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Identification of a Self-Inhibitor from Spores of *Dictyostelium discoideum*

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A self-inhibitor of spore germination has been isolated from spores of *Dictyostelium discoideum*, a cellular slime mold, and chemically characterized as 2-dimethylamino-6-oxypurineriboside.

In many fungi, crowding of spores results in a reduction in the percentage of germination, a phenomenon known as self-inhibition or autoinhibition. Russell and Bonner (11) presented the first evidence for a self-inhibitor in the cellular slime mold *Dictyostelium mucoroides*, and Ceccarini and Cohen (4) described some of its properties. However, since these initial investigations, the chemical nature of the self-inhibitor has remained a mystery.

We report here the isolation, purification, and identification of a self-inhibitor from water extracts of spores of *D. discoideum* which has the properties of the one described (11).

D. discoideum NC-4H was cultured on an agar nutrient medium (5) with *Escherichia coli* as a food source in Pyrex baking dishes. To extract the inhibitor, a modified procedure of Ceccarini and Cohen (4) was used. Seven-day-old spores were removed from the sorocarp with a glass microscope slide, placed in 100 ml of sterile distilled water, and stirred for 1 hr. The suspension was centrifuged at 10,000 × *g* for 10 min, and the spore pellet was resuspended in 100 ml of distilled water, stirred for 15 min, and centrifuged as before; the washings were combined and dried with a rotary evaporator at 45 C. The residue was taken up in 50 ml of water, placed in dialysis tubing, and dialyzed under reduced pressure at 0 C. The dialysate was evaporated to dryness under reduced pressure at 45 C, and the residue was taken up in 5 ml of distilled water. This solution was passed through a Dowex 1-X8 ($HCOO^-$) column (1.5 by 30 cm), and eluted in 3-ml fractions with a continuous gradient of 1 M acetate buffer, *p*H 4.4. Approximately 1.2 g (dry weight) of spores was used for each extraction; the spores from the extraction were washed 10 times more, lyophilized, and stored at 4 C for use in the bioassay. The activity of the fractions was determined by the ability to inhibit the germination of heat-activated spores as described by Cotter and Raper (5) using lyophilized spores (10×10^3 spores/ml). The active fractions were pooled and evaporated to dryness under reduced pressure as before. The residue was dissolved in 5 ml of distilled water.

The self-inhibitor was purified by paper chromatography by streaking 0.5 ml of the above solution onto Whatman no. 1 filter paper (20 by 57 cm). The first solvent system (A) consisted of isopropanol-HCl-water (680:176:144) (12) and the inhibitor was located as a single light-blue fluorescent band at R_F 0.51. This band was eluted with distilled water, spotted on Whatman no. 1 filter paper (20 by 20 cm), and chromatographed two-dimensionally (14). The solvent system for the first dimension consisted of 0.5 N NH_4OH and isobutyric acid (6:10). The second solvent (B) consisted of tertiary butanol and concentrated formic acid (1:1). The fluorescent zone, R_F 0.64, was eluted and chromatographed a third time in solvent system (C), containing ethanol and 1 M ammonium acetate buffer, *p*H 5.5 (7:3) (14).

The ultraviolet absorption spectrum of the self-inhibitor showed absorption maxima at 265, 263, and 265 nm in 0.1 N HCl, 0.1 N KOH, and 10 mM phosphate buffer (*p*H 7.0), respectively. High-resolution mass spectral data were obtained on a CEC-21-110 spectrometer at 70 eV, using a photoplate. These mass spectral data showed the molecular formula to be $C_{12}H_{17}N_5O_5$ with an accurate mass of 311.1280 (calculated 311.1230). The fragmentation pattern showed the following characteristic fragments (*m/e*, mass to charge; M^+, molecular ion; B, base residue; S, sugar fragment): *m/e* 222 (M − 89), *m/e* 208 (B + 30), *m/e* 180 (B + 2), *m/e* 179 (B + 1), *m/e* 178 (B), *m/e* 150 (B +

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H—CH_3N), *m/e* 149 (B − 29), *m/e* 135 (B − 44), and *m/e* 133 (S) (1, 3, 10). These data suggested that the self-inhibitor of spore germination in cellular slime mold was 2-dimethylamino-6-oxypurineriboside (or *N,N*-dimethylguanosine; Fig. 1). Identical fragmentation patterns were obtained from synthetic *N,N*-dimethylguanosine.

Acid hydrolysis (12) of the inhibitor and analysis of the products revealed that ribose was liberated (Table 1). The free base, *N,N*-dimethylguanine, obtained from hydrolysis, was identified both by co-chromatography with synthetic *N,N*-dimethylguanine and by its ultraviolet spectrum. Neither the free base nor ribose possessed inhibitory activity. Bromination of the self-inhibitor resulted in a loss of the anionic maximum, indicating that the inhibitor did not contain adenine (14). A colorimetric phosphate test (13) revealed that the compound did not possess phosphates, i.e., the compound was not a nucleotide.

It was determined that 50 μg of either the synthetic compound or of the native inhibitor per ml produced 100% inhibition in activated spores (10×10^2 spores/ml). Once either the synthetic compound or the native inhibitor was removed, the spores were able to germinate. Furthermore, percent inhibition was proportional to inhibitor concentration using heat-activated spores (10×10^2 spores/ml) (5). Both nutrient agar and nutrient agar with *E. coli* were tested for inhibitory activity and the results were negative. The molar extinction coefficient of the self-inhibitor in 0.1 N HCl was determined to be 7.5×10^{-3} per M per cm which corresponds with published figures for the authentic compound (6, 14). It was calculated that approximately 0.3 mg of this substance can be purified from each gram of spores (dry weight) with this procedure.

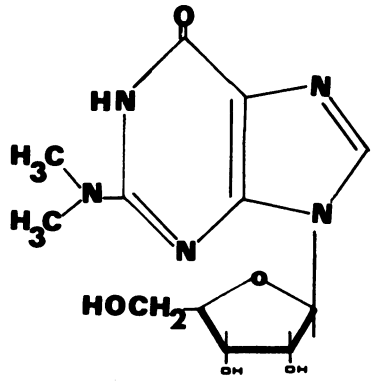

FIG. 1. *Structure of 2-dimethylamino-6-oxypurineriboside.*

TABLE 1. *R_F values[a] and ultraviolet (UV)-absorption maxima of methylated guanines, their ribosides, the self-inhibitor and ribose*

Compounds	Solvent systems[b]				UV maxima in 0.1 N HCl
	A	B	C	D	
N,N-dimethylguanosine	51	64	40		265
Self-inhibitor	51	64	40		265
N,N-dimethylguanine	43	67	28		258
N,N-dimethylguanosine (hydrolyzed)	43	67	28	25	258
Self-inhibitor (hydrolyzed)	43	67	28	25	258
Ribose				25	

[a] R_F values are reported × 100.

[b] Solvent systems A, B, and C are described in the text; solvent system D is specifically used for pentoses (8) and consisted of ethyl acetate-water-acetic acid (3:3:1). The ribose was developed in a naphthoresorcinol reagent (9).

Thus we conclude that the self-inhibitor of spores of *D. discoideum* is *N,N*-dimethylguanosine. This nucleoside is found as a normal constituent of ribonucleic acid from tobacco leaves, sugar beets, and rat liver (14), and it is likely that it is methylated after it is incorporated into ribonucleic acid and deoxyribonucleic acid (2). Although many free nucleosides have been discovered as products of microbial metabolism, and many are inhibitors (7), the biological function for a free methylated base has not been known. However, this study defines one function for such a compound: *N,N*-dimethylguanosine serves to regulate spore development in *D. discoideum*. The mechanism through which this is accomplished is being studied.

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