HERBOXIDIENE, A NEW HERBICIDAL SUBSTANCE FROM *Streptomyces chromofuscus* A7847

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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Screening of microbial fermentation broths for herbicidal activity led to the discovery of a novel polyketide, herboxidiene, from an actinomycete identified as a member of the *Streptomyces chromofuscus* cluster. A 14- to 20-fold increase in fermentation production of herboxidiene was achieved as a result of media optimization. Herboxidiene was purified using successive reverse phase C18 steps and Sephadex LH-20 chromatography. Its molecular formula, $C_{25}H_{42}O_6$, was determined by HRFAB-MS. Herboxidiene demonstrated exceptionally potent, selective, herbicidal activity against a variety of weed species and was inactive against wheat, even at rates as high as 5.6 kg/hectare.

During the course of screening fermentation broths for herbicidal activity, a novel polyketide, herboxidiene, was discovered (Fig. 1) which possesses activity against several annual weed species at use rates comparable with commercial herbicides. In this paper, we describe the isolation, taxonomy and fermentation of the herboxidiene-producing organism, together with the purification, physico-chemical and biological properties of the compound.

Materials and Methods

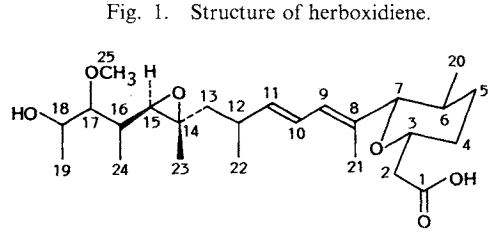
Isolation of Organism

Strain A7847 was isolated from soil collected from a stand of mixed woods from the Stepping Stone Falls Beach Pond State Park, Rhode Island, U.S.A. Baiting techniques as described by COUCH[1] were used. After 7 days incubation, bahia grass baits were examined microscopically for the presence of white, glistening sporangia. Baits with sporangia were transferred to plates which had been overlaid with autoclaved, cellulose ester filter membranes according to the method of HIRSCH and CHRISTENSEN[2]. After 7 days incubation, the filter paper and baits were removed under sterile conditions. Separate, distinct actinomycete colonies, relatively free of contamination, were visible on the plates, and were aseptically transferred to growth medium to obtain pure cultures. These cultures were stored in the vapor phase of liquid nitrogen, as a suspension of spores and mycelia in Nutrient broth (Difco) plus 10% (w/v) glycerol.

Taxonomical Examination

Morphological, cultural and physiological properties of strain A7847 were examined according to the methods described by SHIRLING and GOTTLIEB[3], and WILLIAMS *et al.*[4]. Detailed observation of mycelial and spore morphologies was performed using a long working distance objective on a light microscope (Dialux 20, Leitz) and a

Fig. 1. Structure of herboxidiene.



scanning electron microscope (840, Jeol). Detection of diaminopimelic acid in the cell wall and whole cell sugars was performed using the methods of HASEGAWA *et al.*[5], and SCHAAL[6], respectively.

Medium Optimization

Strain A7847 was grown using different combinations of carbon and nitrogen sources in order to assess the production of the herbicidally active metabolite. The base medium contained $MgSO_4 \cdot 7H_2O$ 0.1%, KH_2PO_4 0.2%, KNO_3 0.2%, NaCl 0.05%, $CaCO_3$ 0.15%, $ZnSO_4 \cdot 7H_2O$ 0.0001%, $FeSO_4 \cdot 7H_2O$ 0.0001%, pH 6.5. Carbon sources (corn starch, cotton seed oil and glucose) were used at 1.5%. Nitrogen sources (soybean flour, casein, Baker's yeast, yeast extract and ProFlo (cotton seed flour from Trader Proteins, U.S.A.)) were used at 1.0%. The medium (No. 32) used in the original fermentation of strain A7847 consisted of glucose 0.05%, dextrin 0.25%, cotton seed oil 0.5%, yeast extract 0.05%, ProFlo 0.25%, soybean flour 0.5%, $MgHPO_4 \cdot 7H_2O$ 0.05%, $ZnSO_4 \cdot 7H_2O$ 0.25%, pH 7. A response surface analysis, RS/DISCOVER (BBN Software Product Corp., U.S.A.) was used to determine the optimum combination of carbon and nitrogen sources for maximum production of herboxidiene. A central composite design with a quadratic model was used to design the experiment, and analyze the data. All experiments were carried out in test tubes (20 × 150 mm) containing 5 ml of medium, shaken at 250 rpm, 30°C for 5 days. The culture filtrates were extracted with *n*-butanol and tested against *Arabidopsis thaliana* by an agar diffusion method[7] and analyzed by HPLC (see analytical procedures). Strain A7847 was then grown under the same conditions in two 250-ml Erlenmeyer shake flasks one containing 50 ml of the optimized medium and the other containing 50 ml of medium No. 32 as a control. A comparision of herboxidiene production, using HPLC methods, was made.

Fermentation

In order to provide sufficient herboxidiene for structure elucidation, strain A7847 was grown in a 2-liter fermenter (Braun Biostat M). Fermentation inoculum was prepared by introducing the contents of one culture vial into a 250-ml flask containing 50 ml of seed medium composed of yeast extract 0.3% and Tryptone 0.5% (Difco). After incubation at 30°C for three days on a rotary shaker at 250 rpm, the resulting cell culture was inoculated into the 2-liter fermenter containing 1.5 liters of production medium. The production medium consisted of corn starch 3.5%, ProFlo 0.8%, $MgSO_4 \cdot 7H_2O$ 0.1%, KH_2PO_4 0.2%, KNO_3 0.2%, NaCl 0.05%, $CaCO_3$ 0.015%, $ZnSO_4 \cdot 7H_2O$ 0.001% and Fe-EDTA 0.018%. The pH of the medium was adjusted to 7.0 before autoclaving. The fermentation was performed at 500 rpm agitation, 25°C, 1 liter per minute aeration. Macol P2000 0.05% v/v (Mazer Chemicals) was used to control foaming. Fermentation production of herboxidiene was monitored daily. The culture was harvested after five days. Large scale production of herboxidiene for advanced biological testing was performed in a 150-liter fermenter (Chemap). Fermentation inoculum was prepared by introducing one culture vial into a two-liter Fernbatch flask which contained one liter of seed medium. After incubation at 30°C for three days on a rotary shaker (150 rpm), the resulting cell culture was inoculated into a 150-liter fermenter (Chemap) containing 100 liters of the production medium. The fermentation was performed at 400 rpm agitation, 30°C, 350 g/cm^2, 50 liters per minute aeration. Macol P2000 0.05% v/v (Mazer Chemicals) was used to control foaming. The fermentation broth was harvested after 96 hours.

Analytical Procedures

Fermentation production of herboxidiene at the 100-liter scale was monitored daily. Broth samples were centrifuged and filtered through a 0.45-μm membrane filter. The filtrates were extracted with equal volumes of *n*-butanol. The resulting *n*-butanol extracts were dried under a stream of nitrogen gas and resuspended in the HPLC solvent (87% methanol in water) at a 10x concentration. Samples were analyzed by C18 reverse phase HPLC (Waters/Phenomenex SP/1067C) employing 87% methanol in water as a mobile phase with a flow rate of 3 ml/minute. Herboxidiene (Rt 6.2 minutes) was monitored by its UV absorption at 236 nm. TLC analysis during isolation was carried out on Kieselgel 60 F_{254} plates (Art. No. 5719, Merck) utilizing $CHCl_3$ - MeOH (9 : 1) as the developing solvent. The Rf value, as determined by UV and *p*-anisaldehyde reaction, was 0.43.

Physico-chemical Characteristics: The UV spectrum was recorded on a Hewlett-Packard 8450A UV/VIS spectrophotometer. The CD spectrum was obtained on a Jasco J-500C spectropolarimeter. The FTIR

spectrum was recorded on a Nicolet 170SX spectrometer, using a Spectra Tech microscope. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 or 500 MHz spectrometer in CD_3OD. The FAB-mass spectrum was obtained on a VG ZAB-HF spectrometer using a glycerol matrix.

Biological Assays

Bacterial and Fungal Assays: *Bacillus subtilis*, *Escherichia coli* ATCC 9723, and *Micrococcus luteus* ATCC 9341 were used in antibacterial screening and were grown at 30°C as a lawn on the surface of Nutrient agar (Difco) plates for two days. *Candida albicans* ATCC 10231, and *Saccharomyces cerevisiae* ATCC 2366, were used in antifungal screening and were incorporated into Vogel Salts medium[8] agar plates for two days at 25°C. *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin Race 4 zoospores and *Pyricularia grisea* (Cooke) Sacc. conidia were grown at 20°C for five days as a lawn on the surface of V8 agar[9] plates for antifungal screening. Similarly, *Gaeumannomyces graminis* (Sacc.) Arx. and Oliver var. *tritici* mycelial macerate was grown as a lawn on quarter strength Potato dextrose agar (Difco) plates. In all cases, a well was cut in the agar using a sterile No. 6 steel cork borer to which 100 μl of sample was added. Assay activity was measured by zones of inhibition.

Whole Plant Greenhouse Assay: A Dupo silt loam soil containing less than 2% organic matter was placed to a depth of 10 cm in an aluminum pan and compacted with furrows to a depth of approximately 1.27 cm from the top of the pan. Seeds of maize, rice, soybeans, annual morning glory, wheat, oilseed rape, wild buckwheat and hemp sesbania were placed in furrows and covered with soil. All pans were watered by subirrigation only. A known amount of herboxidiene was dissolved in 100% acetone such that a 1% solution was obtained. Prior to chemical application, a desired amount of the 1% solution was formulated as an acetone-water (50:50) mixture with 0.1% v/v AG-98 surfactant. When plants reached the 1 to 3 true leaf growth stage, the chemical was applied to the foliage at 5.592, 1.118, 0.279, 0.069, and 0.017 kg/hectare rates. The pan was placed in a greenhouse maintained at day/night temperatures of 30/21°C. At 27 to 30 days after treatment, all pans were rated visually against an untreated control with 0 and 100% representing no injury and complete plant death, respectively.

Results and Discussion

Taxonomy of Strain A7847

Cultural and carbon/nitrogen utilization characteristics of strain A7847 are summarized in Table 1.

Table 1. Cultural and carbon/nitrogen utilization features of strain A7847.

Cultural characteristics:

Media*	Growth	Aerial mycelia	Soluble pigment	Colony reverse**
ISP No. 2	Excellent	Good (white-yellow)	Greenish-yellow	Yellow (95.m.O1.Br)
ISP No. 5	Good	Good (white-yellow)	—	Yellow (95.m.O1.Br)
ISP No. 6	Excellent	Excellent (white-pale yellow)	Brownish	Yellow (95.m.O1.Br)
OYEA	Excellent	Good (white-yellow)	—	Yellow (91.d.gy.Y)
MYA	Good	Poor (white)	—	Yellow (88.d.Y)
BENNETT'S	Good	Scanty (white)	Greenish-yellow	Yellow (95.m.O1.Br)

Carbon/nitrogen utilization:

Carbon sources	D-Glucose, α-lactose, L-arabinose, L-phenylalanine, adonitol, D-galactose, L-tyrosine, salicin, α-(+)-melibiose, (+)-mannose, D-fructose, D-mannitol, D-ribose, (+)-rhamnose, (+)-maltose, L-threonine, L-serine, L-histidine, sucrose, (+)-xylose, *myo*-inositol, dulcitol, L-glutamic acid, (+)-cellobiose
Nitrogen sources	DL-α-Amino-*n*-butyric acid, hydroxy-L-proline
Carbon sources not utilized	L-Valine, D-raffinose, D-xylitol, L-sorbose, L-cystine, inulin, hydroxy-L-proline, DL-α-amino-*n*-butyric acid

* ISP No. 2: yeast extract-malt extract agar; ISP No. 5: glycerol-asparagine agar; ISP No. 6: tyrosine agar; OYEA: oatmeal-yeast extract agar; MYA: malt-yeast extract-peptone agar; BENNETT'S: yeast extract-beef extract agar.

** Colors were recorded as described by the Inter-Society Color Council, National Bureau of Standards.

Table 2. Physiological characteristics of strain A7847.

H_2S production	−
Nitrate reduction	−
Melanin production	−
Degradation of xanthine	−
pectin	+
arbutin	+
allantoin	+
lecithin	+
Growth in the presence of NaCl (7% w/v)	−
sodium azide (0.01%)	−
phenol (0.1%)	−
Resistance to rifampicin	−
neomycin	−
Antibiosis to	
Streptomyces murinus (ATCC 19788)	−
Aspergillus niger (ATCC 36233)	−
Bacillus subtilis (ATCC 6633)	−
Growth at temperature 37°C	−
25~30°C	+
Growth at pH 6.0~9.0	+
10.0	−

Table 3. Effect of carbon and nitrogen sources on the production of herboxidiene by strain A7847.

Carbon source (1.5%)	Nitrogen source (1.0%)	Activity vs. *A. thaliana**
Corn starch	Soybean flour	3
	Casein	2
	Baker's yeast	1
	ProFlo	4
	Yeast extract	1
Cotton seed oil	Soybean flour	0
	Casein	0
	Baker's yeast	0
	ProFlo	0
	Yeast extract	0
Glucose	Soybean flour	3
	Casein	0
	Baker's yeast	0
	ProFlo	0
	Yeast extract	0

* Activity was rated from 0 to 4; 0 is no activity, and 4 is complete inhibition of germination.

Observations by light microscopy revealed well branched substrate and aerial mycelia. No fragmentation was observed on the substrate mycelia. Aerial hyphae bore long chains of arthrospores forming simple spirals (S)[3]. Spores were short, bacilli shaped, and $0.8 \sim 1.0 \times 0.7 \sim 0.9\,\mu m$ in size. Scanning microscopy demonstrated smooth spore ornamentation. Aerial mass color of the strain was white-yellow to white depending on the medium. The reverse side color of the colonies was yellow. Diffusible pigment was formed on ISP No. 2, tyrosine and BENNETT's media. The whole cell acid hydrolysate contained LL-diaminopimelic acid. Whole cell sugars were found to be ribose and rhamnose. No diagnostic sugars were detected. Based on these data, strain A7847 was classified as wall type I as described by LECHEVALIER and LECHEVALIER[10]. Physiological characteristics of strain A7847 are summarized in Table 2. There was no melanin production, growth at 7% NaCl, or growth at 37°C.

Based on morphological and chemotaxonomic characteristics, strain A7847 was determined to belong in the genus *Streptomyces*. Studies of the spore surface ornamentation, carbon utilization and physiological characteristics suggest that it is a member of the *Streptomyces chromofuscus* cluster as described in BERGEY's Manual of Systematic Bacteriology[11].

Media Optimization

Originally, *S. chromofuscus* A7847 was grown in medium No. 32 and produced approximately 2~5 mg/liter of herboxidiene. The combination of corn starch and ProFlo proved to be optimal for production of herboxidiene (Table 3) and was used in a response surface analysis. Results provided in Fig. 2 indicate that maximum product formation was obtained with high corn starch levels and low ProFlo levels. Increases in corn starch concentration beyond those shown in Fig. 2 (>3.5%) did not result in increased production. The optimum combination of carbon and nitrogen sources for maximum cell growth differed from that for maximum product formation. The optimized medium for production of herboxidiene (3.5% corn starch and 0.8% ProFlo), was used in all subsequent large scale fermentations. *S. chromofuscus* A7847 produced approximately 40~70 mg/liter of herboxidiene when grown in this medium in a shaken flask. This represents a 14- to 20-fold increase in production compared to medium No. 32.

Fig. 2. Effect of corn starch and ProFlo on the growth and production of herboxidiene by strain A7847.
Solid line: herboxidiene, mg/liter, dash line: cell wet weight, mg/ml.

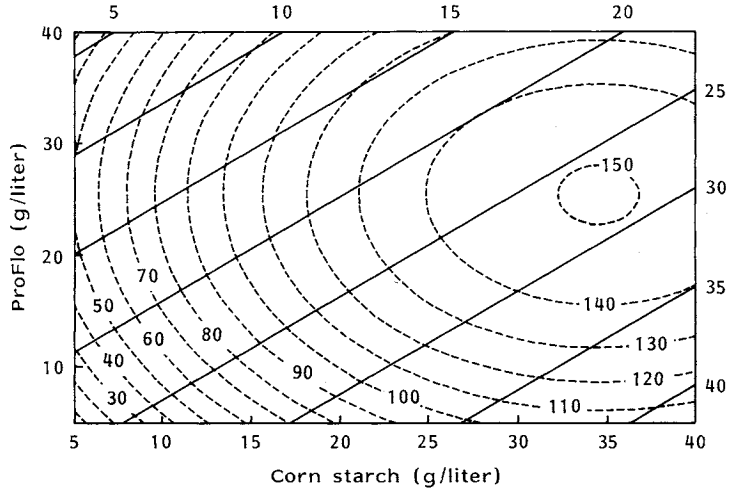
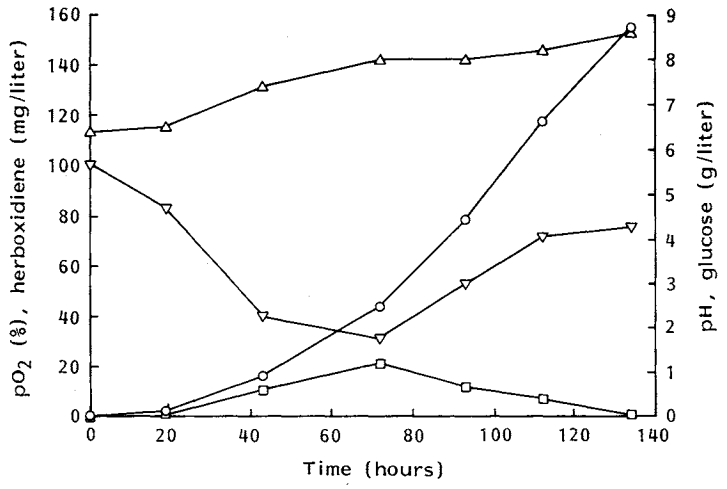


Fig. 3. Time course of herboxidiene production and other growth parameters of strain A7847 in 2-liter fermenter.

○ Herboxidiene, △ pH, ▽ pO_2, □ glucose.



Fermentation

The data presented in Fig. 3 indicate that the synthesis of herboxidiene by *S. chromofuscus* A7847 began after approximately 20 hours of growth. Levels continued to increase with time until harvesting. The majority of herboxidiene was produced when the level of dissolved oxygen in the medium began to increase, an indication that cell growth was entering stationary phase. The medium became basic (pH > 8.0) after four days. The level of glucose resulting from starch hydrolysis was low throughout the fermentation, and was essentially zero after five days. When grown in optimized medium in a 2-liter fermenter, *S. chromofuscus* A7847 produced 155 mg/liter of herboxidiene after five days as estimated by HPLC analysis.

Table 4. Post-emergence, whole plant activity of herboxidiene.

Rate (kg/hectare)	% Inhibition							
	Soybean	Rape	Wild buckwheat	Morning glory	Wheat	Rice	Maize	Hemp sesbania
5.592	100	100	100	100	0	95	100	100
1.118	75	100	100	100	0	75	100	100
0.279	60	100	98	99	0	25	100	99
0.069	20	98	98	99	0	10	90	90
0.017	10	75	75	20	0	10	50	75

Biological Properties

Herboxidiene was inactive against the fungi, *G. graminis* var. *tritici*, *P. grisea*, *C. albicans* and *S. cerevisiae*. Slight activity against *P. megasperma* var. *glycine* was noted. The growth of bacterial strains, *B. subtilis*, *E. coli* and *M. luteus*, was not affected by herboxidiene.

The herbicidal activity of herboxidiene was at commercial levels for many of the monocotyledonous and dicotyledonous species tested (Table 4). Of particular significance is the selective activity of this compound against weeds in wheat. Rape, wild buckwheat, morning glory, maize and hemp sesbania were effectively controlled at a rate of 0.069 kg/hectare while rice, soybean and wheat exhibited little to no phytotoxicity.

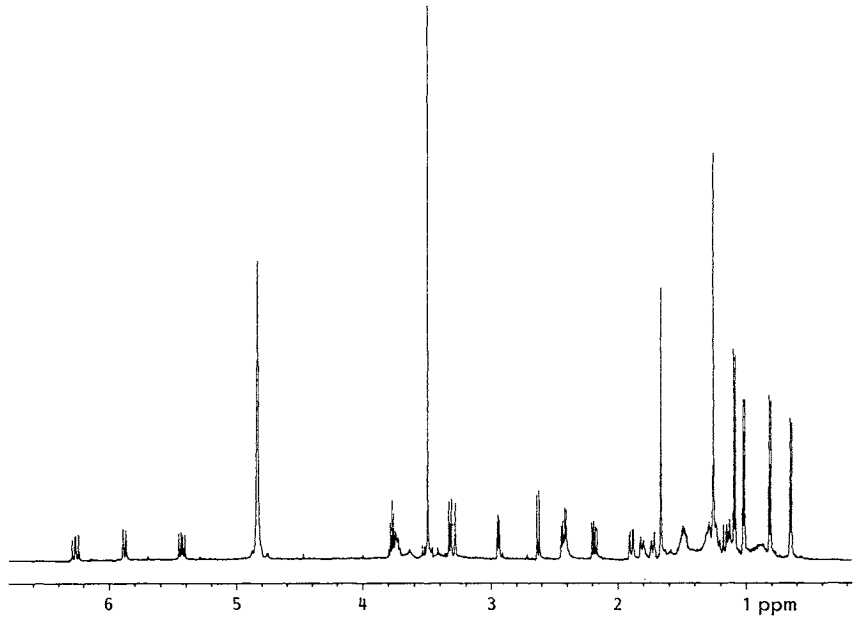
Isolation

The culture filtrate from a 100-liter fermentation was concentrated by lyophilization to 8.7 liters and extracted with an equal volume of *n*-butanol. The organic solvent was concentrated *in vacuo* and chromatographed in two successive steps by reverse phase (C18) flash chromatography (RPFC) using a 10% step gradient from 100% water to 100% methanol. The first column (500 ml) employed multiple application of the crude *n*-butanol extract and batch elution. As revealed by TLC, herboxidiene eluted with 60:40 and 50:50 water-methanol to give approximately 8.5 g of crude active material which was further purified by RPFC (5 × 20 cm, 10 ml fractions). Herboxidiene containing fractions (Nos. 100~195, 4.1 g) were further chromatographed on a Sephadex LH-20 column (2.5 × 83 cm, 5 ml fractions), eluting with water-methanol (3:1). Fractions 63~86, which contained herboxidiene as shown by TLC, were combined (2.8 g) and dissolved in methanol (50 ml), to which was added neutral decolorizing carbon (5.6 g). Following filtration through Celite, the carbon effluent plus methanol washes (800 ml) were concentrated *in vacuo* to give 2.29 g of pure herboxidiene as an off-white powder.

Physico-chemical Properties

Herboxidiene is soluble in water, methanol, *n*-butanol, acetone, and ethyl acetate, but insoluble in hexane: UV (MeOH) λ_{max} 238 nm (ε 21,060); CD (*c* 0.4 mg/ml, CH_3OH): $[\theta]_{199\,nm}$ −23,021 cm^2/dM, $[\theta]_{236\,nm}$ +24,597 cm^2/dM; IR (neat) ν_{max} 1650, 1570, 1450, 1400 cm^{-1}. The molecular formula of herboxidiene, $C_{25}H_{42}O_6$ (MW 438), was determined by HRFAB-MS ($(M-H)^-$ *m/z* 437.2902 for $C_{25}H_{41}O_6$, Δ +0.1 mmu). The ^1H NMR spectrum of herboxidiene is shown in Fig. 4. The ^{13}C NMR spectrum (CD_3OD, 75 MHz) displayed signals at δ 179.77, 140.46, 136.52, 129.51, 126.59, 92.18, 88.58, 76.99, 69.78, 67.82, 62.55, 61.87, 48.12, 46.35, 36.52, 36.44, 33.69, 33.48, 33.11, 22.73, 19.88, 18.19, 16.80, 12.08, and 11.71 ppm.

Fig. 4. ^1H NMR spectrum of herboxidiene (300 MHz, CD_3OD).



In conjunction with 2D NMR data (to be detailed elsewhere), the physico-chemical properties and spectroscopic data described herein enabled the deduction of the structure of the phytotoxic metabolite of *S. chromofuscus* A7847 as shown in Fig. 1. Herboxidiene is a novel, essentially linear, polyketide possessing exceptional herbicidal activity.

Acknowledgments

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