds, such as annual bluegrass (*Poa annua*), without significantly affecting the growth of established grass seedlings and mature plants or germination of the seeds of broadleaf plant species (dicots). Previously, *Pseudomonas fluorescens* WH6 and over 20 other rhizosphere bacteria were isolated and selected for their ability to arrest germination of *P. annua* seeds. The germination-arrest factor (GAF, **1**) responsible for this developmentally specific herbicidal action has now been isolated from the culture filtrate of *P. fluorescens* WH6. Purification of this highly polar, low molecular weight natural product allowed its structure to be assigned as 4-formylaminooxy-L-vinylglycine on the basis of NMR spectroscopic and mass spectrometric data, in combination with D/L-amino acid oxidase reactions to establish the absolute configuration. Assay results for *P. annua* inhibition by related compounds known to regulate plant growth are presented, and a cellular target for **1** is proposed. Furthermore, using bioassays, TLC, and capillary NMR spectroscopy, it has been shown that GAF (**1**) is secreted by all other herbicidally active rhizosphere bacteria in our collection.

Pseudomonad bacteria are known to produce and secrete a broad array of small-molecule metabolites. Many of these bacteria are found in association with plants,[1] where they may exert either deleterious (pathogenic) or beneficial (growth-promoting) effects on plant growth in complex interactions that are frequently mediated by small-molecule bacterial products. These compounds have been reviewed from a genomics perspective by Gross and Loper[2] and may act as quorum sensing agents (e.g., *N*-acylhomoserine lactones and 4-quinolones), antifungals and antibacterials (polyketides, phenazines, and cyclic lipopeptides), surfactants (rhamnolipids and lipopeptides), siderophores (pyoverdines), or phytotoxins (coronatine and amino acids[3]). The complexity and specificity of plant−microbial interactions are exemplified by the isolation from soybean leaf of *Pseudomonas syringae* pv *syringae*, which suppresses the bacterial leaf blight caused by its close relative, plant pathogen *Pseudomonas syringae* pv *glycinea*, by secretion of 3-methylarginine.[4] A number of pseudomonad bacteria have been investigated for their potential direct use as biocontrol agents against microbial plant pathogens in agricultural applications,[5] and several strains of *Pseudomonas* and other bacteria are marketed for the control of bacterial or fungal plant diseases. Similarly, a number of deleterious rhizosphere bacteria (DRB) have been investigated as potential herbicidal biocontrol agents.[6] However, microbial biocontrol agents often require specific environmental and biotic conditions to establish and maintain a critical threshold population sufficient to produce the levels of active metabolites needed to sustain biocontrol activity for agricultural applications in the field and may also lack host specificity.[3,5]

In the grass seed industry, contaminating seeds of annual grassy weeds such as *Poa annua* (annual bluegrass) dramatically reduce the commercial value of the perennial grass seed crop (e.g., ryegrass). Similarly unwanted "weed" grasses that disrupt commercial turfs (e.g., golf courses) are a problem to treat without damaging the turf itself. Therefore, we are investigating the herbicidal activity of a collection of rhizosphere bacteria established at the USDA/ARS National Forage Seed Production Research Center (NFSPRC) in Corvallis, OR. These bacterial strains were isolated originally from soils of the Willamette Valley in Oregon and selected for their potential to inhibit the germination of grassy weeds in grass seed cropping systems and commercial turf management settings. Our efforts have focused on the purification and characterization of the active herbicidal natural products produced by these bacteria and the potential of these metabolites for direct use as selective herbicides. We have recently reported the biological activity[6] and some physical and chemical properties[7,8] of a low molecular weight, hydrophilic, ninhydrin-reactive acid present in the culture filtrate of NFSPRC isolate *Pseudomonas fluorescens* WH6. This compound was shown to be responsible for the observed herbicidal activity of the culture filtrate. It acts as a "germination arrest factor" (GAF, **1**) to block the germination of annual bluegrass (*P. annua*) seeds in a developmentally specific manner, exerting little if any effect on established (crop) grass seedlings or mature plants.[6] In general, GAF-containing culture filtrate from *P. fluorescens* WH6 strongly inhibited the germination of seeds of graminacious plants (grasses and cereals), while the seeds of broadleaf (dicotyledonous) plants were relatively insensitive to GAF at the same concentrations. Testing was carried out on eight different grassy weeds (bluegrasses, bromes, jointed goat grass, and rattail fescue), seven crop grasses grown for seed (rye grasses, fescue, and orchard grass), and the cereal crops wheat, barley, and corn. Among these graminaceous species, only corn appeared relatively resistant to the effects of GAF. Seeds of broadleaf species tested included cabbage, lettuce, alfalfa, tobacco, poppy, carnation, amaranth, and carrot. GAF was clearly distinct in its mode of action and molecular structure from previously reported herbicidal DRB compounds, such as the pseudophomins,[9] phenazine-1-carboxylic acid,[10] and the momilactones.[11] The purification (from isolate WH6) and structure elucidation of GAF are presented here. In addition, 28 other *P. fluorescens* strains present in the USDA-ARS NFSPRC collection have been examined for evidence of GAF production, and the results of that survey are also presented here.

The hydrophilicity and lability of GAF presented a significant challenge in its purification from the aqueous culture filtrate of *P. fluorescens* isolate WH6. However, after some experimentation, 90% EtOH extraction of dried culture filtrates under carefully controlled conditions, followed by preparative thin-layer chromatography (TLC) on cellulose, and exhaustive ion exclusion chro-

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Table 1. NMR Spectroscopic Data for GAF (**1**) in D_2O (300 MHz, 298 K)

position	δ_C mult.	δ_H, mult. (*J*/Hz)	COSY[a]	HMBC[b]
1	173.7 C			
2	53.7 CH	4.26, d (10.0)	5.22	97.0, 153.2, 173.7
3	97.0 CH	5.22, dd (12.2, 10.0)	6.96, 4.26	53.7, 153.2, 173.7
4	153.2 CH	6.96, d (12.2)	5.22	53.7, 97.0
5	160.5 C	7.70, s		

[a] $^1H-^1H$ COSY correlations are from proton(s) stated to the indicated proton(s). [b] HMBC correlations, optimized for $^{2/3}J_{CH} = 8$ Hz, are from proton(s) stated to the indicated carbon(s).

matography on Sephadex G15 in deionized water yielded sufficiently pure GAF for structural analyses. The reactivity of GAF with ninhydrin suggested its identity as an amino acid or small oligopeptide and enabled analytical TLC to be used for the detection of this UV-inactive compound in bacterial culture filtrates. However, for detection of GAF during preparative TLC purification of this compound from 90% EtOH extracts of the culture filtrate, irreversible reaction with ninhydrin was not desirable. Fortunately, TLC conditions could be established under which the GAF-containing band was bound on the lower side by a bright blue fluorescent compound and on the upper side by a weakly fluorescent blue band. Thus, visualization of these bands under UV light could be used to identify the location of the GAF-containing band on the cellulose TLC plate and enable its elution for further purification. At this stage the partially purified GAF was contaminated with inorganic phosphate from the culture medium, as evidenced by mass spectrometric analysis of acidified aqueous solutions of the recovered TLC band. Therefore, ion exclusion chromatography on Sephadex in deionized water was optimized to separate the weakly ionic GAF molecules from the strongly charged phosphate ions. This procedure takes advantage of residual negative charges left on the Sephadex beads in their manufacture. To rapidly assess the distribution of phosphate as well as GAF in the dilute aqueous fractions eluted from the Sephadex column, an alternative method to TLC or bioassay was also needed for detection of GAF. The observation that GAF chelates Cu^{2+} but not Fe^{2+}, while phosphate binds only Fe^{2+}, permitted the use of Cu-Chrome Azurol S and Fe-Chrome Azurol S reagents[12] in microtiter colorimetric assays of small aliquots of the eluting column fractions. Thus, fractions could be combined according to their reactivity with either reagent. As expected (Figure S1), Fe^{2+}-binding phosphate eluted from a Sephadex G15 column before a Cu^{2+}-binding eluant that was confirmed to be GAF by its ninhydrin-reactive analytical TLC profile and herbicidal activity in our standard grass seed (*Poa annua*) assay.[6] The latter fraction was rechromatographed on Sephadex G15 twice more to provide sufficiently pure, phosphate-free GAF (3.0 mg) for mass spectrometric and NMR spectroscopic analysis.

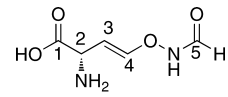
Low-resolution ESIMS of GAF (**1**) provided a tentative molecular mass assignment of *m/z* 183 for $[M + Na]^+$, although the acquisition of MS data was complicated by the delayed passage of **1** through the mass spectrometer, as well as by the formation of polymeric ions during electrospray ionization. The ^{13}C NMR data (in D_2O) for **1** were consistent with a small molecule possessing five carbons, and the ^1H NMR spectrum showed only four resonances, all of equal integration. The latter signals comprised a singlet (δ_H 7.70), two doublets (δ_H 6.96 and 4.26), and a closely overlapped doublet of doublets (δ_H 5.22). Coupling constant analyses and COSY data revealed coupling of each doublet to the midfield double doublet, consistent with a $-CHCH{=}CH-$ spin system ($J_{H\text{-}2,3}$ and $J_{H\text{-}3,4} = 10$ and 12 Hz, respectively). All four relatively deshielded ^1H chemical shifts were HSQC-correlated (Table 1) to four deshielded olefinic or heteroatom-substituted methine ^{13}C resonances (δ_C 53.7, 97.0, 153.2, and 160.5). An alpha amino acid was inferred from HSQC-correlated ^1H and ^{13}C chemical shifts characteristic of an alpha methine (δ_C 53.7, δ_H 4.26, d; CH-

Figure 1. Partial structures for GAF (**1**): (A) the vinylglycine core; (B) the *N*-formylaminooxy moiety.

2) and two- and three-bond HMBC correlations from this α-H and the δ 5.22 double doublet (H-3) to a carbonyl ^{13}C resonance (δ 173.7, C-1). Furthermore, in combination with COSY data and HMBC correlations (Table 1) from H-2 to δ 97.0 (C-3) and δ 153.2 (C-4), H-3 to C-2 and C-4, and H-4 (δ_H 6.96) to C-2 and C-3, these data quickly established a vinylglycine core for GAF (partial structure A, Figure 1). Thus it remained to assign the isolated methine (δ_C 160.5, δ_H 7.70, s) in the molecule and additional NMR-silent atoms (NH, O) to account for the formula of $C_5H_9O_2N_4$ implied by the accurate mass (HR CIMS (+) $[M + H]^+$ *m/z* 161.05195). The deshielded chemical shift of vinyl C-4 (δ_C 153.2) is strongly indicative of an oxyvinyl linkage;[13] an alternative aminovinyl linkage would be indicated by a C-4 shift between δ_C 125 and 140. In addition, no three-bond HMBC correlations from aldehyde H-4 to C-5, or from H-5 to C-4, in support of an (*N*-hydroxy)aminovinyl moiety could be discerned in HMBC experiments optimized for a range of heteronuclear coupling constants ($J_{H,C}$ 2−12 Hz; see Supporting Information). Therefore, the assembly of partial structures A and B to provide an oxyvinyl four-bond connectivity between H-4 and C-5 (commonly inaccessible by HMBC analysis) was indicated.

A *trans* geometry of the olefin at C-3 was consistent with the observed $J_{H\text{-}3/H\text{-}4} = 12$ Hz, which is decreased from the typical range for J_{trans} (14−19 Hz) due to the electronegativity of the oxy substituent.[13] Neither a 2D NOESY correlation between H-3 (δ_H 5.22) and H-4 nor a reciprocal enhancement of H-3 upon selective excitation of H-4 in a 1D double pulsed field gradient spin echo NOESY experiment was observed, as would be expected for a *cis* olefin. Instead, a selective NOE enhancement between H-4 (δ_H 6.96) and H-2 (δ_H 4.26) confirmed the presence of a *trans* olefin. The planar structure of GAF (**1**) could thus be assigned as 2-amino-4-formylaminooxy-but-3*E*-enoic acid, or trivially, 4-formylaminooxy-vinylglycine.

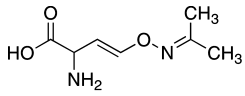
The configuration of this alpha amino acid was investigated by testing it as a substrate for D- and L-amino acid oxidase (from porcine kidney and *Crotalus atrox*, respectively). GAF-containing WH6 extract was treated with each amino acid oxidase in turn, in the presence and absence of control substrates D-Ala and L-Phe as appropriate. The metabolism of GAF and the control amino acids by each enzyme was assessed by analytical TLC with ninhydrin staining, which showed loss of the GAF band in the reaction with L-amino acid oxidase, but not with D-amino acid oxidase, or in control reactions lacking the L-amino acid oxidase (Figure S2). Therefore, we have assigned GAF (**1**) as an L-vinylglycine, similar to the handful of reported alkoxyvinylglycine natural products, which include methoxyvinylglycine, aminoethoxyvinylglycine (AVG), and rhizobitoxine (oxyvinylglycine conjugated with a serinol moiety). These and other vinylglycine natural products have been reviewed, together with synthetic approaches to alkoxyvinylglycines, by Berkowitz et al.[14]



1

While *Pseudomonas* peptidic secondary metabolites regularly incorporate amino acid residues with *N*-formyl and *N*-hydroxy substituents (e.g., N^5-formyl-N^5-hydroxyornithine in pyoverdines), no aminooxyvinylglycine natural products appear in the English-

language peer-reviewed literature. However, a single 1993 Japanese patent reports the isolation of dimethyliminooxyvinylglycine (**2**) and guanidinooxyvinylglycine (**3**) from cultured *Streptomyces* strain WAP5044 as fungicides.[15] Comparison of the ^{1}H and ^{13}C NMR data for GAF (**1**) with those in the patent literature for compounds **2** and **3** revealed an exact match for all ^{1}H and ^{13}C chemical shifts of the oxyvinylglycine core.



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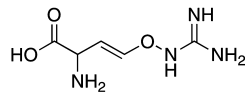

3

The GAF (**1**) biosynthetic pathway may be proposed by considering the studies published for AVG[16] and rhizobitoxine.[17] Homoserine (aspartic acid-derived) has been shown, by gene disruption experiments, to be a direct precursor of these oxyvinylglycines. In the case of rhizobitoxine biosynthesis, homoserine is functionalized at the γ-OH by addition of a serinol group to produce dihydrorhizobitoxine, in which the double bond is introduced via the action of a desaturase.[17] For the biosynthesis of **1**, amination of the homoserine γ-OH by an aminotransferase would form canaline (2-amino-4-[aminooxy]butyric acid), which could be converted to GAF by subsequent formylation (by a formyltransferase) followed by desaturation.

The unusual aminooxy linkage is present in saturated nonproteinogenic amino acids canavanine[18] (2-amino-4-[guanidinooxy]-butyric acid, the dihydro analogue of **3**) and canaline[19] (4-aminooxybutyric acid). As oxy analogues of arginine and ornithine, respectively, both of these legume natural products are important antimetabolites that have been investigated as antimicrobials,[20] as antivirals,[21] and in a variety of human cancers[22] and immunological[22] and neurological[23] diseases, either as potential medicinals or as molecular research tools that potentiate the disease state of interest. Canavanine may be incorporated into proteins in place of arginine, causing misfolding or altered catalytic activity due to its relatively decreased basicity.[24] In contrast, canaline inactivates pyridoxal phosphate-dependent enzymes by reaction with the pyridoxal phosphate carbonyl moiety to form covalently bound oximes.[19] For example, the antimalarial activity of canaline (and other aminooxy compounds) is attributable to inhibition of plasmodial ornithine aminotransferase.[25] The unsaturated aminoalkoxyvinylglycine AVG is also known to bind covalently to the pyridoxal phosphate moiety of enzyme cofactors and mediates developmental effects on plants by inhibition of aminocyclopropane carboxylic acid (ACC) synthase, a component of the ethylene biosynthetic pathway from methionine in plants.[26] Aminooxyacetic acid (AOA) is another plant growth regulator known to inhibit pyridoxal phosphate-dependent enzymes.[27] Commercially available L-AVG hydrochloride (Sigma-Aldrich 359629) and AOA hemihydrochloride (Sigma C13408) were tested in our standard *Poa* seed germination assay described previously[6] and produced complete GAF-like arrest of germination (an assay score of 1.0) at 0.1 and 1 mM, respectively (equivalent to estimated 3.7 μM GAF). Therefore, the observed activity of GAF and synthetic analogues as selective herbicides is being investigated in the context of the inhibition of pyridoxal phosphate-dependent enzymes.

The NFSPRC collection of *Pseudomonas* isolates includes, besides the five GAF-producing *P. fluorescens* isolates originally reported,[6] 20 additional field-collected isolates obtained from the roots of Willamette Valley wheat and grasses and four laboratory strains of *P. fluorescens* (A506, D7, Pf-5, and PfO-1). These isolates and strains have been examined for their production of GAF or related natural products (see Supporting Information Table S13−S14 for the taxonomy and source of all isolates). In each case, culture filtrate preparations from these bacteria were tested for their ability to arrest the germination of the seeds of *P. annua*. Crude 90% ethanolic extracts of the solids recovered from 36 mL samples of these culture filtrates were prepared for TLC analyses (at 20× concentration) and capillary NMR (capNMR) profiling (after concentration to dryness and preparation in D_2O, ~200×). The biological assay results, TLC, and capNMR data were consistent for all extracts in indicating the presence or absence of GAF. By capNMR the deshielded alpha (δ_H 4.34−4.42), olefinic (δ_H 5.37−5.43 and 6.98−7.03), and aldehyde (δ_H 8.19−8.25) ^{1}H signals for GAF were readily observed despite the presence of glucose and phosphate media constituents and the single shim set used for all samples. Notably, in these crude extract spectra the four ^{1}H chemical shifts are deshielded relative to those for purified GAF (δ_H 4.26, 5.22, and 6.96, 7.70 ppm). This is due to the electronic effects (induced dipoles) of counterions supplied by the different salt compositions in the crude and purified materials. The culture filtrate extracts from 22 of the 25 field-collected isolates inhibited *Poa* seed germination at the same level as GAF-producing strain WH6, while the remaining three isolates showed only a slight inhibition of germination at the highest concentration tested. In contrast to these inactive filtrates, extracts of the 22 biologically active filtrates all exhibited characteristic ninhydrin-reactive TLC bands on silica gel and cellulose as well as the four downfield ^{1}H NMR signals for GAF.

Examples of these data are illustrated for GAF-producing strain A3422A and nonproducing strain AH7 in Figure 2 (see Supporting Information for data for all isolates listed in Table S13−S14). None of the extracts of the four laboratory isolates produced a GAF-like effect in the *Poa* assay, or exhibited the characteristic GAF band on TLC, or showed ^{1}H NMR signals for GAF. However, for all four extracts, other ninhydrin-reactive TLC bands and ^{1}H NMR chemical shifts for minor constituents were observed (see Supporting Information). Together with the potential agricultural applications of GAF-related compounds, the variable GAF biosynthetic capabilities of closely related *P. fluorescens* strains has prompted the ongoing identification, cloning, and expression of the biosynthesis genes for GAF, as well as genome sequencing of *Pseudomonas fluorescens* strain WH6. These ongoing genetic studies will be reported in subsequent publications.

Experimental Section

General Experimental Procedures. Deionized water and redistilled EtOH were used in the purfication of **1** and the preparation of extracts for the isolate survey. All NMR data were acquired on a Bruker DRX 300 MHz spectrometer. For purified GAF, a 5 mm BBO NMR probe was used and chemical shifts were referenced to an external sample of DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid; δ_H, δ_C = 0.0 ppm) in D_2O. For the survey of isolate extracts, a Protasis/MRM microflow capillary NMR probe (2.5 μL active coil volume) was used, and ^{1}H NMR spectra acquired in 32−256 scans at 298 K were internally referenced to the residual HDO signal (δ 4.80 ppm). Low-resolution ESI data were acquired on a Finnigan LCQ Classic ion trap mass spectrometer and a Waters Micromass LCT mass spectrometer (TOF-ESI). HRCI+ mass data were acquired on a JEOL MSRoute spectrometer. Optical rotation was measured on a JASCO P-1010 polarimeter.

Extraction and Isolation. *Pseudomonas fluorescens* Biotype C WH6 (deposited as NRRL # B-30485) was cultured in *Pseudomonas* minimal salt (PMS) medium as described previously.[6] Two samples of GAF were prepared consecutively for NMR analysis, the first from 1.2 L and the second from 1.8 L of culture filtrate. In each case the following typical purification procedure entailing preparative TLC and repeated Sephadex G15 column chromatography was followed. The culture filtrate was evaporated to dryness *in vacuo* (≤45 °C) in 150 mL aliquots, and the resulting dry solids were extracted with 90% EtOH (3 × 50 mL per 150 mL culture filtrate aliquot). For storage (4 °C) prior to TLC, the combined and concentrated extracts were resuspended in 76% EtOH to give a GAF solution of 20× concentration relative to the original culture filtrate. TLC purification was carried out on Analtech microcrystalline cellulose TLC plates (20 × 20 cm, 1000 μM thick) prewashed by ascending chromatography, twice in deionized water and once in redistilled 95% ethanol, prior to use. An aliquot (3.2 mL) of

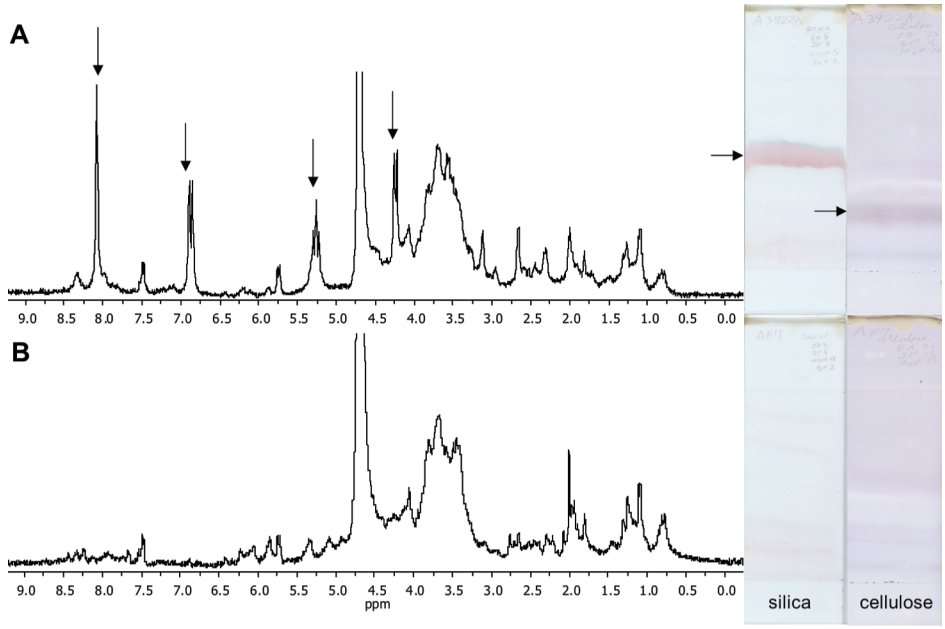

Figure 2. ^{1}H capNMR spectra (D_2O, 256 scans) and corresponding silica and cellulose TLC plates of the crude culture filtrate extracts of two *Pseudomonas fluorescens* isolates. GAF (**1**) is present in isolate A3422A (A), as evidenced by the signals and TLC bands indicated with arrows, but is absent from isolate AH7 (B).

the 20× GAF solution was applied to each prewashed TLC plate using an Analtech TLC sample streaker. The preparative chromatograms were developed in EtOAc−^{i}PrOH−H_2O (5:15:12), dried, and examined under long-wave UV light to visualize the fluorescent bands bounding the GAF-containing zone. The cellulose from each GAF zone was scraped into a 30 mL centrifuge tube and centrifuged in deionized water (16 mL/tube; Sorvall SS-34 rotor, 7500 rpm = 6780*g*) for 10 min. The cellulose pellet was discarded, and the supernatant was filtered through a bacteriological filter (0.2 μm, Acrodisc 25 mm diameter syringe filter, Pall Corporation) into a sterile sample bottle. The resulting sterile aqueous solutions (4× GAF concentration relative to the original culture filtrate) were stored at 4 °C until further processing. Analytical TLC analysis of these 4× solutions demonstrated that they contained a single ninhydrin-reactive compound with the R_f expected for GAF.

The TLC-purified GAF solutions (4×) were evaporated to dryness in 150 mL aliquots, and the recovered solids from each aliquot were dissolved in 6 mL of deionized water (100×) for Sephadex G15 column chromatography. Each 6 mL sample was subjected to repeated open column chromatography on three consecutive Sephadex G15 columns (2.5 × 71 cm, 355 mL bed volumes) in 100% H_2O. After the first column, aliquots of the collected fractions were assayed for Cu^{2+} and Fe^{2+} binding (by Chrome Azurol S assays described below), and fractions with Cu^{2+}-binding activity (containing GAF) were pooled and rechromatographed on two additional consecutive G15 columns. The purified GAF fractions were combined and concentrated to dryness (3.0 and 3.6 mg, respectively, from the 1.2 and 1.8 L culture filtrate preparations) for NMR spectroscopy (D_2O, 450 μL).

Chrome Azurol S Assays of Column Fractions. The Chrome Azurol S reagents were prepared according to Shenker et al.[12] The reagents were composed of 210 μM Crome Azurol S and 200 μM of either $CuSO_4$ or $FeSO_4$, in 40 mM MES buffer. The resulting solutions were adjusted to either pH 5.5 (Cu-CromeAzurol) or 5.7 (Fe-ChromeAzurol) with NaOH. Chome Azurol S and MES [2-(*N*-morpholino)ethanesulfonic acid] were purchased from Sigma. To assay column fractions for metal binding, 75 μL of the appropriate Chrome Azurol reagent was mixed with 75 μL of column fraction in one of the wells of a 96-well microtiter plate (Corning Costar No. 3590). The plates were sealed with Parafilm, and the color changes allowed to develop overnight. Absorbance in the wells was measured at 595 nm in a Bio-Tek EL808 plate reader.

4-Formylaminooxyvinylglycine. (2-Amino-4-formylaminooxy-but-3-enoic acid, GAF, **1**): white amorphous solid; $[\alpha]^{25}_D$ +21.8 (*c* 0.03, EtOH−H_2O, 3:1); NMR data, see Table 1; LRMS, see Supporting Information pp S9−10; HR-CIMS(+) obsd $[M + H]^+$ *m/z* 161.05195 (calcd for $C_5H_9O_2N_4$, 161.05624).

Configuration of GAF (1). Two sets of experiments were performed with either L- or D-amino acid oxidase (AAO) to assay the metabolism of GAF by either enzyme. In each case, four sample treatments incorporating positive and negative controls were set up: (1) GAF-containing WH6 extract, L-AAO (Sigma A9378-5MG, type IV from *Crotalus atrox*) or D-AAO (Sigma A5222-100UN, from porcine kidney), and bovine liver catalase (Sigma C3155-50MG); (2) negative control, GAF-containing WH6 extract, and bovine liver catalase only; (3) positive control for enzyme activity, GAF-containing WH6 extract, L-AAO or D-AAO, L-Phe (with L-AAO) or D-Ala (with D-AAO), and bovine liver catalase; (4) negative control, GAF-containing WH6 extract, L-Phe or D-Ala, and bovine liver catalase only.

In a typical experiment, 8 mL of pH-adjusted GAF solution (~100 μM in H_2O, from a 90% EtOH extract of 330−360 mL of WH6 culture filtrate) was added as appropriate to L-AAO (3.8 units in 0.1 mL) and/or L-Phe (100 μM, 1 mL) plus catalase (80 units, 0.9 mL), or D-AAO (3.8 units, 0.5 mL) and/or D-Ala (100 μM, 1 mL) plus catalase (380 units, 0.5 mL). In experiments in which the AAO or standard amino acid substrate was omitted, an equal volume of H_2O was added for a final volume of 10 mL. Each sample mixture was incubated at 35 °C for 30 h, after which the enzymes were separated from the reaction mixtures by ultrafiltration using Pall Life Sciences Macrosep centrifugal devices (10 000 MWCO, 15 mL size, centrifugation for 2 h at 4000*g*). Each filtrate was concentrated *in vacuo* and redissolved in 76% EtOH at 32× concentration for TLC analysis on Analtech microcrystalline cellulose (EtOAc−PrOH^i−H_2O, 3:6:4) and Analtech GHL silica (EtOAc−PrOH^i−MeOH−H_2O, 5:5:18:2) plates.

USDA-ARS NFSPRC Culture Collection Survey: Taxonomic Identification, Culture, Preparation, and Extraction of Culture Filtrates. All bacterial isolates were cultured on PMS agar slants and submitted to Microcheck Inc. (Northfield, VT) for taxonomic characterization by fatty acid methyl ester (FAME) analysis and 16S rDNA gene sequencing. FAME analysis used a Sherlock Microbial Identification system (Microbial Identification Inc., Newark, DE) for all culturing methods, extraction of fatty acids, GC protocols, and database analyses. An Applied Biosystems' MicroSEQ Microbial Identification system was used for the 16S rDNA gene sequencing. *Pseudomonas* isolates stored in cryovials (50% glycerol, −60 °C) were inoculated (10 μL) into 100 mL Erlenmeyer flasks containing PMS medium (60 mL) and allowed to grow (27 °C, 200 rpm rotary shaker) for 7 days prior to harvest.[28] The cultures were then centrifuged (3000*g*, 15 min), and the supernatant was passed through a bacteriological filter (Millipore GP Express Steritop, 0.22 μM pore size) to give a sterile culture filtrate that was stored at 4 °C until extraction. A sample of the culture filtrate

was taken for bioassay, and a 36 mL aliquot of the remaining filtrate was concentrated *in vacuo* (≤45 °C) and then extracted (3 × 12 mL) with 90% EtOH, which solubilizes ~60% of the GAF present and minimizes the coextraction of culture medium salts and glucose. In each case, the combined EtOH extracts were concentrated to dryness *in vacuo* (≤45 °C) and then redissolved in 76% EtOH to give a 20× solution (1.8 mL), which was stored at 4 °C prior to TLC and chemical analyses (all completed within two weeks of extract preparation).

Biological Assay of Isolate Extracts, Aminoethoxyvinylglycine (AVG) Hydrochloride, and Aminooxyacetic Acid (AOA) Hemihydrochloride. The *Poa* seed germination assay was carried out as described previously.[6] All compounds were tested over a range of concentrations, and for each test concentration, 200 μL of the test solution was added to each of three wells of a sterile tissue-culture plate (Corning Costar 3548 48-well plates). Three surface-sterilized seeds were added to each well. The plates were sealed with Parafilm and incubated (20 °C) for 7 days (8 h light, 50 $\mu mol/m^2/s$; 16 h dark). At 7 days, seed germination was scored using the system described previously.[6] AOA hemihydrochloride was tested at concentrations of 0.03, 0.1, 0.3, 1.0, and 3.0 mM, while AVG hydrochloride also tested at 0.003 and 0.01 mM in addition to the concentrations listed above.

Chemical Profiling of Isolate Extracts. Analytical TLC of each isolate extract was carried out on Avicel microcrystalline cellulose and Avicel silica gel GHL plates (5 × 20 cm, 100 μM thick layers) in EtOAc−^{i}PrOH−H_2O (7.5:15:10) and EtOAc−^{i}PrOH−MeOH−H_2O (5:5:18:2), respectively. Each test extract solution was streaked (using a 50 μL B&B Bang-Levy micropipet) across the cellulose and silica plates (200 μL per plate). The plates were developed over 12 cm, air-dried, and stained with ninhydrin (see Supporting Information), as done previously to visualize GAF in the *P. fluorescens* WH6 extract.

^{1}H NMR spectra with moderate presaturation (zgpr, pl9 = 60 dB) of the HDO signal were acquired for each isolate extract. The remainder of each extract solution (1.4 mL 20× concentration, equivalent to 28 mL culture volume) was concentrated *in vacuo* at 37 °C and placed under high vacuum for up to 24 h before being resuspended in 20−50 μL of D_2O (dissolution not complete in all cases). These extremely concentrated solutions were filtered through a 2 μm Upchurch Scientific Mini Microfilter before 5−10 μL of the resulting filtrate was manually injected through a second inline microfilter into the capillary NMR probe.

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Supporting Information Available: Full experimental and TLC plates for amino acid oxidase experiments. ^{1}H, ^{13}C, COSY, HSQC, HMBC, and 1D selective NOESY NMR spectra in D_2O and ESIMS and tandem MS spectra for 4-formylaminooxyvinylglycine (GAF, **1**). Table of *P. fluorescens* isolates in the USDA-ARS collection. TLC plates and ^{1}H capNMR spectra for USDA-ARS *P. fluorescens* isolates. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Preston, G. M. *Philos. Trans. R. Soc. London, B* **2004**, *359*, 907–918.
(2) Gross, H.; Loper, J. E. *Nat. Prod. Rep.* **2009**, *26*, 1408–1446.
(3) Sands, D. C.; Pilgeram, A. L. *Pest Manag. Sci.* **2009**, *65*, 581–587.
(4) Braun, S. D.; Volksch, B.; Nuske, J.; Spiteller, D. *ChemBioChem* **2008**, *9*, 1913–1920.
(5) Stockwell, V. O.; Stack, J. P. *Phytopathology* **2007**, *97*, 244–249.
(6) Banowetz, G. M.; Azevedo, M. D.; Armstrong, D. J.; Halgren, A. B.; Mills, D. I. *Biol. Control* **2008**, *46*, 380–390.
(7) Banowetz, G. M.; Azevedo, M. D.; Armstrong, D. J.; Mills, D. I. *Biol. Control* **2009**, *50*, 103–110.
(8) Armstrong, D. J.; Azevedo, M. D.; Mills, D. I.; Baileey, B.; Russell, B.; Groenig, A.; Halgren, A. B.; Banowetz, G. M.; McPhail, K. L. *Biol. Control* **2009**, *51*, 181–190.
(9) Pedras, M.; Soledade, C.; Ismail, N.; Quail, J. W.; Boyetchko, S. M. *Phytochemistry* **2003**, *62*, 1105–1114.
(10) Gealy, D. R.; Gurusiddaiah, S.; Ogg, A. G., Jr. *Weed Sci.* **1996**, *44*, 383–392.
(11) Fukuta, M.; Xuan, T. D.; Deba, F.; Tawata, S.; Khanh, T. D.; Chung, I. M. *J. Plant Interact.* **2007**, *2*, 245–251.
(12) Shenker, M.; Chen, Y. *Soil Sci. Soc. Am. J.* **1995**, *59*, 1612–1618.
(13) Pretsch, E.; Buehlmann, P.; Affolter, C. *Structure Determination of Organic Compounds: Tables of Spectral Data*. 3rd ed.; Springer-Verlag: Berlin, 2000.
(14) Berkowitz, D. B.; Charette, B. D.; Karukurichi, K. R.; McFadden, J. M. *Tetrahedron: Asymmetry* **2006**, *17*, 869–882.
(15) Hirata, H.; Kato, A.; Nakatani, S.; Aiba, T.; Oohashi, Y.; Goto, M. (Wakamoto Pharma Co Ltd, Japan). Antibiotics WAP-5044C and WAP-5044A, WAP-5044C derivative, their manufactures with Streptomyces, and fungicides containing them. JP Patent 05105657, April 27, 1993. Scifinder Scholar 1993:537536.
(16) Fernández, M.; Cuadrado, Y.; Aparicio, J. F.; Martín, J. F. *Microbiology* **2004**, *150*, 1467–1474.
(17) Yasuta, T.; Okazaki, S.; Mitsui, H.; Yuhashi, K.-i.; Ezura, H.; Minamisawa, K. *Appl. Environ. Microbiol.* **2001**, *67*, 4999–5009.
(18) Rosenthal, G. A. *Plant Physiol.* **1990**, *94*, 1–3.
(19) Rosenthal, G. A. *Life Sci.* **1997**, *60*, 1635–1641.
(20) Berger, B. J.; English, S.; Chan, G.; Knodel, M. H. *J. Bacteriol.* **2003**, *185*, 2418–2431.
(21) Archard, L. C.; Williamson, J. D. *J. Gen. Virol.* **1974**, *24*, 493–501.
(22) Bence, A. K.; Worthen, D. R.; Adams, V. R.; Crooks, P. A. *Anti-Cancer Drugs* **2002**, *13*, 313–320.
(23) Teichberg, V. I. (Yeda Research and Development Co. Ltd.). Methods and compositions using glutamate-modifying enzymes, cofactors of these enzymes, and glutamate synthesizing enzyme inhibitors for protecting neuronal tissue from damage induced by elevated glutamate levels. US Patent 7767204, August 3, 2010. United States Patent and Trademark Office Web site. http://patft.uspto.gov (accessed Oct 20, 2010).
(24) Bence, A. K.; Crooks, P. A. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 383–394.
(25) Gafa, C.; Wilson, J.; Berger, L. C.; Berger, B. J. *Mol. Biochem. Parasitol.* **2001**, *118*, 1–10.
(26) Adams, D. O.; Yang, S. F. *Proc. Natl. Acad. Sci. U. S. A.* **1979**, *76*, 170–174.
(27) Hoagland, R. E. *Weed Technol.* **1996**, *10*, 651–674.
(28) A 7-day culture period was selected initially to be certain that the cultures had reached and maintained stationary phase for some period of time because peak production of many *Pseudomonas* secondary metabolites occurs in stationary phase.

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