Ribofuranosyl Triazolone: A Natural Product Herbicide with Activity on Adenylosuccinate Synthetase Following Phosphorylation

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2,4-Dihydro-4-(β-D-ribofuranosyl)-1,2,4(3*H*)-triazol-3-one (**2**) was identified as the principal phytotoxic component of a fermentation broth derived from an *Actinomadura*. The compound is a new natural product, but known by synthesis. Broad-spectrum herbicidal activity was demonstrated in greenhouse tests. Metabolite reversal studies suggested the target site was adenylosuccinate synthetase, which was confirmed by direct measurement of the activity of the 5′-phosphorylated derivative on the isolated enzyme.

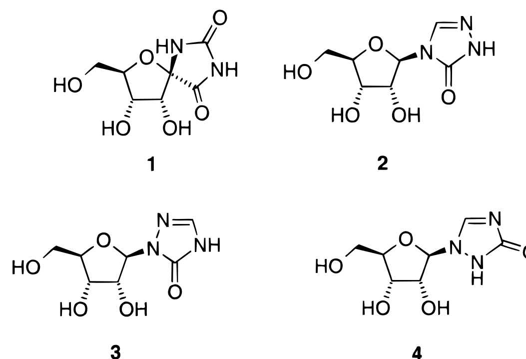
The recent commercialization of sulcotrione, a synthetic optimization of the plant-derived natural product leptospermone, has further validated the importance of natural products as starting points for herbicide discovery.[1] Sulcotrione is unique in combining high levels of activity with a novel mode of action, inhibition of quinone biosynthesis.[2] The large number of patent applications on this and closely related areas of chemistry suggest this family of compounds, known as the triketones, will have a major role in shaping the future of weed-control technology.

Another natural product with excellent herbicidal activity and a target site not represented in commercial herbicides, hydantocidin (**1**), has also been the subject of intense research. This compound was first described from *Streptomyces hygroscopicus* SANK 63584 by Sankyo[3] but has subsequently been identified in a number of different *Streptomyces* strains at Novartis,[4] Mitsubishi,[5] and Dow AgroSciences (unpublished). It has been pursued synthetically by several agricultural chemical companies including Sandoz,[6] Ciba Geigy,[7] and American Cyanamid.[8] The mode of action was examined by three groups, including ours, and found to be inhibition of adenylosuccinate synthetase (AdSS) following bioactivation by phosphorylation of the 2′-hydroxyl group.[9−12] Commercialization of hydantocidin and synthetic analogues is uncertain, with at least part of the barrier being attributed to the cost of synthesis and to the difficulty of identifying active analogues that are more easily accessible.[13] In the course of our screening program for new phytotoxins, we discovered a ribofuranosyl triazolone (**2**) that shares the same target site as hydantocidin, is a new natural product but known by synthesis, is readily approachable synthetically, and also appears bioactivated by phosphorylation of the primary hydroxyl. In this report we describe the discovery of this compound as a natural product and detail its synthesis, herbicidal activity, and mode of action.

Results and Discussion

The organism was identified using PCR amplification of a 357 base pair region of DNA encoding 16s ribosomal RNA and indicated the producing strain closely resembled *Actinomadura madurae.* The *Actinomadurae* are known to produce a range of antibiotics, including specifically the carbazole antibiotics carbazomadurins A and B,[14] an unusual ene-diyne madurapeptin,[15] and a series of xanthone antibiotics, the simaomicins.[16,17] No previous reports of a ribofuranosyl triazolone exist from any biological source.

MS and UV data had suggested that **2** was a nucleoside. This was confirmed by NMR data, which also indicated a β-D-ribofuranose sugar. LC/MS analysis of **2** revealed a $[M + H]^+$ peak at *m/z* 218. Negative ion LC/MS produced the $[M - H]^-$ and $[M + \text{acetate} - H]^-$ adduct ions at *m/z* 216 and 276. LC/MS/MS of the $[M + H]^+$ peak at *m/z* 218 produced a neutral loss of 132 daltons, consistent with loss of a ribose ring. With the ribose ring accounting for 133 amu, a base unit of 84 amu remained. The most likely formula arising from this is $C_2H_2N_3O$, consistent with a triazole ring system. The proton NMR indicated a single C−H bond, implying the existence of a single carbonyl group in the molecule. This molecular formula may be assembled in three different ways: **2**, **3**, and **4**.



Two of these molecules (**2** and **3**) have been described,[18] the third (**4**) is unknown. To distinguish these molecules, the long-range coupling from the anomeric proton to the protonated carbon atom was measured using 1D INEPT giving a value of 4 Hz, thus discounting structure **3** as the identity of the natural product. To distinguish between **2** and **4**, the chemical shift of the protonated carbon atom was predicted (Advanced Chemical Development, Toronto). For **2**, the predicted chemical shift was 134 ppm; whereas, for **4** it was 148 ppm. The actual value of the chemical shift was 136 ppm. Thus, the structure of the natural product was assigned as **2**.

To confirm this structure and to provide more of the compound for biological assessment, **2** was synthesized according to the procedure of Haines et al.[18] In this TMS

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Table 1. Phytotoxicity of Ribofuranosyl Triazolone (**2**) to Several Plant Species in the Greenhouse

		visual injury (%)							
application	rate kg ha^{-1}	HELAN[a]	IPOHE	ECHCG	AVEFA	ABUTH	AMARE	SETFA	ALOMY
postemergence	4.0	95	90	90	60	80	70	85	80
	2.0	95	80	85	70	80	50	60	70
	1.0	100	80	85	60	70	50	70	70
	0.5	90	75	70	50	85	0	50	40
preemergence	4.0	60	40	0	0	65	0	0	40
	2.0	20	40	0	0	30	0	0	35
	1.0	10	25	0	0	30	0	0	30
	0.5	0	15	0	0	15	0	0	0

[a] HELAN, *Helianthus annuus*; IPOHE, *Ipomoea hederacea;* ECHGG, *Echinochloa crusgalli*; AVEFA, *Avena fatua*; ABUTH, *Abutilon theophrasti*; AMARE, *Amaranthus retroflexus*; SETFA, *Setaria faberi*; ALOMY, *Alopecurus myosuroides.*

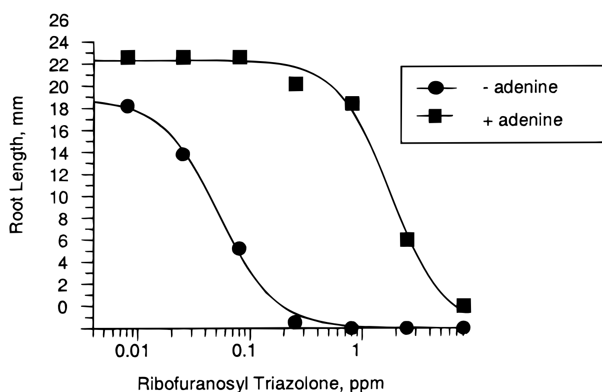

Figure 1. Root-growth inhibition of *Arabidopsis* elicited by ribofuranosyl triazolone (**2**) in the presence and absence of adenine.

procedure, the bis-TMS-protected triazole is coupled with protected α-D-ribofuranose-1-acetate. This reaction gives a number of products, which were separated by liquid chromatography (Scheme 1, Supporting Information). Deprotection of the benzoyl groups furnished the appropriate nucleosides, one of which was shown to be identical with the natural product **2** both by LC and NMR analyses.

The ribofuranosyl triazolone **2** demonstrated broad-spectrum phytotoxicity in the greenhouse, with activity on all test species (Table 1). Symptoms included stunting, systemic chlorosis, and necrosis of apical meristems. In the postemergence test, the ribofuranosyl triazolone had high levels of phytotoxicity, with activity on all species except *Amaranthus retroflexus* at 500 g ha^{-1}, the lowest rate tested. The activity was much less after preemergence applications, most likely due to poor soil stability.

Root growth of *Arabidopsis thaliana* was inhibited by ribofuranosyl triazolone with an I_{50} of 0.04 ppm, or 0.2 μM (Figure 1). When the medium was supplemented with a combination of adenine and guanine, the effect of the herbicide on *Arabidopsis* declined markedly. The I_{50} for the inhibition of *Arabidopsis* roots in the presence of adenine and guanine increased to 2.5 ppm, or 11.5 μM. A test of the individual purines indicated that adenine reversed the effects of the ribofuranosyl triazolone, with an I_{50} of 1.8 ppm, or 8.3 μM (Figure 1), while guanine had no effect (data not shown). Alleviation of the herbicidal activity of the ribofuranosyl triazolone by adenine is the same metabolite reversal reported by Heim et al.[9] for hydantocidin (**1**). Therefore, the reversal data implicated AdSS as a potential target site of **2**.

Direct measurement of the effect of **2** on isolated AdSS isolated from both maize cell culture and rabbit-muscle acetone powder indicated no inhibition (Figure 2). Given the *Arabidopsis* reversal data and the structural similarities of **2** to **1**, the hypothesis was made that **2** required phosphorylation to inhibit AdSS in a manner analogous

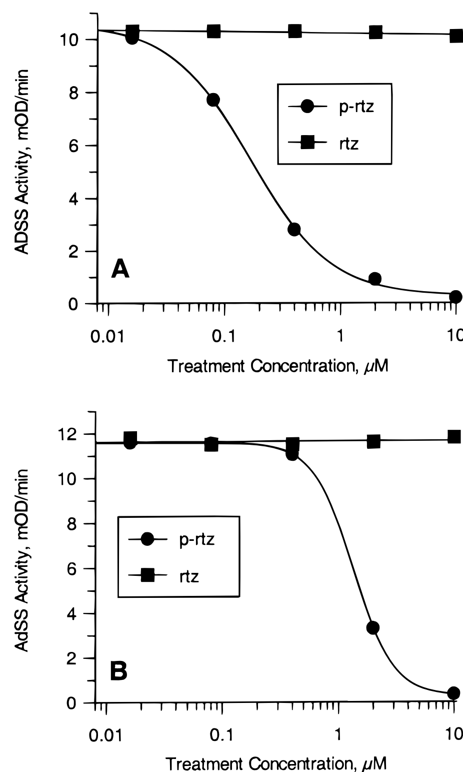

Figure 2. Dose-response curves for ribofuranosyl triazolone (**2**) (rtz) and phosphoribofuranosyl triazolone (p-rtz) on (**A**) mammalian AdSS and (**B**) plant AdSS; reaction time, 5 min.

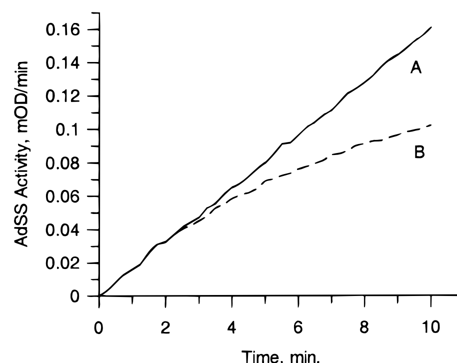

Figure 3. Time course of the plant AdSS reaction (A) without any treatment and (B) with 0.1 ppm phosphoribofuranosyl triazolone (**2**).

to the required bioactivation of **1**. The 5′-phosphate derivative of **2** was prepared synthetically and found to be a potent inhibitor of both mammalian and plant AdSS in a time-dependent manner (Figure 3). The time-dependent nature of the inhibition was also observed in inhibition of AdSS by phosphohydantocidin.[10] Using a linear part of the inhibition curve, the phosphorylated **2** demonstrated I_{50}

values of 0.18 μM and 0.98 μM for rabbit-muscle AdSS and maize cell culture AdSS, respectively (Figure 2).

Ribofuranosyl triazolone (**2**) could represent an important new lead for herbicide discovery. Its synthetic approachability and apparent overlap with the structure–activity relationships governing hydantocidin (**1**) suggest opportunities for further optimization/hybridization of these structures. It is the second reported compound that must be bioactivated by phosphorylation in order to inhibit the activity of AdSS. Compound **2** is a new natural-product phytotoxin that is herbicidal via inhibition of AdSS, a target site not represented among commercial herbicides today.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker DRX400 spectrometer, operating at 400.13 MHz (^{1}H) and 100.62 MHz (^{13}C), and equipped with either a 3-mm micro inverse probe or a 3-mm micro dual probe (Nalorac). Samples were dissolved in 140 μL of D_2O. All spectra were referenced to the residual proton solvent resonance at 4.7 ppm. Carbon data were externally referenced to sodium 3-trimethylsilylpropionate. LC/MS analyses were conducted using a Hewlett-Packard 1050 HPLC coupled to a Finnigan LCQ mass spectrometer. Mass spectra were obtained using both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

Identification of Organism by DNA Sequencing. DNA was isolated from 0.25 mL of liquid vegetative culture using the QIAamp Tissue Kit (Quiagen, Valencia, CA) and eluted in a final volume of 400 μL. The 16s rRNA gene was amplified by PCR using a Gene Amp Kit (PE Applied BioSystems, Foster City, CA). Each 100-μL reaction contained 10 μL of the bacterial DNA and 20 pmol each of the 16s sense (5′>AGAGTTTGATCCTGGCTCAG<3′) and 16s anti-sense (5′>AAGGAGGTGATCCAGCCGCA<3′) primers. Amplification was carried out in a DNA Thermal Cycler 480 (PE Applied Biosystems), after a 5-min soak at 94 °C, 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min, followed by a 7-min extension at 72 °C. The reaction product was purified using Quiagen's QIAquick Kit and eluted in 30 μL of H_2O. DNA-sequencing reactions were carried out using a Perkin-Elmer ABI PRISM Dye Terminator Ready Reaction Kit supplemented with 10% DMSO. The eluted PCR product (1 μL) was sequenced in a 20-μL reaction using 1 pmol of the 519 primer (5′>GWATTACCGCGGCKGCTG<3′). Unincorporated nucleotides were removed using Centriflex Gel Filtration Cartridges (Edge BioSystems, Inc., Gaithersburg, MD) and then analyzed on an Applied Biosystems 373A DNA sequencer.

The resulting sequence was compared with the public database at the National Center for Biotechnology Information using the search tool BLAST 2.0 (Washington, DC). The query sequence consisted of 357 bases. There was no identical sequence in the public database (Genbank Database, May 1998). However, it was similar to members of the genus *Actinomadura* (98% identity). Within the *Actinomadura*, the sequence most closely matched *A. madurae* (Vincent 1894) Lechevalier and Lechevalier 1970 (349 out of 357 bases).

Culture and Fermentation of Organism. The culture (M05053-G8) was isolated from a soil sample according to standard methods[19] and deposited at Dow AgroSciences (9330 Zionsville Road, Indianapolis, IN 46268). The culture was fermented for 72 h in a vegetative medium (50 mL) containing glucose (5.0 g), yeast extract (Difco, 2.5 g), soluble starch (10.0 g), N–Z Amine (casein, 2.5 g), and $CaCO_3$ (0.5 g) per liter of deionized H_2O. Incubation was maintained at 28–30 °C with shaking at 195 rpm for 3 days. This seed inoculum (30 μL) was then added to small rectangular bottles with vented cap closures each containing 14 mL of production medium. The production medium was composed of corn steep powder (5.0 g), dextrose (5 g), lactose (50 g), $CaCO_3$ (3 g), soybean flour nutrisoy (10 g), bacto peptone (5 g), NH_4SO_4 (2 g), $FeCl_2{\cdot}4H_2O$ (0.1 g), $ZnCl_2$ (0.1 g), $MnCl_2{\cdot}4H_2O$ (0.1 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), and hexaglycerol dioleate (15.0 g),per liter of deionized H_2O, and incubated for 8 days at 28–30 °C with shaking at 195 rpm. After 8 days, the culture was harvested and processed as described below.

Isolation and Identification of Ribofuranosyl Triazolone (2). The material from two 13.5-mL fermentations were combined, centrifuged at 10000*g* for 30 min, and the supernatant transferred to a 100-mL separating funnel. The supernatant was extracted three times with an equal volume of *n*-BuOH, and the combined extracts were evaporated *in vacuo*. Compound **2** was isolated by liquid chromatography (Hypercarb S graphite) using 5% aqueous acetonitrile containing 0.05% trifluoroacetic acid, to give an off-white solid (ca. 0.5 mg). Two separate chromatography runs were required to isolate **2** at >98% purity: colorless solid; ^{1}H NMR (D_2O) δ 7.9 (1H, s, H-2), 5.4 (1H, d, H-1′), 4.4 (1H, dd, H-2′), 4.2 (1H, dd, H-3′), 4.0 (1H, ddd, H-4′), 3.7 (H, dd, H-5′a), 3.6 (1H, dd, H-5′b); ^{13}C NMR (D_2O) δ 136 (d, C-2), 86 (d, C-1′), 85 (d, C-4′), 73 (d, C-2′), 70 (d, C-3′), 61 (d, C-5′); positive ion ESIMS *m/z* 218 $[M + H]^+$; negative ion ESIMS *m/z* 216 $[M - H]^-$ 276 $[M + \text{acetate} - H]^-$.

Synthesis of Ribofuranosyl Triazolone (2) and the Corresponding Phosphoribofuranosyl Triazolone. Structure confirmation was achieved by independent synthesis of **2** following the method of Haines et al.[18] (see Schemes 1 and 2, Supporting Information). Experimental variations are noted below.

4-Imidazolin-2-one. Semicarbazide hydrochloride (10 g) was refluxed in an excess of triethyl orthoformate (100 mL) for 2 h. The resultant homogeneous solution was concentrated *in vacuo* to give the desired product, which was used without any further purification.

1-(Trimethylsilyl)-2-[(trimethylsilyl)oxy]imidazole. The reaction was run on 4-imidazolin-2-one prepared above as described by Haines et al.[18] After the solvent was removed *in vacuo*, the residue was sublimed at 60 °C, 0.01 mmHg, to give the title compound as a white solid.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranoyl)-4-triazol-3-one. According to the published procedure,[18] tin tetrachloride was added last over 1 h to a mixture of imidazole and ribofuranose at 0 °C. After 3 h of stirring at 0 °C, the aqueous workup was performed as described. Reversed-phase gradient HPLC (C_{18} fully end-capped ODS) using H_2O and acetonitrile containing 0.05% trifluoroacetic acid, gave five products that were identified by NMR analysis as alkylation on N5 (5%), alkylation on N2 (30%), acylated N2 derivative (15%), the ribose starting material (15%), and bis-ribosylated material (35%).

Deprotection of *O*-Benzoylated Ribonucleosides. Deprotection of both the second and third products by the published procedure of Haines et al.[18] yielded **2**, which was identical in all respects to the material produced by fermentation.

Phosphorylation of 2. To a solution of **2** (8.1 mg, 0.04 mmol) in DMF (125 μL) and THF (1.25 mL) was added tetrazole (7 mg, 0.085 mmol) and dibenzyl diethyl phosphoramidite (32 μL, 0.1 mmol). The reaction was stirred for 4 h and quenched with oxone (93 mg, 0.15 mmol) in H_2O (1 mL). The reaction was stirred for an additional 30 min and partitioned between EtOAc and H_2O. The organic fraction was evaporated and the residue purified by reversed-phase gradient HPLC (C_{18}), using H_2O and acetonitrile containing 0.05% trifluoroacetic acid, to produce the protected phosphate derivative (2 mg, 10%); ESIMS, *m/z* 477.

The protected phosphate (2 mg) was dissolved in EtOH (1.5 mL) and cyclohexene (2.5 mL). After addition of palladium-on-carbon catalyst (1 mg), the reaction was heated to reflux for 5 h. The reaction was filtered, evaporated, and chromatographed by reversed-phase HPLC as described above to produce the desired phosphorylated ribofuranosyl triazolone (ca. 1.0 mg, 76%); ESIMS, *m/z* 297.

Evaluation of Herbicidal Activity. Whole plant activity was assessed on several plant species using foliar-applied (postemergence) and soil-applied (preemergence) applications. Seeds of sunflower (*Helianthus annuus*, HELAN), morningglory (*Ipomoea hederacea*, IPOHE), velvetleaf (*Abutilon theo-*

phrasti, ABUTH), pigweed (*Amaranthus retroflexus*, AMARE), barnyardgrass (*Echinochloa crusgalli*, ECHCG), giant foxtail (*Setaria faberi*, SETFA), wild oats (*Avena fatua*, AVEFA), and blackgrass (*Alopecurus myosuroides*, ALOMY) were planted in mineral soil (sandy clay loam, 51% sand, 26% silt, 23% clay, 2.8% O. M., pH 7.8) and Metro-mix (Bulk Sak, Inc., Malvern, AR) for preemergence and postemergence treatments, respectively. Pots receiving preemergence treatments were watered immediately after application. Prior to postemergence treatment, plants were grown in the greenhouse (16-h photoperiod, 27 °C) and thinned to a density of 2−15 plants per pot, depending on species. At the time of postemergence applications, plants were 8−12 days old, 3−10 cm in height, and in the one- to three-true-leaf stage. Natural light in the greenhouse was supplemented with metal halide lights that provided an average photosynthetic photon flux of 500 μmol m^{-2} s^{-1}.

A sample of ribofuranosyl triazolone was dissolved in 4 mL of distilled H_2O followed by 10 mL of formulation stock "B" [200 mL of 2-propanol, 20 mL of crop oil concentrate, 0.4 g of Triton X-155 (Sigma Chemical Co., St. Louis, MO 62718), and 78 mL of H_2O]. This was followed by three 1:1 (v/v) serial dilutions using formulation stock "C" (100 mL of 2-propanol, 10 mL of crop oil concentrate, 0.2 g of Trition X-155, 390 mL of H_2O, 485 mL of acetone, and 15 mL of DMSO).

Treatments were made at four different doses (4000, 2000, 1000, and 500 g ha^{-1}). For preemergence treatments, 1.75 mL of spray solution was applied to the soil surface in each pot using a glass Cornwall syringe fitted with a Teejet SS8001E flat-fan nozzle (Spraying Systems Co., Wheaton, IL 60189-7900). For postemergence applications, the foliage of the test plants was sprayed using a DeVilbiss atomizer (DeVilbiss Health Care, Inc., Somerset, PA 15501) driven by compressed air at a pressure of 22 kPa. Untreated controls were included.

Pots were placed in the greenhouse after compound application. Pots from postemergence and preemergence treatments were sub-irrigated and top-watered, respectively, as needed for the duration of the test. Visual injury ratings were taken 9 and 16 days after treatment for postemergence and preemergence applications, respectively, on a 0−100 scale, where 0 represents no injury and 100 represents complete necrosis. Results are summarized in Table 1.

Metabolite Reversal Studies. Metabolite reversal studies were conducted on *A. thaliana* as previously described by D. R. Heim et al.[9] with minor changes. The *Arabidopsis* growth medium was supplement with 0.8% sucrose and 0.45% agarose. A 10-mL aliquot of this melted medium, maintained at 62 °C, was used for each treatment. The inhibitors and/or metabolites were added to the medium as concentrated solutions in either H_2O or DMSO. Controls included the appropriate amount of DMSO added without the inhibitor and/or metabolite. The final concentration of DMSO varied but never exceeded 0.8%. We established that DMSO concentrations in this range did not alter *Arabidopsis* growth, development, or response to common herbicidal agents (data not shown). Adenine was added to a final concentration of 185 μM, and guanine was added to a final concentration of 165 μM. After thoroughly mixing, 8 mL of the treated medium was transferred into a 60 × 15-mm sterile Petri dish (Falcon 1007; Becton-Dickinson, Lincoln Park, NJ) and allowed to solidify. Approximately 25 μL of sterilized seed was then applied onto the solidified medium and spread evenly over the plate using the remaining 2 mL of the treatment medium. These plates were incubated for 7 days at 23 °C under continuous fluorescence lighting (50 μE m^{-2} s^{-1}).

Treatment effects were recorded as root measurements. Roots of *Arabidopsis*, when grown as above, characteristically elongate as a single tap root with little or no branching. Root measurements involved carefully extracting individual plants from the medium and measuring the length of the tap root. Each reported value is the average of five such measurements. All values were rounded to the nearest millimeter, and averages are reported as percentages of control. Visual measurements of shoot development were recorded as percentages of control (Figure 1).

Mammalian AdSS Extraction. Rabbit-muscle acetone powder was extracted by gently shaking 3−5 g in 20 mL of AdSS extraction buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 5 mM DTT. This suspension was filtered through four layers of cheesecloth. The residue was rinsed twice with 10 mL of extraction buffer. The combined filtrate was centrifuged for 20 min at 25000*g*. Protamine sulfate (88 μL of a 5% solution) was added to each milliliter of the supernatant while it was stirring at 4 °C. The precipitate was removed by centrifugation for 10 min at 10000*g*. Saturated ammonium sulfate (pH 7.0) was added to the supernatant to 35% saturation and stirred for 30 min at 4 °C. The precipitate was removed by centrifugation for 10 min at 10000*g*. The saturated ammonium sulfate solution was added to the supernatant to 60% saturation and stirred for 30 min at 4 °C. The precipitate was collected by centrifugation at 10000*g* for 10 min. The resulting pellet was resuspended in 8 mL of dialysis buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 0.5 mM DTT and dialyzed overnight against 1 L of the same buffer.

Maize AdSS Extraction. Maize (*Zea mays*) AdSS was extracted from stationary-phase BMS cells (day 7). A 20 mL packed-cell volume was added to a Waring blender with 80 mL of AdSS extraction buffer (see above) and blended on high for 2 min. The ground tissue was filtered through four layers of cheesecloth. The filtrate was centrifuged for 20 min at 25000*g*. Protamine sulfate (88 μL of a 5% solution) was added to each milliliter of the supernatant while it was stirring at 4 °C. The precipitate was removed by centrifugation for 10 min at 10000*g*. Saturated ammonium sulfate (pH 7.0) was added to the supernatant to 35% saturation and stirred for 30 min at 4 °C. The precipitate was removed by centrifugation for 10 min at 10000*g*. The saturated ammonium sulfate solution was added to the supernatant to 60% saturation and stirred for 30 min at 4 °C. The precipitate was collected by centrifugation at 10000*g* for 10 min. The resulting pellet was resuspended in 8 mL of dialysis buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 0.5 mM DTT and dialyzed overnight against 1 L of the same buffer.

The dialyzed sample was centrifuged for 10 min at 10000*g* before being placed on a monoQ 10/10 column that had been equilibrated with 10 mM Tris-HCl, pH 7.4. The column was subjected to a 0−300 mM KCl gradient to remove the AdSS activity. Fractions containing AdSS activity were pooled and concentrated.

AdSS Assay. The AdSS assay was conducted as described by Heim et al.[9] The AdSS reaction mixture contained 50 mM tricine, pH 8.0, 5 mM $MgCl_2$, 0.04 mM GTP, 0.15 mM IMP, 0.04 mM ATP, 0.4 mM phosphoenolpyruvate, 4 U/ mL pyruvate kinase, and enzyme extract. The reaction was initiated with the addition of aspartate (2 mM final concentration). Reaction volumes were 1 mL each. The treatments were diluted into the reaction mixture from concentrated solutions in DMSO. DMSO concentration never exceeded 0.1%. The assays were followed in a Beckman diode-array spectrophotometer at 30 °C. The formation of adenylosuccinate was monitored as a difference in absorbance between 282 and 320 nm. All rates were calculated from the linear part of the reaction curves (Figures 2 and 3).

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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