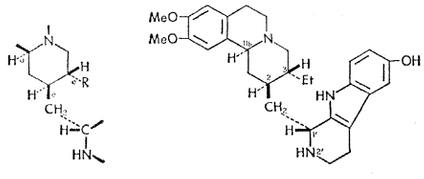
Structural Basis for the Inhibition of Protein Biosynthesis: Mode of Action of Tubulosine

Abstract. *A structural model for the inhibition of protein biosynthesis was previously formulated on the basis of a topochemical analogy between the ipecac alkaloids and the glutarimide antibiotics. The structure of tubulosine satisfies the requirements of this model. The prediction that such a compound would exhibit amebicidal activity and act by selectively inhibiting the transfer reaction in protein biosynthesis is confirmed.*

The numerous antibiotics which act by inhibiting the biosynthesis of protein, DNA, or RNA (*1*) have been discovered almost exclusively by empirical screening procedures. Although relations between structure and activity of such antibiotics have been established, it has been concluded (*2*) that "no common chemical characteristics are evident which would suggest a priori to the knowledgeable investigator that a particular activity of the type observed should be possessed by the molecule." I now describe the inhibition of protein biosynthesis by tubulosine and the amebicidal activity of this alkaloid—properties which were predicted on the basis of structural considerations.

Emetine, an ipecac alkaloid, and cycloheximide, a glutarimide antibiotic, are potent amebicides which act by inhibiting the transfer reaction in protein biosynthesis (*3, 4*). Configurational and functional similarities between these compounds led to the proposal (*3*) that structure I contained the topochemical requirements for the inhibition of protein biosynthesis. Structure I comprises part of the indole alkaloid tubulosine (II), whose configuration has been established (*5*), but whose biological properties have not been reported. Tubulosine thus provides an opportunity to experimentally test the proposal that amebicidal activity and inhibition of protein biosynthesis are properties which can be ascribed to structure I.



(I) (II) TUBULOSINE

The methods used for measurement of macromolecular synthesis in HeLa cells and for protein biosynthesis by cell-free preparations from rabbit reticulocytes, yeast, and bacteria have been described (*3, 6*). The isolation and characterization of the sample of tubulosine used for these experiments has been reported (*7*).

As illustrated in Fig. 1, tubulosine produces 50-percent inhibition of protein synthesis in intact HeLa cells at a concentration in the media of $3 \times 10^{-8}M$ and 98 percent inhibition at $1 \times 10^{-6}M$. The alkaloid partially inhibits DNA synthesis, while RNA synthesis is unaffected. The effects on protein and DNA synthesis are essentially complete within 2 minutes after the addition of tubulosine to the medium. In contrast to cycloheximide, inhibition could not be reversed by washing and resuspending the cells in fresh medium. The rate of hemoglobin synthesis by suspensions of intact rabbit reticulocytes is also inhibited by 50 percent at a tubulosine concentration of $1 \times 10^{-7}M$.

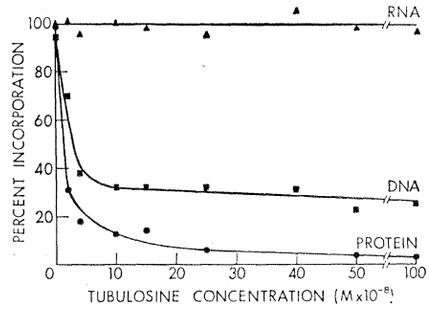


Fig. 1. Effect of varying concentrations of tubulosine on the synthesis of protein, RNA, and DNA in HeLa cells. HeLa cells (4×10^5 cell/ml) were suspended in leucine-depleted Eagle's medium containing horse serum (5 percent). Portions (3 ml) were incubated for 30 minutes, and tubulosine was added at the indicated final concentrations. After 2 minutes, 1 mμmole of ^{14}C-leucine (100,000 count/min), 10 mμmole of ^{14}C-thymidine (100,000 count/min), or 5 mμmole ^{14}C-uridine (60,000 count/min) was added. After 60 minutes, duplicate portions of 0.5 ml were transferred to tubes containing cold Earle's solution, and the cells were prepared for the determination of radioactivity (*6*). The incorporation of radioactivity into material insoluble in trichloroacetic acid was used to calculate the linear rate of protein, RNA, and DNA synthesis, which, in the control reaction, was 4305, 1110, and 915 count min^{-1} ml^{-1} hr^{-1}, respectively. The percentage of incorporation is relative to these controls. The experiment is representative of eight others.

Experiments with cell-free preparations (Table 1) indicated that inhibition of protein synthesis by tubulosine occurs after the formation of aminoacyl-sRNA. The rate of leucine incorporation from leucyl-sRNA into peptide-bound form on reticulocyte ribosomes is inhibited by 50 percent at a concentration of $5 \times 10^{-6}M$. The rate of polyphenylalanine synthesis from phenylalanyl-sRNA, stimulated by the addition of polyuridylic acid to the same preparation, is inhibited by 50 percent at the same concentration. Inhibition of polyphenylalanine synthesis by tubulosine was also observed with ribosomal preparations from *Saccharomyces fragilis* but S-30 (see *3*) extracts from *Escherichia coli* were unaffected at $10^{-3}M$ (Table 1).

The effects on the attachment of nascent peptide to polyribosomes was studied in HeLa cells and rabbit reticulocytes by exposing intact cells to $10^{-6}M$ tubulosine before and after the addition of a single radioactive amino acid to the medium. The cells were lysed, and the cytoplasm was subjected to sucrose density gradient analysis (*8*). A 50-percent decrease in the number of single ribosomes with a concomitant increase in the amount of polyribosomes occurred within 2 minutes after the addition of tubulosine. When the alkaloid was added before the addition of the radioactive amino acid, no nascent peptide was found in the polyribosome area of the gradient. If the inhibitor was added 2 minutes after the addition of the radioactive amino acid, nascent peptide remained attached to the polyribosomes for at least 1 hour.

Structural specificity was tested by determining the effects of certain closely related isomers of tubulosine on protein synthesis in intact HeLa cells, in suspensions of rabbit reticulocytes, and in the cell-free reticulocyte preparations. The molar concentrations of each isomer required to inhibit the initial rate of protein synthesis by 50 percent was compared to that of tubulosine. Deoxytubulosine (*9, 10*) which lacks the hydroxyl group at the 6′-carbon, was slightly less active than tubulosine. Isotubulosine (*11*), which differs from tubulosine only in the configuration at the 1′-carbon, and 2′,3′-dehydro-6′-deoxytubulosine (*9*) showed less than 0.5 percent of the inhibitory activity of the parent compounds.

Tubulosine was amebicidal against

Table 1. Effect of tubulosine on protein and polyphenylalanine synthesis in cell-free preparations from different species. Reticulocytes: Hemoglobin synthesis was measured in reaction mixtures containing, in a final volume of 0.5 ml, 5 μmole of glutathione; 33 μmole of KCl; 16 μmole of tris-HCl, *p*H 7.4; 3.3 μmole of $MgCl_2$; 5 μmole of phosphoenolpyruvate; 0.033 μmole of guanosine triphosphate (GTP); 1.5 μg of pyruvate kinase; 93 μg (11,980 count/min) of ^{14}C-leucyl-sRNA; 0.33 μmole of ^{12}C-L-leucine; amino acid mixture containing 0.05 μmole of each of the following L-amino acids: alanine, arginine, aspartic acid, glycine, glutamine, glutamic acid, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, hydroxyproline, cysteine, and valine; 0.3 mg of reticulocyte ribosomes, and tubulosine at the final concentration shown. Reactions mixtures were incubated at 37°C for 5 minutes. *Saccharomyces fragilis*: Polyphenylalanine synthesis was measured in reaction mixtures containing, in a final volume of 0.5 ml, 50 μmole of imidazole, *p*H 7.0; 3 μmole of magnesium acetate; 50 μmole of NH_4Cl; 0.4 μmole of spermine; 1 μmole of GTP; 50 μg of polyuridylic acid; 70 μg of ^{14}C-phenylalanyl-sRNA (4950 count/min); 2 μmole of ^{12}C-L-phenylalanine and 0.34 mg of *Saccharomyces fragilis* ribosomes. Reaction mixtures were incubated at 25°C for 10 minutes. *Escherichia coli*: The reaction mixture for polyphenylalanine synthesis was composed of 25 μmole of tris-HCl, *p*H 7.4; 5.5 μmole of magnesium acetate; 15 μmole of KCl; 1.5 μmole of adenosine triphosphate; 0.1 μmole of GTP; 5.6 μmole of mercaptoethanol; 5 μmole of phosphoenolpyruvate; 0.02 μmole of each of the 19 amino acids listed above; 0.02 μmole of ^{14}C-phenylalanine (191,000 count/min); 16 μg of pyruvate kinase; 8 μg of polyuridylic acid, and 0.5 mg of preincubated S-30 fraction (*3*) in a final volume of 0.5 ml. Reaction mixtures were incubated at 25°C for 10 minutes. All reactions were terminated by the addition of trichloroacetic acid, and radioactivity in the fraction insoluble in hot acid was then determined (*6*).

Tubulosine (mole/liter)	Rabbit reticulocytes		*S. fragilis*		*E. coli*	
	Incorporation (count/min)	Inhibition (%)	Incorporation (count/min)	Inhibition (%)	Incorporation (count/min)	Inhibition (%)
10^{-3}			125	94	15,130	0
10^{-4}	89	98	626	70	15,012	0
10^{-5}	1201	73	1522	27	15,186	0
10^{-6}	4005	10	2002	4		
10^{-7}	4461	0	2085	0		

several strains of *Entamoeba histolytica*. This ambicidal activity was compared (*12*) to that of emetine as tested by published methods (*13*); the lowest concentration of either tubulosine or emetine which produced 100 percent killing of the organisms was $1 \times 10^{-5}M$.

The data indicates that the action of tubulosine is: (i) species-specific, being active against certain mammalian cells, protozoa, and yeast but inactive against preparations of *Escherichia coli*; (ii) structurally specific, requiring a secondary nitrogen atom at the 2′-position and the (R) configuration at the 1′-carbon for activity; (iii) selective, as RNA synthesis is unaffected at concentrations of inhibitor which totally inhibit protein synthesis; and (iv) exerted during the enzymatic transfer of amino acids from aminoacyl-sRNA to the growing peptide chain. The foregoing properties are common to the ipecac alkaloids (*3*) and the glutarimide antibiotics (*4*), and they were predicted (*3*) for compounds, such as tubulosine, which contain configurational features of structure I.

The steric and electronic effects of substituents at other positions in tubulosine have not been evaluated. Nevertheless, the data reported are consistent with the structural basis assigned for the inhibition of the transfer reaction of protein synthesis (*3*) and represent a successful prediction of antibiotic activity based on structural considerations. Tubulosine should be expected to have the antitumor (*14*) and antiviral (*15*) properties shown by the ipecac alkaloids, which probably result from their action as potent inhibitors of protein biosynthesis in mammalian cells.

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16. I thank Dr. A. R. Battersby for a sample of 2′,3′-dehydro-6′-deoxytubulosine, Dr. C. J. Djerassi for deoxytubulosine, Dr. H. T. Openshaw for synthetic isotubulosine, and Dr. A. Popelak for the tubulosine. I thank Mrs. Maria Walsh for technical assistance. Supported in part by grants from the USPHS, NSF, and American Cancer Society. This is paper III in the series *Inhibitors of Protein Biosynthesis*.
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Sex Attractant of the Black Carpet Beetle

Abstract. *The principal component of the sex attractant of the black carpet beetle* Attagenus megatoma (*Fabricius*) *is* trans-*3*, cis-*5-tetradecadienoic acid.*

We report the isolation, identification, and synthesis of the principal component of the sex attractant of the black carpet beetle *Attagenus megatoma* (Fabricius) [*A. piceus* (Olivier)]. The response of the male beetle to the female has been described by Burkholder (*1*), whose laboratory bioassay was used to monitor the following isolation steps.

Thirty thousand unmated female beetles were extracted in a Waring Blendor with benzene (four 300-ml portions), and the benzene-soluble material (9.4 g) was distilled in a short-path still onto a condenser (cooled with dry ice) at 100°C and 0.01 mm-Hg. A solution of the distillate (3.8 g) in 100 ml of diethyl ether was extracted with ice-cold 0.1*N* solution of sodium hydroxide (two 100-ml portions) which, after acidification to *p*H 1.5 with 1*N* hydrochloric acid, was extracted with 200 ml of diethyl ether. The acidic fraction thus obtained (1.7 g) was chromatographed on silica gel (Gallard Schlesinger; 90 to 200 mesh; 175 g in a 3.5 by 30 cm water-cooled column); used successively were 400 ml of benzene, 800 ml of diethyl ether, 325 ml of acetone, and 600 ml of methanol. The active ether fraction (0.8 g from 17,000 female beetles) was chromatographed on an anion-exchange column [AGl-X4, chloride form, 200 to 400 mesh (Bio-Rad Laboratories); 4.6 by 28 cm column]; we used successively 500 ml of a 3 : 1 mixture of methanol and water, 550 ml of 0.1*M* solution of sodium chloride in the same mixture, and 300 ml of a saturated solution of sodium chloride in the same mixture. The active component (0.6 g), which