Mode of Action of Illudin S

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The effect of the antitumor antibiotic illudin S on bacterial macromolecular synthesis was investigated. Illudin S was found to be inhibitory to *in vivo* deoxyribonucleic acid (DNA) synthesis from thymidine. Ribonucleic acid (RNA) synthesis was inhibited only at a concentration of illudin S 10 times that which inhibited DNA synthesis. The rate of protein synthesis remained the same except for a brief initial inhibition. When thymidine triphosphate was used for *in vitro* DNA synthesis, inhibition by illudin S did not occur, as tested with partially purified DNA polymerase II from *Escherichia coli* pol A₁⁻, with *E. coli* DNA-dependent RNA polymerase, with *E. coli* pol A₁⁻ spheroplasts, and with frozen and thawed *Bacillus subtilis* cells. A protein fraction isolated from *B. subtilis* capable of forming thymidine mono-, di-, and triphosphates from thymidine was not inhibited by illudin S. Furthermore, ¹⁴C-illudin S taken up by *B. subtilis* cells was reisolated unchanged, making an intracellular activation of illudin S unlikely. Therefore, an attractive hypothesis is that illudin S inhibits DNA synthesis from thymidine which does not proceed through deoxyribonucleoside triphosphates, the generally accepted substrates for DNA synthesis.

Illudin S is an antibiotic produced by *Clitocybe illudens* (1). Its structure was shown (8, 9, 11) to be that illustrated in Fig. 1. Illudin S was independently isolated from *Lampteromyces japonicus* (12) and was called lampterol. In addition to inhibiting the growth of gram-negative and gram-positive bacteria and mycobacteria, this agent also showed antitumor activity (9, 13).

Illudin S possesses some unique structural features: a cyclopropane ring and an α,β -unsaturated ketone function. Both functionalities are potentially good alkylating sites.

This paper describes the effect of illudin S on protein and nucleic acid synthesis in *Bacillus subtilis* and *Escherichia coli* cells, in bacterial cells made permeable to deoxyribonucleoside triphosphates, and in cell-free nuclei acid biosynthetic systems.

MATERIALS AND METHODS

Organisms. *C. illudens* (ATCC 11719) was grown at 25°C in stationary cultures in corn steep medium (2) or in potato-dextrose broth. *E. coli* (ATCC 14948) was grown in nutrient broth at 37°C on a rotary shaker (260 rpm). *B. subtilis* (ATCC 6633) was grown in the medium described by Reusser (14). *E. coli* pol A₁⁻ (6, 7), a mutant that lacks deoxyribonucleic acid (DNA) polymerase I, was a gift from T. Kornberg, and was grown in Penassay Broth (7).

Chemicals. Thymidine-methyl-³H (³H-T) was purchased from International Chemical and

Nuclear Corp. Adenosine-8-¹⁴C-5'-triphosphate (¹⁴C-ATP), thymidine-methyl-³H-5'-triphosphate (³H-TTP), uridine-5-³H (³H-U), and leucine-U-¹⁴C were obtained from Amersham/Searle Corp.

Some of the illudin S used in the inhibition studies was generously supplied by M. Anchel; the remainder was isolated from the culture medium of *C. illudens* (1). ¹⁴C-labeled illudin S was prepared by feeding 3- to 4-week-old cultures of *C. illudens* with mevalonic acid-2-¹⁴C (2). The ¹⁴C-illudin S was extracted with chloroform (four times with an equal volume) from the culture medium and the homogenized mycelium. The combined extracts were concentrated to a small volume and purified by partition between water and chloroform on a Celite column. Moist Celite 545 was boiled with 1 N HCl, washed with deionized water to neutrality, suspended in chloroform, and poured into a column (2 by 50 cm). The infrared spectrum of the ¹⁴C-illudin S eluted from the column with chloroform was found to be identical to that of a known illudin S sample. Furthermore, no difference of migration of the isolated ¹⁴C-illudin S and an authentic sample could be observed on thin-layer chromatography plates (silica gel G). Two solvent systems were employed: chloroform-acetone, 1:1, and benzene-95% ethanol, 2:1. In both cases, the radioactivity coincided with the illudin S used as standard material.

Synthesis of macromolecules *in vivo*. Cellular nucleic acid and protein synthesis was studied by following the incorporation of the appropriate radioactively labeled precursors into acid-insoluble material. A 1-ml sample from a *B.*

Fig. 1. Structure of illudin S.

subtilis culture, grown overnight, was used to inoculate 25 ml of medium. After 1 h at 37 C, radioactive precursor was added ($^3\text{H-T}$ for DNA synthesis, $^3\text{H-U}$ for ribonucleic acid [RNA] synthesis, and leucine- $U\text{-}^{14}\text{C}$ for the formation of protein); 10 min thereafter, the culture was divided. Half was used as a control, and to the other half illudin S (5 to 150 $\mu\text{g/ml}$) was added. Samples were withdrawn from the cultures at appropriate time intervals (see figures). The reaction was stopped by the addition of ice-cold 10% trichloroacetic acid (equal volume), and the acid-insoluble material was collected on a 0.45- μm membrane filter (Millipore Corp.). The precipitate was washed three times with ice-cold 5% trichloroacetic acid, dried, and counted in 10 ml of Bray's solution in a Beckman LS-100 liquid scintillation counter, with a counting error of 2%.

$^3\text{H-T}$ uptake by the cells was compared with $^3\text{H-T}$ incorporation into DNA by performing the experiment in the same manner as described above except that the samples taken from the incubation mixture were filtered immediately without trichloroacetic acid treatment. The collected cells were washed three times with fresh medium; they were then dried and radioactivity was determined as above.

In some *in vivo* incorporation experiments, ^{14}C -illudin S (12.5 $\mu\text{g/ml}$, 1.26×10^5 counts/min) together with $^3\text{H-T}$ was fed to a 60-ml *B. subtilis* culture, inoculated with 5 ml from an overnight culture. After 2 h at 37 C in a shaker, the cells were harvested by centrifugation, suspended in 0.1 M potassium phosphate buffer, pH 7.4, and treated at 0 to 4 C in a sonifier cell disruptor (Heat System Co.) for 1 min/ml at a maximal output setting. The broken cell suspension was extracted three times with equal volumes of ethylacetate and three times with equal volumes of chloroform. The extracts were combined, concentrated, and analyzed by thin-layer chromatography (see above). The aqueous phase was passed through a G-150 Sephadex column (2 by 45 cm), and the radioactivity was determined in each of the 6-ml fractions collected.

Spheroplast formation. Preparation of frozen *B. subtilis* cells and the incorporation of $^3\text{H-TTP}$ into DNA were carried out as described by Billen et al. (3). Illudin S was added to the incubations at a concentration of 40 $\mu\text{g/ml}$. The method of Smith et al. (16) was used to prepare spheroplasts from

E. coli pol A₁⁻ and to study incorporation of $^3\text{H-TTP}$ into DNA with and without illudin S.

Synthesis of DNA and RNA *in vitro*. DNA-dependent RNA polymerase from *E. coli* was isolated by one of the two methods described by Burgess (5). The enzyme obtained after the two glycerol gradient centrifugations (see 5) was utilized and assayed (5) with and without illudin S. DNA polymerase II from *E. coli pol A₁*⁻ was partially purified (7). The active protein fraction, after the two glycerol gradient centrifugations (low and high salt), was used as an enzyme source and assayed according to Kornberg and Geffter (7), with and without illudin S.

Phosphorylation of thymidine *in vitro*. The cells (5 g, wet weight) from an overnight culture of *B. subtilis* were washed twice with 0.1 M tris-(hydroxymethyl)aminomethane - hydrochloride, pH 7.5, containing 1 mM ethylenediaminetetraacetate (EDTA) and 5 mM mercaptoethanol (one-tenth the volume of the original medium). After being suspended in 3 ml of the same buffer per 100 ml of original culture medium, the cells were sonically treated for 30 s/ml (output setting: 7). The sonically treated solution was centrifuged at $13,000 \times g$ for 30 min, and the supernatant fluid was used for the phosphorylation of thymidine experiment. All operations were carried out at 0 to 4 C.

The assay mixture was identical to that described by Masui and Garren (10); however, the amount of phosphorylated thymidine derivatives produced was determined by use of diethylaminoethyl-cellulose paper disks, and the washing procedure of Shiosaka et al. (15) was used. The products of this reaction were identified by descending paper chromatography with the system isobutyric acid-ammonia-EDTA (4) and were found to be mainly thymidine monophosphate (TMP) and small amounts of thymidine di- and triphosphates (TDP and TTP).

RESULTS

Effect of illudin S on protein and nucleic acid synthesis *in vivo*. Incorporation of leucine into acid-insoluble material (Fig. 2) was inhibited temporarily upon addition of illudin S. However, after 10 min, protein synthesis continued at a rate identical to that in the control incubations. RNA synthesis, as measured by the incorporation of uridine into acid-insoluble material, was inhibited 95% at rather high concentrations of illudin S (Fig. 3). Incorporation of thymidine into DNA, however, was almost completely stopped by illudin S at a concentration of 10 $\mu\text{g/ml}$ (Fig. 4). Furthermore, inhibition occurred immediately after the addition of illudin S. Similarly, DNA synthesis was inhibited by illudin S in the *E. coli pol A₁*⁻ mutant (Fig. 5).

FIG. 2. Effect of illudin S on the formation of protein in *B. subtilis* cells, as measured by the incorporation of ^{14}C -leucine into acid-insoluble material. The addition of illudin S is shown by the arrow, and the concentrations of illudin S are given after the appropriate curves.

This mutant has been reported to lack DNA polymerase I (6, 7).

To test the possibility that illudin S was inhibiting the transport of thymidine into *B. subtilis* cells, we measured thymidine uptake as well as incorporation of thymidine into acid-insoluble material. Although illudin S immediately inhibited incorporation of thymidine into DNA (Fig. 6), the uptake of thymidine continued for 10 min after addition of illudin S. DNA synthesis from TTP in *B. subtilis* cells frozen for 11 h was found to be no longer sensitive to illudin S. As can be seen in Fig. 7, the incorporation of ^3H -TTP into DNA was not inhibited by 100 µg of illudin S/ml but rather was slightly stimulated.

Spheroplasts of *E. coli* pol A_1^- were prepared (16), and DNA synthesis was measured with and without illudin S. The results in Table 1 showed no inhibition by illudin S of ^3H -TTP incorporation into acid-insoluble material, even at twice the illudin S concentrations which

FIG. 3. Synthesis of RNA in *B. subtilis* cells, as measured by the incorporation of uridine-5- ^3H into acid-insoluble material. The addition of illudin S is shown by the arrow, and the concentrations of illudin S are given after the appropriate curves.

inhibited thymidine incorporation into DNA in intact cells (Fig. 5).

In vitro inhibition studies. DNA-dependent RNA polymerase, isolated from *E. coli* (5), was not affected by illudin S (Table 2). For comparison, actinomycin D readily inhibited the partially purified enzyme at a concentration one-tenth that of illudin S. A number of variations were incorporated into the experiment: preincubation of illudin S with the RNA polymerase, preincubation of the illudin S with different amounts of calf thymus DNA, used as template, and incubations with illudin S at different stages of enzyme purification. In none of the above experimental designs was inhibition of DNA-dependent RNA polymerase by illudin S observed.

DNA-dependent DNA polymerase II from the DNA polymerase I-deficient *E. coli* mutant

FIG. 4. Synthesis of DNA in *B. subtilis* cells, as measured through the incorporation of thymidine-methyl- ^3H into acid-insoluble material. The addition of illudin S is shown by the arrow, and the concentrations of illudin S are given after the appropriate curves.

FIG. 5. Inhibition of DNA synthesis from thymidine-methyl- ^3H in *E. coli* $\text{pol } A_1^-$, a mutant which lacks DNA polymerase I. The tritiated substrate was added at zero time, and the illudin S was added to one culture after 10 min (arrow) at a concentration of 10 $\mu\text{g/ml}$.

$\text{pol } A_1^-$ was not inhibited by illudin S at a concentration of 50 $\mu\text{g/ml}$ (Table 3), which was five times the concentration causing virtually complete inhibition of in vivo DNA synthesis

FIG. 6. Effect of illudin S on thymidine-methyl- ^3H ($^3\text{H-T}$) uptake and incorporation into acid-insoluble material in *B. subtilis*. $^3\text{H-T}$ was added at zero time, and after 10 min illudin S (50 $\mu\text{g/ml}$) was added (arrow). Thereafter, samples were taken and immediately filtered (uptake of $^3\text{H-T}$, $\circ$); identical samples were pipetted into equal volumes of 10% trichloroacetic acid (incorporation of $^3\text{H-T}$, $\triangle$).

(Fig. 5). Variations in addition of enzyme, template, and illudin S and use of different preincubation times (see above for RNA polymerase) showed no inhibition of DNA synthesis by illudin S.

To eliminate the possibility that illudin S inhibits the conversion of thymidine to TMP, TDP, or TTP, we prepared a cell-free preparation of *B. subtilis* which could carry out this conversion. However, as shown in Table 4, the conversion was not inhibited by illudin S.

Fate of ^{14}C -illudin S in *B. subtilis* cells. Of the ^{14}C -illudin S fed to a *B. subtilis* culture, approximately 70% was absorbed by the cells (Table 5). Most of the absorbed illudin S (41.3%) was bound to macromolecules, as shown by molecular sieve chromatography (Sephadex G-150), and could not be extracted with either ethylacetate or chloroform. Some of the illudin S absorbed by the *B. subtilis* cells was apparently

FIG. 7. Effect of illudin S on DNA synthesis in *B. subtilis* spheroplasts, formed by freezing in a sodium citrate buffer according to Billen et al. (3). Assay mixtures were identical to those of Billen et al. (3), with the exception that 0.5 μ Ci of 3 H-TTP was used, and to one incubation 100 μ g of illudin S/ml was added at zero time ($\odot$).

TABLE 1. Effect of illudin S on DNA synthesis in *E. coli* pol A₁⁻ spheroplasts^a

Sample	TTP incorporation (counts per min per sample)	Percentage of control
Control	1,289	100.0
Illudin S		
2 μ g/ml	1,676	130.0
20 μ g/ml	1,250	96.9
100 μ g/ml	1,794	139.2

^a Spheroplast formation and assay mixtures (0.2 ml) were those described by Smith et al. (16), with the following exceptions: 0.01 M mercaptoethanol (instead of 0.1 mM dithiothreitol) and 0.5 μ Ci of 3 H-TTP as substrate.

present in a free form in the cell and could be extracted with ethylacetate and chloroform. Chromatographic comparison of the extracted 14 C-illudin S with the original fed 14 C-illudin S showed no difference in migration in two different solvent systems.

DISCUSSION

Illudin S inhibited macromolecular synthesis in intact *B. subtilis* cells. From the observation

that DNA synthesis was preferentially and immediately inhibited compared with RNA and protein synthesis, we concluded that illudin S has a direct effect on the incorporation of thy-

TABLE 2. Effect of illudin S on cell-free RNA synthesis^a

Sample	Incorporation (counts per min per sample)	Percentage of control
Control	558	100.0
Illudin S		
2 μ g/ml	535	95.9
20 μ g/ml	554	99.3
100 μ g/ml	570	102.2
Actinomycin D		
5 μ g/ml	112	20.0

^a RNA polymerase was isolated from *E. coli* and assayed according to Burgess (5). Activity was measured by the incorporation of 14 C-ATP (0.15 mM, 1 μ Ci/ μ mole).

TABLE 3. Effect of illudin S on cell-free DNA synthesis^a

Sample	Incorporation (pmoles of 3 H-TTP)	Percentage of control
Control	11.45	100.0
Illudin S		
15 μ g/ml	13.85	121.0
50 μ g/ml	13.90	121.4

^a DNA polymerase II was isolated from *E. coli* pol A₁⁻ and assayed according to Kornberg and Gefter (7).

TABLE 4. Effect of illudin S on the phosphorylation of thymidine in cell-free extracts of *B. subtilis*^a

Sample	3 H-thymidine incorporation (nmoles/mg of protein)	Percentage of control
Control	32.52	100.0
Illudin S		
10 μ g/ml	32.56	100.1
25 μ g/ml	33.40	102.7
50 μ g/ml	31.44	96.7
100 μ g/ml	31.92	98.2

^a A sonic extract of *B. subtilis* served as the enzyme source. The incubation mixture was identical to that described by Masui and Garren (10). Phosphorylated products of 3 H-thymidine were determined by absorption on diethylamino-ethyl-cellulose paper disks (15).

TABLE 5. Distribution of ^{14}C -illudin S in *B. subtilis* cells^a

Sample	Radioactivity (counts/min)	Percentage of original
^{14}C -illudin S fed	12.6×10^4	100
^{14}C recovered in medium	2.2×10^4	18.0
^{14}C in organic phase of cell sonic extract	3.73×10^4	29.6
^{14}C in aqueous phase of cell sonic extract	5.2×10^4	41.3

^a ^{14}C -illudin S was added to a *B. subtilis* culture; after 2 h, the cells were harvested, sonically treated and extracted three times with equal volumes of ethylacetate and chloroform (^{14}C in organic phase of cell sonic extract). The residue (aqueous phase) of this extraction was used to determine ^{14}C in aqueous phase of cell sonic extract. Similarly, the medium was extracted three times with equal volumes of ethylacetate and chloroform (^{14}C recovered in medium). Negligible amounts (less than 2%) of radioactivity were obtained from the aqueous residue of the medium.

midine into DNA. However, this inhibition was no longer apparent when *B. subtilis* cells were made permeable to TTP and when TTP was utilized for DNA synthesis. Furthermore, isolated RNA polymerase and DNA polymerase II were not inhibited by illudin S, which excluded inhibition of DNA synthesis through the alkylation by illudin S of the DNA template used by both enzymes. Since one of the differences between the in vivo and in vitro experiments was the partial (spheroplast formation) or complete (isolated RNA and DNA polymerase) removal of the cell wall of *B. subtilis*, the possibility existed that the uptake of thymidine was inhibited by illudin S, or that during the uptake of illudin S by *B. subtilis* cells the compound underwent a metabolic change and the product of this reaction was the active inhibitor. Both possibilities could be excluded because uptake of thymidine continued for at least 10 min after addition of illudin S, whereas DNA synthesis was immediately inhibited, and because ^{14}C -illudin S taken up by *B. subtilis* cells was found to be unchanged. This, therefore, focused attention on the other difference between the in vivo and in vitro experiments, namely, the difference in substrates. In the in vivo experiments, thymidine was used as substrate for DNA synthesis, whereas TTP served as precursor for in vitro experiments (including spheroplasts). Although the incorporation of thymidine into DNA was inhibited

by illudin S, no inhibition was observed when TTP served as the substrate. This difference was not due to the phosphorylation of thymidine, because a cell-free preparation which could catalyze the formation of TMP, TDP, and TTP from thymidine was not inhibited.

From the above results, therefore, it is tempting to postulate the existence of two DNA-synthesizing systems in *B. subtilis*: one utilizing thymidine directly for DNA synthesis through a so far unknown DNA precursor and the conventional DNA-synthesizing system utilizing TTP. In this case, illudin S could inhibit the formation of the unknown DNA precursor from thymidine or its incorporation into DNA. Similar conclusions have been drawn by others (3), from the observation that in *B. subtilis* ultraviolet light inhibits thymidine incorporation into DNA, but not synthesis of DNA from TTP. Similarly, Werner (17) concluded from experiments with *E. coli* that thymidine is incorporated into DNA by a different route than the deoxyribonucleotide triphosphates.

Similar results have been obtained with the bactericidal drug nalidixic acid (18). This agent appears to inhibit in vivo DNA synthesis in *E. coli* and *B. subtilis*, but does not affect cell-free synthesis of DNA by DNA polymerase I or incorporation of TTP into DNA in bacterial cells made permeable to the deoxynucleoside triphosphates (16).

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