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ORIGINAL ARTICLE

Albucidin: a novel bleaching herbicide from *Streptomyces albus* subsp. *chlorinus* NRRL B-24108

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A novel nucleoside phytotoxin, albucidin (1), was isolated from the culture broth of *Streptomyces albus* subsp. *chlorinus* NRRL B-24108 using bioassay directed fractionation. The structure of the new natural product, albucidin, was determined by NMR and MS; however, the compound has been reported earlier in the literature following synthetic modification of oxetanocin. This is the first report of herbicidal activity for compounds of this structural type. Albucidin shows high levels of broad spectrum activity following post-emergence applications as well as moderate levels of pre-emergence activity. Accordingly, albucidin could be an important new lead for herbicide discovery.
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INTRODUCTION

Natural products have served as leads or starting points for the development and commercialization of numerous agricultural chemicals. In most cases, synthetic optimization has been necessary to address limitations in potency or field translation, although there are exceptions. For example, the commercial insecticide, spinosyn, is derived directly from the fermentation of *Saccharopolyspora spinosa*.[1] However, both the strobilurin fungicides and the pyrethroid insecticides are the products of synthetic optimization of natural products with poor photostability.[2,3] Particularly significant among herbicides derived from natural products are glufosinate, a phosphorylated amino acid present in the microbial tripeptides, phosalacine and bialaphos, and the triketone herbicides that were derived from the synthetic optimization of leptospermone.[4] In both cases, these herbicides introduced new modes-of-action for commercial weed control, an important success factor for new products. In the course of screening for new natural product herbicides, we identified a novel phytotoxic metabolite, albucidin (**1**), from the culture broth of an actinomycete (Figure 1). This metabolite showed herbicidal activity against a broad spectrum of weeds. The isolation and biological activity of the metabolite as well as physiochemical properties and structural elucidation are provided in this report.

RESULTS

Fermentation and isolation

Streptomyces albus subsp. *chlorinus* NRRL B-24108 was fermented in shake flasks. The production of **1** began at 6 days and increased to a maximum at 12–13 days. Greater than 90% of the phytotoxic activity in shake flask cultures was localized in the broth and could be partitioned from the aqueous broth into butanol. Isolation was achieved by semi-preparative chromatography under isocratic conditions in which the activity eluted at 6.9 min. A strong absorbance was recorded at this retention time with absorbance maxima of 200 and 257 nm. The total recovered yield was approximately 2 mg l^{-1} fermentation. Interestingly, the activity eluted later under neutral pH conditions than under acidic conditions, which gave an early indication of the presence of an amine. The compound was not stable under acidic conditions ($<$50% recovery at pH 2, 24 h; $\sim$100% recovery at pH 6.5, 24 h).

Structural assignment

The ESI LC/MS analysis of **1** indicated a molecular weight of 221, with accurate mass CI giving the molecular ion $[M+H]^+$=222.0988, $C_9H_{12}N_5O_2$ requiring 222.0991. With initial NMR analysis suggesting a nucleoside, ESI LC/MS/MS was then used to probe the nature of the nucleoside base. Non-selective fragmentation of the molecule yielded an ion at *m/z* 136, the MS/MS spectrum of this fragment ion was acquired, and compared with that from standard samples of adenosine and 2-aminopurine riboside. The spectra for **1** and adenosine were nearly identical, whereas those from **1** and 2-aminopurine riboside gave peak intensities that were significantly different. This indicated that the position of the amino group for **1** was located at C-6′.

The NMR analysis of **1** in D_2O exhibited broad signals, but revealed a nucleoside with fewer aliphatic signals than expected for adenosine. An adenine base was suggested by the two downfield signals (between 8.5 and 8.0 p.p.m.), and confirmed as described by MS. The aliphatic protons resonated as broad signals, not allowing the measurement of any coupling constants (Table 1). Analysis of an HMQC experiment

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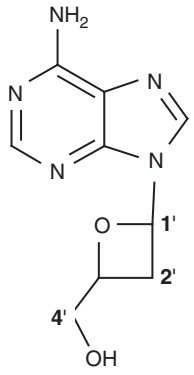


Figure 1 Structure of albucidin (**1**).

Table 1 1H NMR data for 1 and acetylated product 2

Assignment	*1* (D_2O)	*2* (CD_3OD)	*J (for 2)* (Hz)
Adenine-H	8.5	8.6	—
Adenine-H	8.2	8.2	—
1	6.6	6.7	6.9, 6.9
2	3.4	3.5	12.4, 6.9, 6.9
	3.3	3.3	12.4, 6.9, 6.9
3	4.9	5.0	m
4	3.9	4.5	12.8, 5.1
	3.7	4.4	12.8, 2.9
Acetyl Me	—	2.2	—

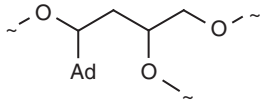
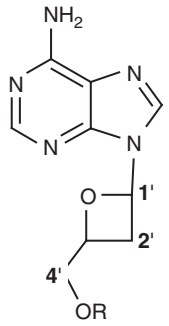


Figure 2 Identified spin system from 2D NMR spectroscopy, Ad=adenine.

indicated the presence of two methylene and two methine groups, only one methylene of which was not substituted with an oxygen atom. The molecular formula of $C_9H_{11}N_5O_2$ allowed for seven unsaturations, six of which were accounted for by the adenosine base, indicating one unsaturation for this portion of the molecule, suggesting the presence of one ring, and thus one hydroxy group. Analysis of the 2D COSY spectrum indicated that the upfield methylene was indeed located between the two methines giving the spin system shown in Figure 2. With only two oxygen atoms accounted for by the molecular formula, the final ring may be assembled through ether linkages to give three possible structures for the molecule—a 3-, 4- or 5-member ring—two of which were described in the literature. Comparison with this literature data suggested that the compound contained an oxetane ring,[5] rather than a furan ring [6] or an epoxide.

Confirmation of this assignment came from the acetylated derivative (**2**), which was readily prepared from **1** with acetic anhydride in pyridine (Figure 3). Comparison of the ^1H NMR spectrum of **2** in $CDCl_3$ indicated a large downfield shift for the terminal methylene protons confirming the site of the hydroxy group in albucidin, and thus the oxetane ring.



1 R=H
2 R=Acetyl

Figure 3 Structure of acetylated albucidin (**2**).

Table 2 Pre-emergence activity of 1[a]

Rate g per ha	*AVEFA*	*ECHCG*	*HELAN*	*IPOHE*
	Visual injury (%)			
2000	80	95	95	85
1000	50	90	90	80
500	45	60	80	80
250	20	20	75	75
125	0	0	75	75
62.5	0	0	65	50

[a]See text for species abbreviations.

Biological activity

Plants emerging from albucidin-treated soil (pre-emergence test) were severely stunted. Broadleaf weeds were more sensitive than grasses and most did not develop beyond the cotyledonary stage. At lower rates, the plants did develop some new growth that was bleached. The pre-emergence activity listed in Table 2 suggests that the mode of action is a metabolic perturbation not limited to bleaching, as most plants did not develop beyond the cotyledonary stage.

The post-emergence activity of albucidin is shown in Table 3. The onset of symptoms after post-emergence application was extremely slow, as only the new growth developed subsequent to the application initially appeared bleached. Most plants continued to grow following treatment. Pre-existing growth on the plants at the time of treatment did not show symptoms until many weeks after treatment. Symptoms progressed in severity with time, and chlorotic new growth ultimately became necrotic. Most plants treated at higher rates (>100 g per ha) died 28–35 days after application.

DISCUSSION

This is the first report of the natural product, albucidin (MW 221.0913; $C_9H_{11}N_5O_2$), isolated from a novel strain of *S. albus* subsp. *chlorinus* (NRRL B-24108). The structure was discovered earlier as a semisynthetic derivative of the natural anti-viral oxetanocin from *Bacillus megaterium*.[5,7] The IC_{50} of 2′-desmethoxy oxetanocin A (**1**) against HIV was approximately 10-fold better than that of oxetanocin A. However, subsequent synthetic efforts indicated that carbocylic analogs of oxetanocin were more potent anti-viral agents than analogs with an oxetanosyl *N*-glycoside;[8–10] therefore, **1** was not investigated further. It is not known whether the anti-viral activity reported for

Table 3 Post-emergent activity of 1, 21-day grading[a]

Conc. (p.p.m.)	220	110	55	27	14
	Visual injury (%)				
GOSHI	70	45	40	25	25
BRSNN	85	80	65	65	45
GLXMA	80	60	60	60	45
BEAVA	85	70	70	70	65
STEME	90	75	70	50	45
XANST	98	90	85	80	80
CHEAL	75	55	65	50	40
IPOHE	85	75	70	70	45
AMARE	90	75	70	50	60
ABUTH	85	80	75	65	55
VIOAR	75	50	50	30	20
POLCO	90	85	75	70	65
ZEAMX	95	75	65	20	0
ORYSA	65	55	40	60	35
TRZAX	88	85	45	20	10
ALOMY	98	75	60	20	0
ECHCG	85	80	70	65	30
DIGSA	90	75	65	65	60
SORVU	95	90	80	60	10
AVEFA	98	90	75	40	35
CYPES	75	65	0	0	0

[a]See text for species abbreviations.

oxetanocin and related compounds is linked to the herbicidal activity discovered for **1**.

Albucidin is a very potent herbicide (lethality at rates less than 100 g per ha in some species) that induces chlorosis and bleaching in many grass and broadleaf weeds. Although potent, the onset of symptoms after post-emergence application is very slow and effects are seen principally in new growth. The mode of action has not been pursued, but the speed of action and progression of symptoms are relatively unique and suggest that the mode of action may be new. It seems possible that the slow onset of symptoms from **1** reflects a requirement for *in planta* activation. Hydantocidin, a nucleoside-type phytotoxin, is phosphorylated *in planta* to the active inhibitor of adenylosuccinate synthase.[11] Whether phosphorylation resulting in bioactivation of albucidin may similarly occur is unknown and under investigation.

EXPERIMENTAL SECTION

Taxonomy of the producing organism

The albucidin producing organism, strain LW030448, was isolated from soil at Lilly Research Laboratories in Indianapolis, IN, USA. Strain LW030448 produced well-branched vegetative mycelia and aerial hyphae, which were flexous or loose spirals. The spores were round with a smooth surface (Figure 4). Substrate mycelium was yellow to brown and aerial mycelial color was gray to green-yellow. The strain could use adonitol, inositol, mannitol and xylose for growth and could not grow at temperatures below 25 °C. The partial 16S rDNA sequence (accession number DQ069278) as well as morphological and growth characteristics of strain LW030448 were highly similar to *S. albus* ATCC 3004. Therefore, strain LW030448 was identified as a unique subspecies, *S. albus* subsp. *chlorinus*, distinguishable by a characteristic brilliant green-yellow pigment produced in the aerial mycelium on Oatmeal Agar (ISP-3). Strain LW030448 has been deposited in the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research, Peoria, Illinois, USA under the accession number NRRL B-24108.

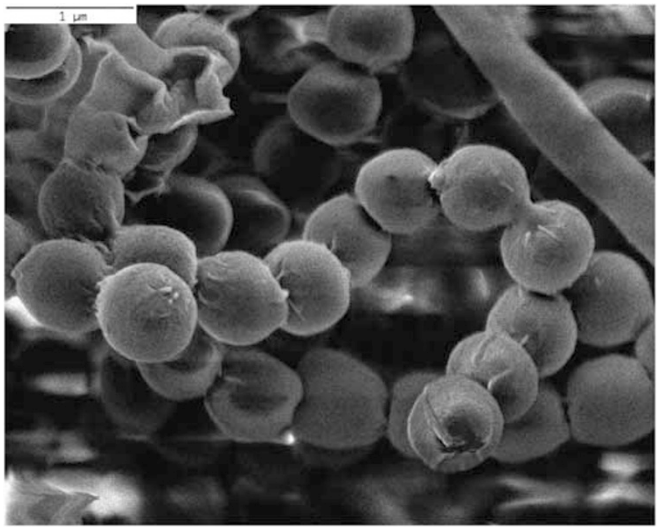


Figure 4 Scanning electron micrograph of *Streptomyces albus* subsp. *chlorinus* LW030448.

Fermentation and isolation

Strain LW030448 was fermented in Medium G (500 ml per 2.8 l-baffled Fernbach flask (Bellco, Vineland, NJ, USA)). Medium G contained soybean flour Nutrisoy (ADM, Decatur, IL, USA) 5 g, dextrose 10 g, glycerin 10 g, starch solubles 5 g, potato dextrin 20 g, corn steep solids 5 g, $CaCO_3$ 3 g, phytic acid 1 g, cane molasses 10 g, $FeCl_2 \bullet 4\ H_2O$ 0.1 g, $ZnCl_2$ 0.1 g, $MnCl_2 \bullet 4\ H_2O$ 0.1 g and $MgSO_4 \bullet 7\ H_2O$ 0.5 g in 1 l of deionized water; pH 7.0. The flasks were inoculated with plugs from freshly sporulated agar culture and incubated at 29 °C, 150 r.p.m. for 13 days. Fermentation broth (50 l) was separated from the mycelium by centrifugation and the phytotoxic activity partitioned from the aqueous broth into butanol (150 l).

Isolation was achieved by re-dissolving the dried butanol extract in 25% aqueous acetonitrile and fractionating by semi-preparative chromatography on reversed-phase C-18 (column 10 mm×25 cm, 5 µm particle size, flow rate 5 ml min^{-1}) under isocratic conditions of 6% acetonitrile in water at pH 6.5. The active metabolite eluted at a retention time of 6.9 min based on bioassay against *Echinochloa crus-galli* and *Helianthus annuus* using procedures described earlier.[12]

NMR sectroscopy

The NMR spectra were acquired on a Bruker 400 MHz spectrometer (Bruker BioSpin, Billerica, MA, USA), operating at 400.13 MHz. The sample was dissolved in 0.25 ml of D_2O, and placed in a 5 mm Shigemi micro-tube (Shigemi Inc, Allison Park, PA, USA). For the 1H/1H COSY, 256 experiments consisting of 1 K data points were collected with 96 transients collected per experiment. ^{13}C data were acquired using a 2D inverse-detected HMQC experiment. For this data set, 256 experiments were acquired over 2 K data points, with 160 transients per experiment.

Mass spectrometry

The LC/MS analysis was carried out using a Finnigan TSQ-700 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA, USA) equipped with both electrospray (ESI) and atmospheric pressure chemical oxidation (APCI) interfaces. Gradient high-performance liquid chromatography separations were accomplished on a Kromacil C-18 4.5×250 mm column (Eka Chemicals Separation Products, Bohus, Sweden), with simultaneous UV (254 nm) and mass spectral detection (positive ESI and APCI in both MS and MS/MS modes).

Accurate mass analyses were carried out using a Finnigan MAT-95q magnetic sector mass spectrometer (Finnigan-MAT) using both electron ionization, and methane chemical ionization. Samples were introduced through the direct exposure probe, and perfluorokerosene was used as an internal standard.

Acetylation of albucidin

A very small sample (<1 mg) of **1** was dissolved in pyridine (0.25 ml) and acetic anhydride (0.1 ml). The solution was stirred overnight and the excess solvents were removed by evaporation. Purification by high-performance liquid chromatography yielded the monoacetate of albucidin (**2**); MS (+ve ESI); 264 (100%, M+), 136 (40%): NMR, see Table 1.

Biological activity

Pre-emergence and post-emergent herbicide tests were conducted in a manner similar to that described earlier.[13] Whole plant activity was assessed on the following species using one or both of the above means of application: *Gossypium hirsutum* (GOSHI), *Brassica napus* (BRSNN), *Glycine max* (GLXMA), *Beta vulgaris* (BEAVA), *Stellaria media* (STEME), *Xanthium strumarium* (XANST), *Chenopodium album* (CHEAL), *Ipomoea hederacea* (IPOHE), *Amaranthum retroflexus* (AMARE), *Abutilon theophrasti* (ABUTH), *Viola arvensis* (VIOAR), *Polygonum convolvulus* (POLCO), *Zea mays* (ZEAMX), *Orysa sativa* (ORYSA), *Triticum aestivum* (TRZAX), *Alopecurus myosuroides* (ALOMY), *E. crus-galli* (ECHCG), *Digitaria sanguinalis* (DIGSA), *Sorghum vulgare* (SORVU), *Avena fatua* (AVEFA), *Cyperus esculentus* (CYPES) and *H. annuus* (HELAN). Plants were seeded in sandy loam soil (pre-emergence testing) or a commercial potting mix containing 30% organic matter (post-emergence testing). For post-emergence applications, each pot was thinned to 2–25 plants per pot, depending on species. Plants were grown to a height of 3–10 cm before application. Albucidin was dissolved in methanol and diluted with 25% aqueous acetone containing 0.05% non-ionic surfactant. Serial dilutions were carried out in a solvent mixture containing 25% acetone and 0.05% non-ionic surfactant to achieve the desired p.p.m. concentrations. Each spray concentration was applied with an atomizer driven by compressed air at a pressure of 22 kPa, and approximately 1.5 ml of total solution was applied to each pot. The plants were returned to a greenhouse (16-h photoperiod, 27 °C day, 24 °C night) for the duration of the study and watered by sub-irrigation. After 21 days, the treated plants were compared with untreated controls and graded on a scale of 0–100, where 0 represented no effect and 100 indicated complete plant death.

For pre-emergence tests, albucidin was dissolved and diluted as described above and applied to the surface of pots seeded with the desired test species. Applications were made with a hand-held syringe equipped with a hollow cone nozzle to distribute the spray solution (2.5 ml per pot) as a course mist. The pots were watered manually to move the chemical into the soil and initiate seed germination. The pots were returned to the greenhouse (16-h photoperiod, 27 °C day, 24 °C night) for the duration of the study and top-watered daily. After 21 days, the emergence and height of plants from treated pots were compared with those of untreated controls on a scale of 0–100 as described above.

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