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Synthesis and biological evaluation of sparsomycin analogues

Scherer Preston Sanders

SYNTHESIS AND BIOLOGICAL EVALUATION OF SPARSOMYCIN ANALOGUES

by

Scherer Preston Sanders Duke
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Thesis

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VITA

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	amino acid
Ac_2O	acetic anhydride
αMEM	Alpha Modification of Minimum Essential Medium
AraC	4-amino-1β-arabinofuranosyl-2-(1H)-pyrimidinone
BOC	tert-butyloxycarbonyl
bp	boiling point
CDMEM	Complete Dulbecco's Modified Eagle's Minimum Essential Medium
Ci	curie
CPM	counts per minute
DCC	dicyclohexylcarbodiimide
dec	decomposition
DMF	N,N-dimethylformamide
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
ED_{50}	median effective dose
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
g	grams
Δ	heat
^{125}IUdR	5-^{125}I-iodo-2-deoxyuridine
ip	intraperitoneal
IR	infrared
LAH	lithium aluminum hydride
Me_2SO	dimethylsulfoxide
μCi	microcurie
μg	microgram
μL	microliter
mL	milliliter
mmol	millimole
mp	melting point
mRNA	messenger ribonucleic acid
NMR	nuclear magnetic resonance
P388	P388 lymphocytic leukemia cells
P815	P815 mastocytoma cells
RNA	ribonucleic acid
^{75}Se	^{75}selenium
TFA	trifluoroacetic acid
THF	tetrahydrofuran
tRNA	transfer ribonucleic acid
UV	ultraviolet
v/v	volume per volume
X	times normal concentration
Z	benzyloxycarbonyl

ABSTRACT

SYNTHESIS AND BIOLOGICAL EVALUATION OF SPARSOMYCIN ANALOGUES

Scherer Preston Sanders Duke, Ph.D.

Medical College of Virginia -- Virginia Commonwealth University, 1981

Major Director: Dr. M. R. Boots

In 1962, Owen, Dietz, and Camiener reported the isolation of a new antitumor antibiotic from the culture filtrate of Streptomyces sparsogenes. The structure of the crystalline antibiotic, named sparsomycin, remained elusive until 1970, when Wiley and MacKellar reported results of spectroscopic and degradation studies which elucidated the structure. In addition to the molecular structure, investigators have examined the mechanism of action, toxicity, and related analogues, striving to establish sparsomycin or a synthetic analogue's usefulness as an effective chemotherapeutic agent.

The initial pharmacological evaluation of sparsomycin revealed it possessed activity against KB human epidermoid carcinoma cells, a variety of gram-negative and gram-positive bacteria, and fungi. This broad spectrum of activity prompted a closer examination of the biochemical mechanisms. These studies revealed sparsomycin interfered with protein synthesis by inhibiting peptide bond formation near the

enzyme peptidyl transferase.

Ottenheijm, Liskamp, and Tijhuis reported the first total synthesis of sparsomycin in 1979, which provided access to greater quantities of the material for investigational use. Sparsomycin was selected for use by cancer patients in phase I clinical trials, but was found to cause ocular toxicity which hindered its development as an antitumor agent. In an effort to reduce or eradicate the toxic effects while maintaining the antitumor activity, analogues of sparsomycin were prepared.

Using the sparsomycin analogues which were synthesized, studies were performed to determine the effect alteration of key structural parameters had on the efficacy of the compounds. Previous investigators examined analogues which incorporated modifications of the uracil ring, the unique mono-oxodithioacetal moiety, and the stereochemical configuration of the chiral centers. Vince and Lee reported there was an apparent requirement for the D-configuration at the asymmetric carbon atom. Overall, however, the small number of sparsomycin analogues prepared and evaluated limited the definitive statements concerning the functional groups required for antitumor activity. In order to expand and clarify the structure-activity relationships, three series of new sparsomycin analogues were prepared for this project. The compounds of Series I and II, distinguished by the inclusion or exclusion of a hydroxymethyl functional group, were designed to elucidate the effect on activity

of replacing the mono-oxodithioacetal side chain of sparsomycin with 4-substituted benzyl groups. The Series III analogues, which excluded the hydroxymethyl functional group, featured a 4-substituted benzyl amide group in place of the mono-oxodithioacetal moiety of sparsomycin, and were designed to investigate the potential interaction of an amide oxygen in contrast to the sulfoxide oxygen of sparsomycin.

The target compounds synthesized for this project were experimentally examined to quantitate their effects on cell growth, [^{75}Se]-selenomethionine incorporation as an indirect measurement of protein synthesis, and 5-^{125}I-iodo-2-deoxyuridine incorporation as an indirect measurement of DNA synthesis in bone marrow, P388 lymphocytic leukemia, and P815 mastocytoma cells. The results for the Series I and II analogues indicated the removal of the hydroxymethyl functional group as seen in sparsomycin affected activity to varying degrees depending upon the assay and the type of cells used. The results for the Series III compounds suggested the removal of the hydroxymethyl functional group and substitution of the mono-oxodithioacetal side chain of sparsomycin with a substituted benzyl amide moiety was not beneficial for activity. Finally, examination of the collective data revealed that the bromobenzyl-substituted analogues consistently imparted the greatest inhibitory activity, while the methoxybenzyl-substituted analogues displayed the least. The methylbenzyl and the unsubstituted

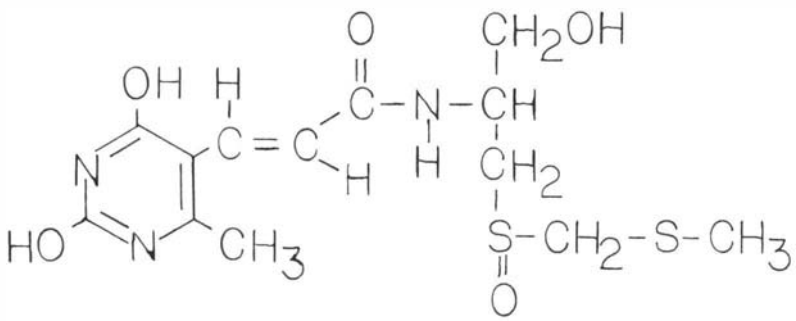
benzyl compounds were intermediate in inhibitory potency. The activity may correspond to the lipophilic and electronic characteristics of the substituents on the benzyl moiety of the analogues. It appears that the bromobenzyl-substituent of hydrophobic and electron withdrawing character is optimal for inhibitory activity, and conversely, the methoxybenzyl substituent of hydrophilic and electron donating character is least desirable.

I. INTRODUCTION

The advent of antibiotics, initiated by the discovery of penicillin's antimicrobial activity, marked a great advance in chemotherapy. Since that time, the development and expansion of screening techniques for chemotherapeutic agents have enabled investigators to isolate several thousand new antibiotics, but few of these have displayed the selective toxicity desired for clinical use (1). To assess the potential therapeutic value, each substance identified required detailed characterization, including investigation of the mechanism of action.

In 1962, Owen, Dietz, and Camiener reported the isolation of a new antitumor antibiotic from the culture filtrate of Streptomyces sparsogenes var. sparsogenes (2). Separation and purification of the antibiotic were achieved by Argoudelis and Herr using partition chromatography and countercurrent distribuition (3). The structure of the crystalline antibiotic, named sparsomycin (Figure I , remained elusive until 1970 when Wiley and MacKellar reported results of spectroscopic and degradation studies which elucidated the structure (4,5). In addition to the trans olefinic bond, the molecule featured one chiral carbon atom which had S-configuration, and one chiral sulfur atom with R-configuration(6).

FIGURE I. SPARSOMYCIN



The initial pharmacological evaluation of sparsomycin revealed it possessed activity against KB human epidermoid carcinoma cells in tissue culture, against a variety of gram-negative and gram-positive bacteria, and against fungi (2, 3,7,8). The biological activity displayed by sparsomycin stimulated the investigation of the biochemical mechanisms responsible for the broad spectrum of activity. Experiments performed to measure DNA, RNA, and protein synthesis in Escherichia coli (E. coli) cells indicated sparsomycin acted primarily by inhibiting protein synthesis and correspondingly, was most cytotoxic to cells during the S phase of the cell cyle (7,9-11).

Many antibiotics, including sparsomycin, accomplish their inhibition of protein synthesis by interfering with the function of ribosomes, the macromolecular complexes on which decoding of the genetic message occurs (12). Prokaryotic and eukarotic ribosomes can be distinguished via sedimentation coefficients (abbreviated S) and are referred to as 70S and 80S ribosomes, respectively. By dialysis with buffers containing magnesium ions, ribosomes can be fractionated into subunits with lower sedimentation coefficients. Bacterial ribosomes dissociate into 50S and 30S fragments and mammalian ribosomes cleave into 60S and 40S subunits (12).

The ribosomes are specifically designed for protein synthesis and it is at these sites that the L-amino acids are assembled and coupled to form the polypeptide chains which compose proteins. The process can be divided into

four stages (13-22)(Figure II). The first stage, the activation step is the only one which takes place entirely in the soluble cytoplasm. In this stage, the amino acids are enzymatically esterified to their corresponding transfer RNA (tRNA) molecules by aminoacyl-tRNA synthetases. The products of these reactions, the aminoacyl-tRNA molecules, are used in the next stage. Prior to the second stage, however, the genetic code of the DNA molecule carrying the information specifying the sequence of amino acids required for synthesis of a particular protein is transcribed onto a newly synthesized single strand of messenger RNA (mRNA) by the action of RNA polymerase. During the second stage, the initiation step, this mRNA and the initial aminoacyl-tRNA of the sequence bind to the small subunit of the ribosome (40S). The large ribosomal subunit, 60S, then attaches to form a functional ribosome, which is ready to translate the transcribed information of the mRNA into the corresponding protein molecule.

Specificity of protein synthesis is incorporated into the binding of the mRNA and the aminoacyl-tRNA by the interaction of specific nucleotide base triplets present on each. The tRNA moiety of the aminoacyl-tRNA molecule contains a triplet of nucleotides, the anticodon, which pairs by hydrogen bonding with a complementary triplet, the codon, on the mRNA molecule. Although ribosomes do not contribute to the specificity of protein synthesis, they provide the appropriate environment for the codon-anticodon interaction

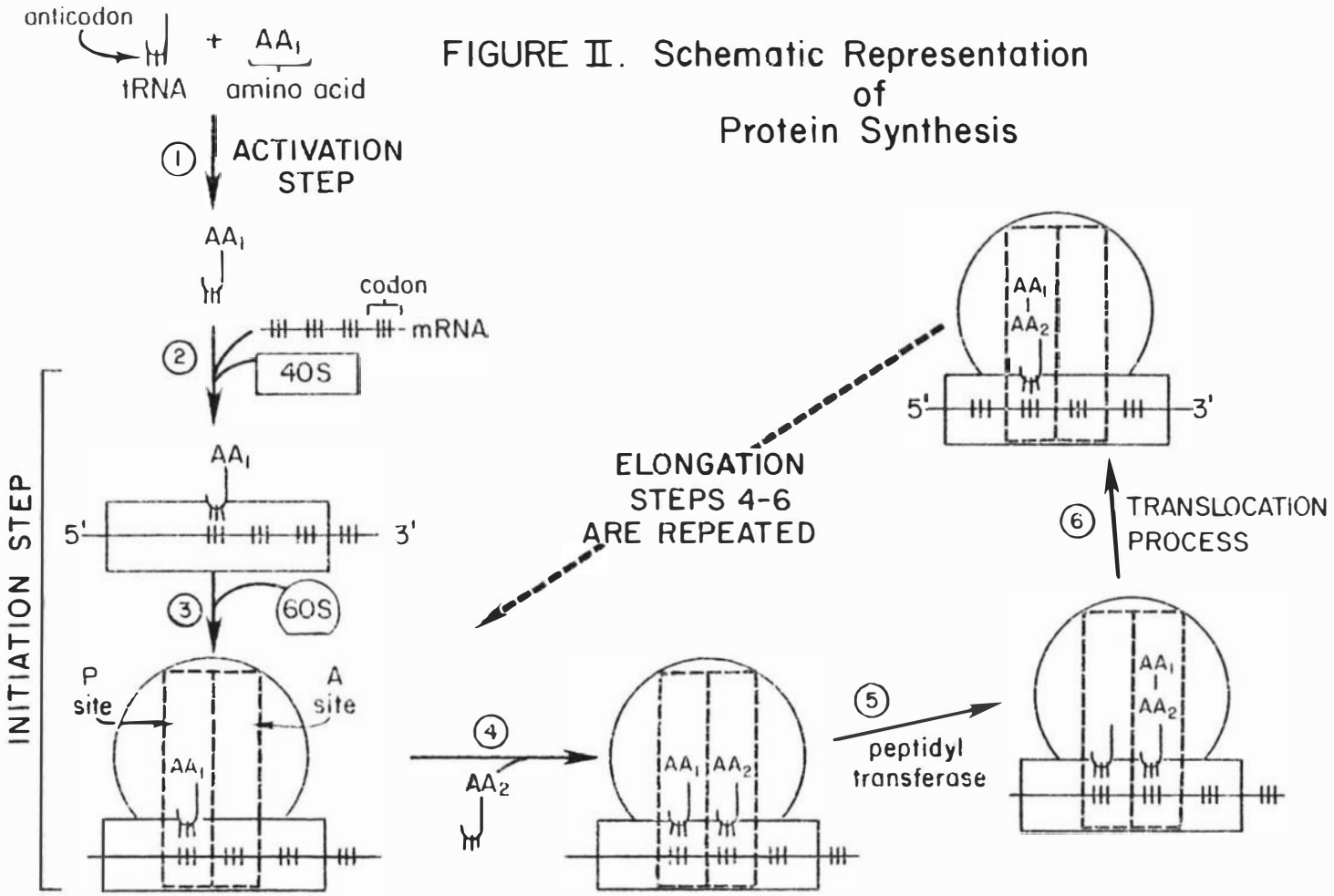
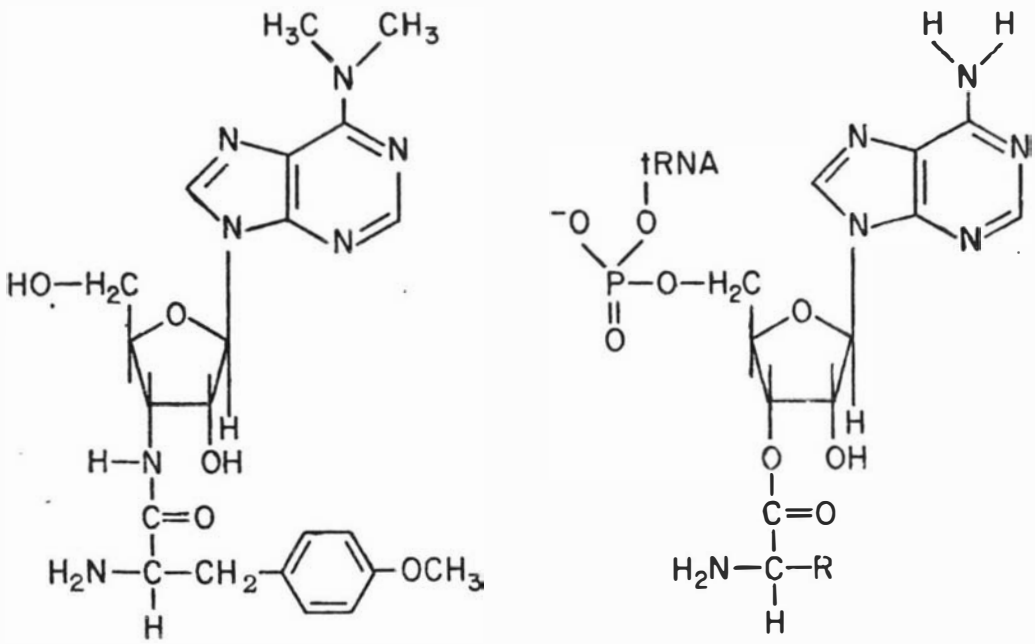


FIGURE II. Schematic Representation of Protein Synthesis

and subsequent peptide bond formation. The ribosome contains two sites on which the tRNA molecules may be located during the third stage of protein synthesis, the elongation cycle. During this stage the peptide bond is formed and the polypeptide chain is lengthened. Peptide bond formation begins with the nascent peptide bound to one of the two sites, the peptidyl donor site or P site, and the aminoacyl-tRNA bound to the other, the aminoacyl acceptor site or A site. The first reaction to occur is the transfer of the nascent peptide to the aminoacyl-tRNA. This reaction is catalyzed by the enzyme peptidyl transferase, an integral part of the larger ribosomal subunit, and the product is a polypeptide lengthened at its carboxy terminus by one amino acid residue. The translocation process follows, which shifts the elongated peptide still attached to tRNA from the A site to the P site. Meanwhile, the deacylated tRNA, which had carried the nascent peptide, is ejected from the P site and the ribosome moves toward the 3' end of the mRNA by shifting over one codon unit. Repetition of the process results in a polypeptide chain lengthened by sequential addition of new amino acids from the aminoacyl-tRNA esters, each bound to the ribosome in response to a specific codon in the mRNA molecule. After the formation of each new peptide bond, the ribosome moves along the mRNA aligning the next codon in the correct orientation to bind with the next aminoacyl-tRNA anticodon. This continues until the final stage of protein synthesis, the termination step, in which appropriate

codons in the mRNA signal the completion of the polypeptide chain and the product is released from the ribosome.

The mechanism of peptide bond formation on ribosomes was clarified through research performed to elucidate the mode of action of the antibiotic puromycin (12,13,23). Puromycin is a structural analogue of aminoacyl-adenosine (Figure III), the 3' terminal nucleotide of aminoacyl-tRNAs. In contrast to the normal ester linkage between the 2'- or 3'-hydroxyl group of the ribose moiety and the carboxyl group of the amino acid of the aminoacyl-tRNA, puromycin contains an amide linkage between its sugar component and the carboxyl group of the 4-methoxyphenylalanine portion of the molecule. Despite this variation, puromycin can bind to the ribosomal A site in a manner analogous to the 3' terminus of aminoacyl-tRNA and can form a peptide linkage with the nascent peptide chain. Puromycin interrupts peptide chain elongation, however, as it forms a covalent peptidyl-puromycin derivative. The formation of peptidyl-puromycin can be measured directly by determining the incorporation of [^{3}H]-puromycin into nascent polypeptide chains (24). Since the peptidyl-puromycin which forms does not have the precise structure for recognition by the translocation apparatus to move it to the P site, the derivative dissociates from the ribosome. The similarities between the puromycin reaction and the true peptide bond forming reaction have promoted the puromycin reaction as a useful model for studying the mechanism of peptide bond formation as well as the mode of action of other



PUROMYCIN

AMINOACYL-ADENOSINE
TERMINAL NUCLEOTIDE

FIGURE III. COMPARISON OF PUROMYCIN AND THE TERMINAL NUCLEOTIDE OF t-RNA

antibiotics.

Puromycin has played a key role in determining the mechanism of action of the antibiotic sparsomycin, one of the few agents found to specifically inhibit protein synthesis in both 70S and 80S ribosomal centers (7). In an attempt to identify the precise location of interaction, the puromycin reaction has been used to accumulate evidence which suggests that sparsomycin inhibits the peptide bond forming step of protein synthesis (25). In the puromycin reaction, the transfer of the nascent peptide chain from the P site of the ribosome to the amino group of puromycin results in the release of the nascent peptide from the ribosome as peptidyl-puromycin. This reaction is readily blocked by sparsomycin (16,23,26-32) (Figure IV).

Experiments performed to verify its inhibitory effect have shown that sparsomycin blocks the puromycin-induced release of polylysine from polylysyl-tRNA bound to Escherichia coli ribosomes (32,33). The formation of polylysyl-puromycin serves as a model for the formation of a single peptide bond. Sparsomycin does not cause the deacylation of polylysyl-tRNA nor does it prevent the binding of aminoacyl-tRNA, but it does act as a competitive inhibitor of the peptidyl transferase reaction with respect to puromycin (25,27, 28,34). To further clarify the action of sparsomycin, its effect on the limited addition of labeled lysyl-tRNA to polylysyl-tRNA bound to ribosomes was studied. This reaction has been shown to involve the addition of one lysyl

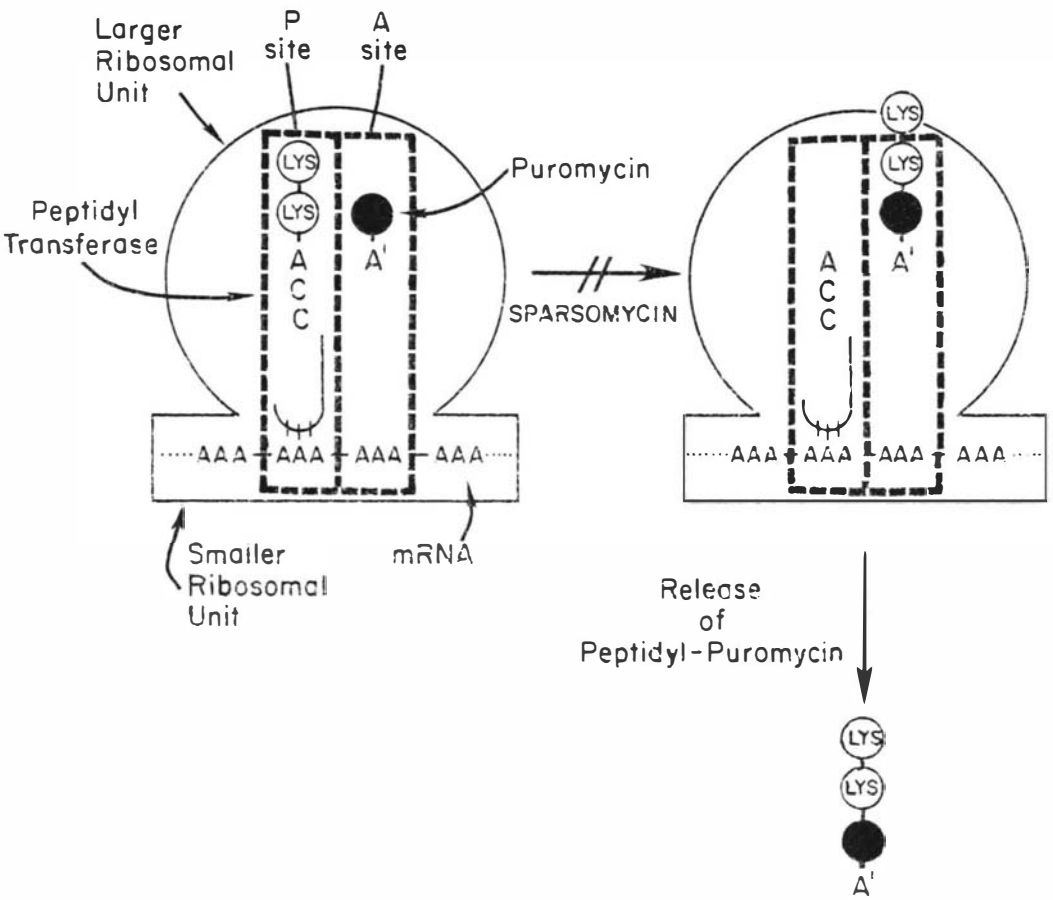


FIGURE IV. THE PUROMYCIN REACTION

residue per reacting peptide chain and is extremely sensitive to inhibition by sparsomycin. Increasing the concentration of lysyl-tRNA, for which puromycin serves as an analogue, does not overcome the inhibition. This result is expected since aminoacyl-tRNA depends primarily on codon recognition and interaction with the smaller ribosomal subunit for its binding to the ribosome, whereas puromycin interaction occurs at another location in the A site of the ribosome, presumably with the peptidyl transferase itself. The data showing that sparsomycin competes with the puromycin but not with aminoacyl-tRNA binding suggest that sparsomycin, like puromycin, acts at or close to the site where peptide bond formation takes place, the peptidyl transferase (33).

Additional support for the supposition that sparsomycin inhibits peptide bond formation has been generated from experiments which examined the formation of a single peptide bond isolated from the other steps of polypeptide synthesis. One of the reactions studied, the "fragment reaction", utilized a short fragment of transfer RNA, the 3' terminal cytidylic-cytidylic-adenylic acid residue (C-C-A). In this reaction, an N-substituted aminoacyl or peptidyl moiety attached to the short fragment similar to the 3' terminus of tRNA was transferred to puromycin or another acceptor in the presence of only the larger ribosomal subunit (50S) (33,35-41). In an experiment investigating the "fragment reaction", acetylleucine was transferred from C-C-A to puromycin, forming acetylleucine-puromycin and C-C-A (36)(Fig-

ure V). This "fragment reaction" has the advantage that interactions between substrates and ribosomes are confined to the immediate vicinity of peptidyl transferase. Sparsomycin inhibits this "fragment reaction", providing further evidence that its target for inhibiting protein synthesis is the peptidyl transferase.

Supplementary reports indicate that sparsomycin interacts with ribosomal peptidyl transferase to form an inert complex between the 50S subunit and the C-C-A-peptidyl moiety, thereby "freezing" the peptidyl-tRNA in the P site on the ribosome (30,33,40,42-44). Low concentrations of sparsomycin stimulate the binding of peptidyl-tRNA to ribosomes whereby it cannot be released by puromycin. The "freezing" effect of sparsomycin evident in the "fragment reaction" may involve the steps shown in Figure VI (30,38). In the first step, a C-C-A-peptidyl group binds to the P site on the 50S subunit, in the vicinity of the peptidyl transferase catalytic center. In the second step, the bound fragment either reacts with puromycin or interacts with sparsomycin to give the noninterconvertible products, peptidyl-puromycin or the sparsomycin complex, respectively. The formation of either product irreversibly blocks the normal process of peptide elongation.

The preponderance of evidence substantiates the hypothesis that sparsomycin interferes with protein synthesis by inhibiting peptide bond formation by peptidyl transferase. As discussed earlier, Goldberg and Mitsugi (25,27) and

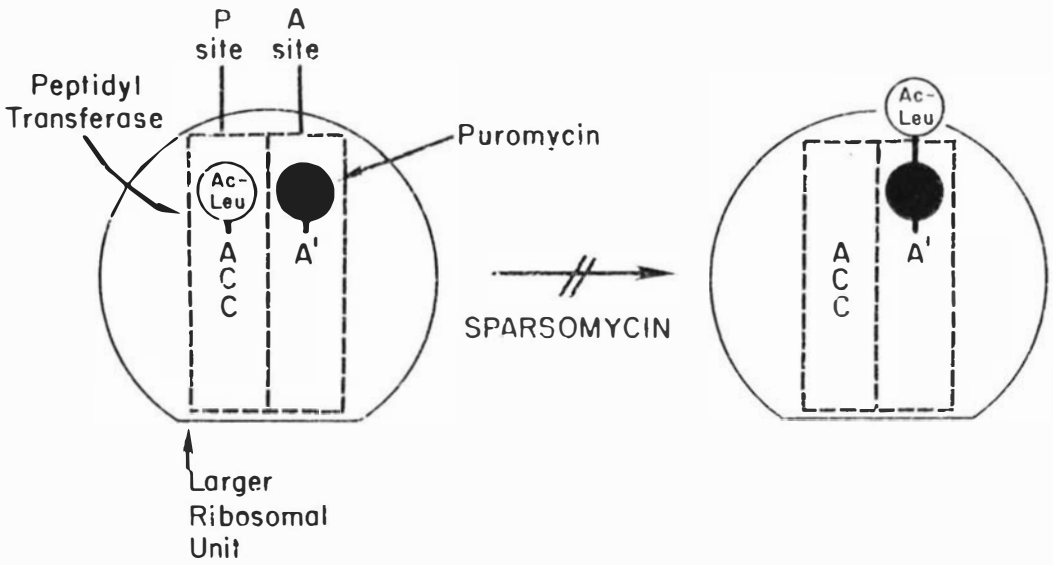


FIGURE V. THE FRAGMENT REACTION

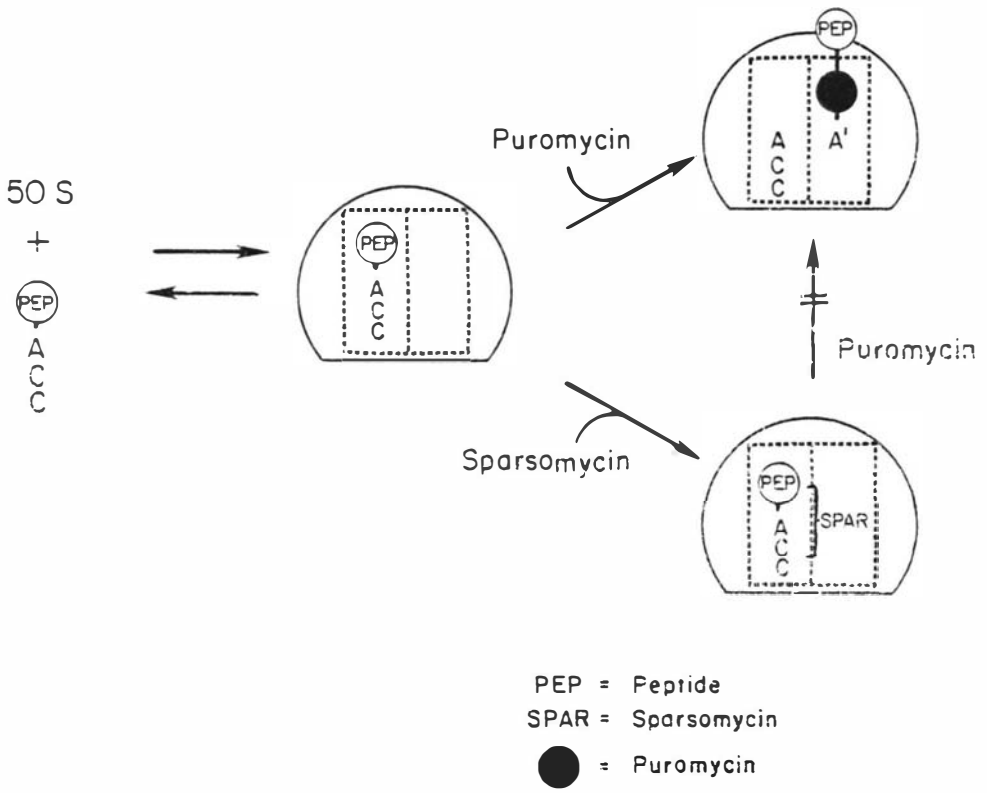


FIGURE VI. PROPOSED SCHEME OF SPARSOMYCIN'S MECHANISM OF ACTION

Pestka (28,24) have observed that inhibition by sparsomycin of the reaction between ribosome-bound aminoacyl-tRNA and puromycin displays competitive kinetics. This suggests that sparsomycin interferes with the peptide bond forming step either directly or by an allosteric mechanism.

In order to facilitate the study of sparsomycin, its biochemical mode of action, pharmacology, and toxicology, a synthetic route for production of the drug was sought. Until 1979, sparsomycin had to be obtained by isolation from bacteria followed by purification. In that year, Ottenheijm, Liskamp, and Tijhuis reported the first total synthesis of the enantiomer of naturally occurring sparsomycin (45).

The sparsomycin molecule was divided into two intermediate segments (A and B), which were coupled in the final step of a convergent synthesis (Figure VII). Two procedures were developed for the preparation of intermediate A, the β-uracilylacrylic acid 6, with a trans configuration (46) (Scheme I). Both preparations began with 5-hydroxymethyl-6-methyluracil (2) made from 6-methyluracil (1) with formaldehyde and aqueous sodium hydroxide. When the hydroxymethyl uracil 2 was treated with hydrobromic acid in glacial acetic acid, the alkyl bromide 7 resulted, which could be converted into the phosphonium salt 8 by reacting 7 with triphenylphosphine in dimethylformamide. A Wittig reaction of the phosphonium salt 8 and n-butyl glyoxylate afforded a low yield of 9. This approach was abandoned when an alternative method was found to result in superior yields. The

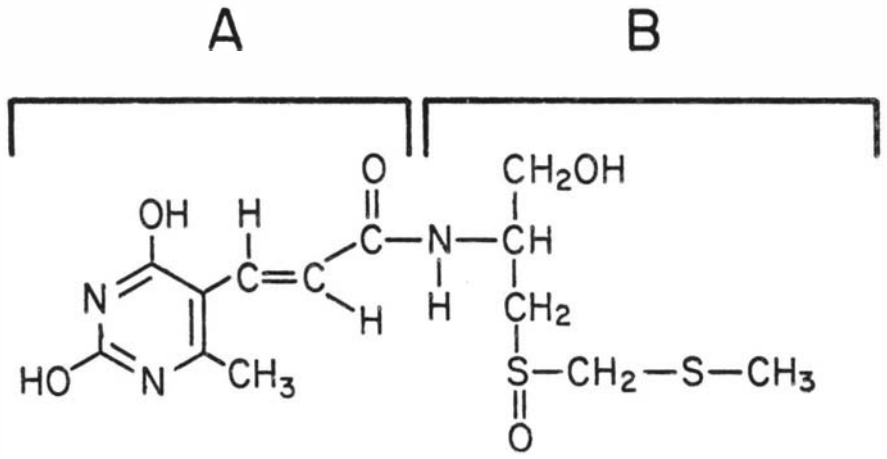
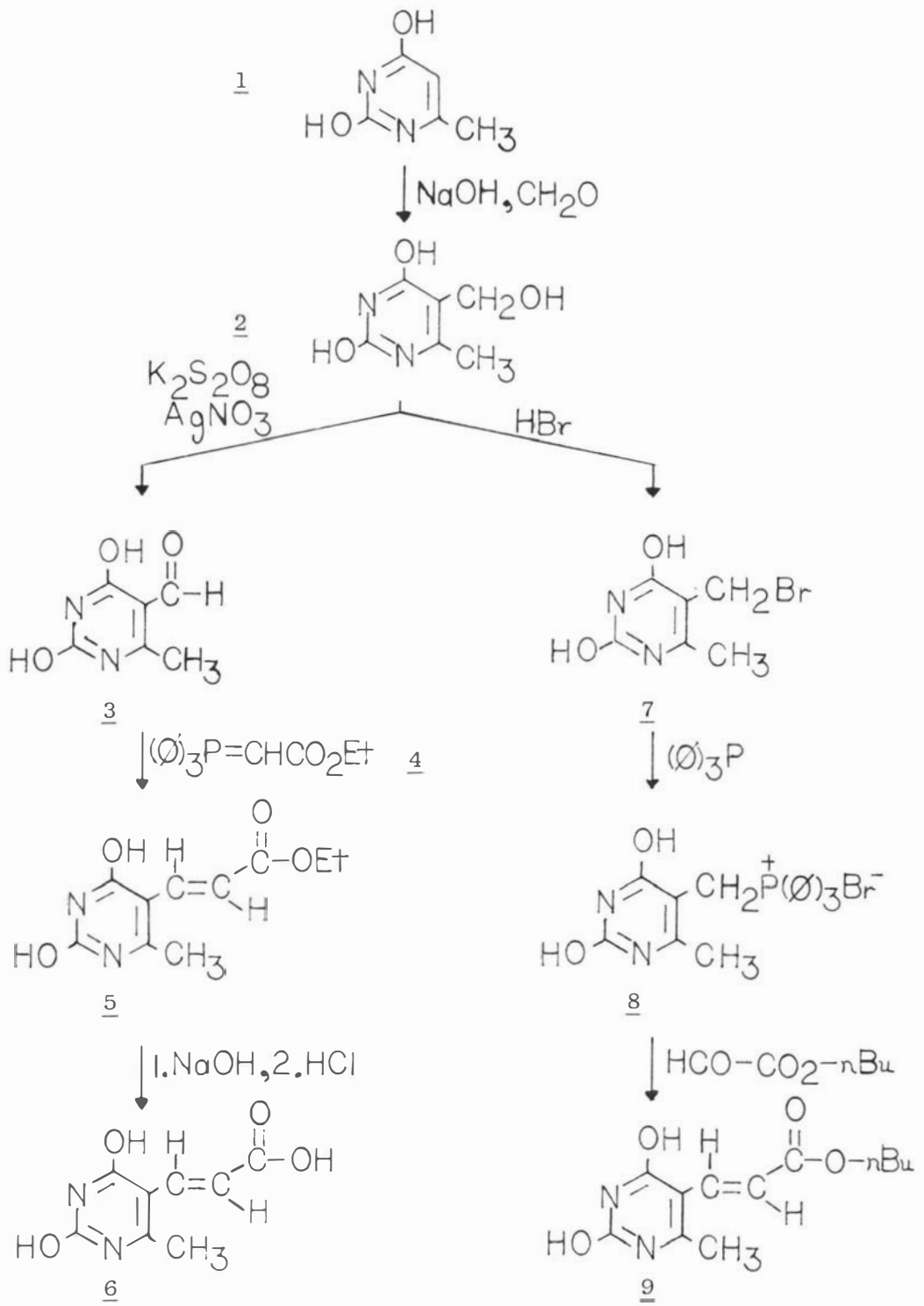


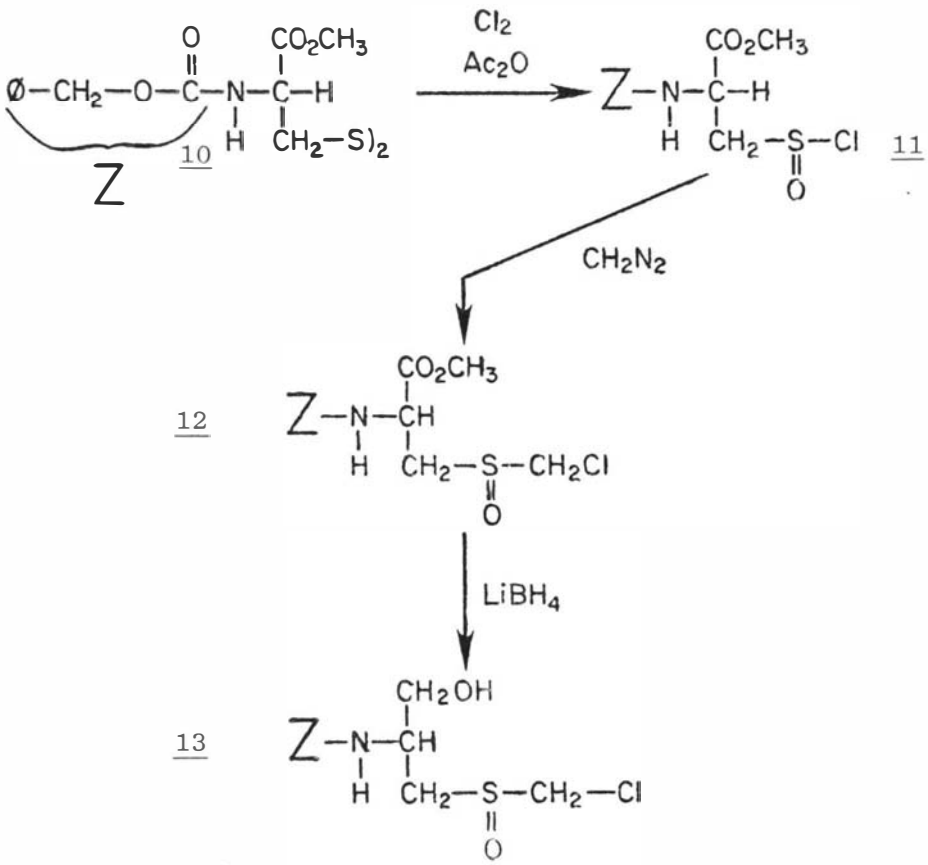
FIGURE VII. SPARSOMYCIN


1
NaOH, CH2O
2
K2S2O8
AgNO3
HBr
3
7
(Ø)3P=CHCO2Et
4
(Ø)3P
5
8
1.NaOH, 2.HCl
HCO-CO2-nBu
6
9

SCHEME I

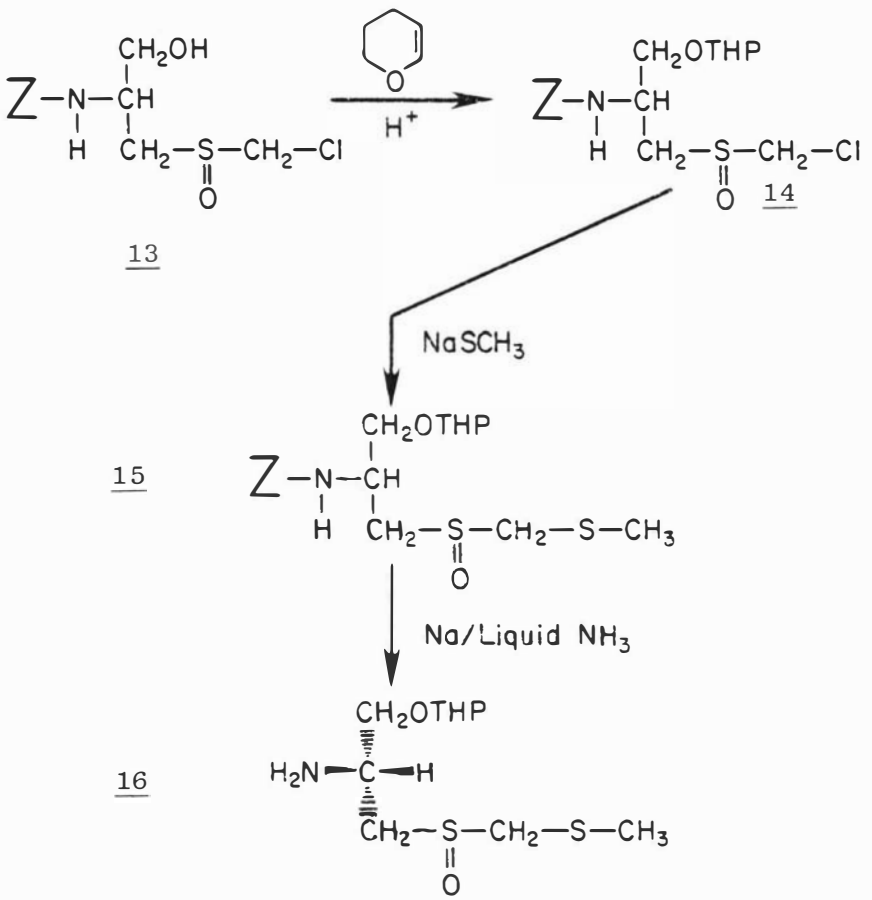
alcohol 2 was oxidized to the aldehyde 3 with potassium persulfate and a trace of silver nitrate. In an inverse Wittig reaction, the aldehyde 3 was coupled with carbethoxymethylenetriphenylphosphorane 4 to form the ester 5 in the trans configuration. Alkaline hydrolysis of the ester in dioxane, methanol, and water, followed by acidification gave the acid 6 (Scheme I).

The second key intermediate B, the protected amino alcohol moiety (16) of sparsomycin, was considered as an S-alkylated derivative of cysteine (Scheme II and III) (45,47). Synthesis of this portion of the molecule began by treating N-benzyloxycarbonyl L-cystine methyl ester 10 (R configuration) with chlorine in the presence of acetic anhydride to give the sulfinyl chloride 11. The sulfinyl chloride 11 was reacted with diazomethane to give the chloromethylsulfoxide 12. Substitution of the chloro function in 12 with methyl mercaptide had to occur after reduction of the ester function, to avoid cleavage of the thioacetal side chain. The ester function of 12 was reduced with lithium borohydride producing the alcohol 13. To circumvent problems with cyclization, the alcohol function of 13 was protected with the tetrahydropyranyl group forming 14. Treatment of the protected alcohol 14 with sodium methylmercaptide in ethanol gave the desired mono-oxodithioacetal 15. The problem of selective removal of the benzyloxycarbonyl group was surmounted by treating a solution of 15 in liquid ammonia with sodium. The desired amine 16 was isolated by column chromatography.



SYNTHESIS OF FRAGMENT B

SCHEME II

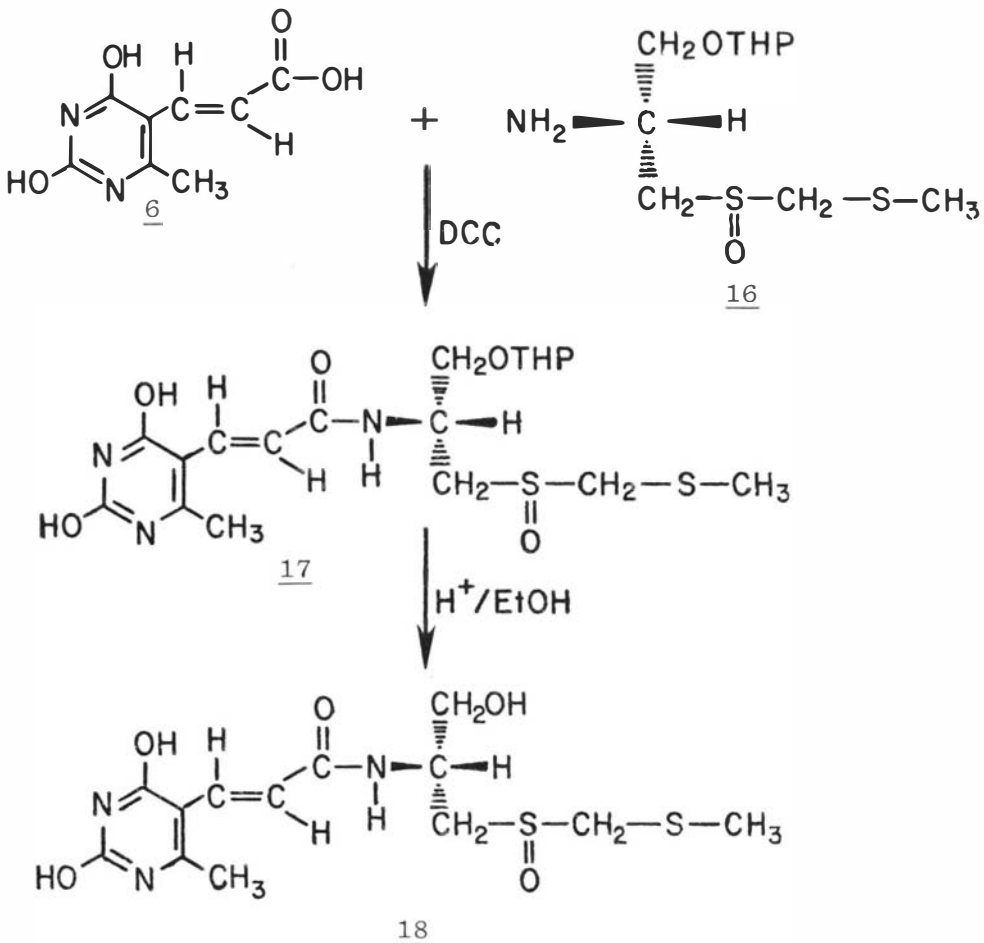


SYNTHESIS OF FRAGMENT B

SCHEME III

The two intermediates of the convergent synthesis, the acrylic acid 6 and the amine 16, were coupled using dicyclohexylcarbodiimide and hydroxybenztriazole to give 17 (Scheme IV). The tetrahydropyranyl group of 17 was removed by heating at reflux an acidified ethanol solution of 17 for fifteen minutes to give the final product, enantiomeric sparsomycin, 18.

The completion of the total synthesis of sparsomycin was a significant accomplishment as it provided access to greater quantities of the material for investigational use. Sparsomycin has aroused the interest of investigators not only for its synthetic challenge and value as a tool for biochemical studies probing the mechanism of protein synthesis, but also for its activity as an antitumor agent. Sparsomycin has been of limited value in the treatment of cancer in man, however, due to its toxicity (33,48). During phase I clinical trials, sparsomycin caused unusual ocular toxicity which hindered its development as an antitumor agent. Analogues of sparsomycin, obtained by structurally modifying the chemical composition of the molecule, may reduce or eradicate the toxic effects of sparsomycin while maintaining its antitumor activity. Preparation and evaluation of such agents are the aim of this research.



CONVERGENT SYNTHESIS OF SPARSOMYCIN

SCHEME IV

II. RESEARCH AIM

Pharmacological studies have revealed that sparsomycin possesses a broad spectrum of biological activity, including antitumor, antifungal, and antibacterial properties. On the basis of its significant antitumor activity, sparsomycin was selected for phase I clinical trials. As discussed in the previous chapter, the study was terminated due to the occurrence of ocular toxicity (48). The desire to nullify the toxic properties and clarify the biochemical mode of action prompted the development of sparsomycin analogues (49-52).

The first active analogue of sparsomycin was reported by Vince, Brownell, and Lee (52) in 1977. The structure of this analogue (Figure VIII) incorporated an S-deoxo-S-propyl moiety instead of the mono-oxodithioacetal side chain of sparsomycin. The S-deoxo-S-propyl sparsomycin analogue significantly inhibited protein synthesis by E. coli ribosomes. The activity exhibited by this simplified sparsomycin molecule led to the synthesis of additional analogues to establish a more extensive structure-activity relationship for the antibiotic.

Lee and Vince (50) reported the synthesis of several sparsomycin analogues which contained modifications of the amino alcohol portion of sparsomycin (Table I). In their

FIGURE VIII. S- DEOXO-S-PROPYL SPARSOMYCIN ANALOGUE

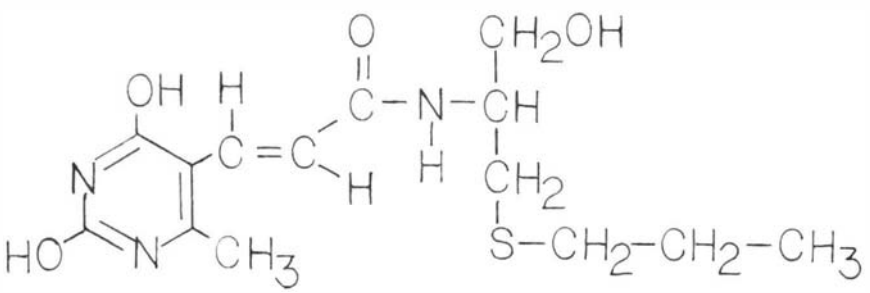
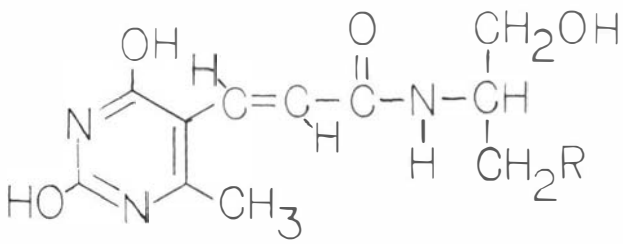


TABLE I: COMPETITIVE INHIBITION OF N-ACETYL[^{14}C] PHENYLALANYL-PUROMYCIN SYNTHESIS BY E. COLI RIBOSOMES WITH SPARSOMYCIN ANALOGUES (49)



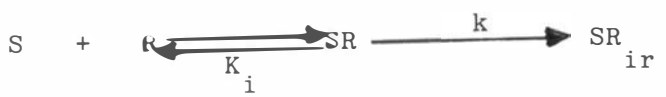
R	STEREO-CHEMISTRY	K_i (μM)	COMPOUND
SPARSOMYCIN	D	0.50 ± 0.01	18
SCH_2SCH_3	DL	1.09 ± 0.13	18a
$S(CH_2)_2CH_3$	DL	2.00 ± 0.19	18b
$S(CH_2)_2CH_3$	D	1.10 ± 0.13	18c
$S(CH_2)_2CH_3$	L	INACTIVE	18d
$SCH_2C_6H_5$	DL	1.21 ± 0.14	18e
H	DL	INACTIVE	18f
$(CH_2)_3CH_3$	DL	2.30 ± 0.20	18g

work, Lee and Vince assessed the stereochemical specificity of binding of the S-deoxo-S-propyl analogue by preparing the D and L isomers 18b, 18c, 18d. Since the chirality of the asymmetric carbon of sparsomycin was identical to the chirality of D cysteinol (5), Vince and Lee anticipated only the D isomer of the racemic analogues would be capable of ribosomal binding. As expected, the D isomer 18c of the S-deoxo-S-propyl analogues was about twice as active as the DL mixture 18b in inhibiting the puromycin reaction in E. coli ribosomes. Lin and Dubois (51) substantiated the conclusion that the activity of sparsomycin analogues had a D configurational dependency.

In addition to the stereochemical requirements, Lee and Vince studied the contribution to ribosomal binding by the sulfur atom of the $-CH_2-S-CH_3$ portion of sparsomycin (50). A comparison of S-deoxosparsomycin (18a) and the S-deoxo-S-propyl analogue (18b) showed a twofold decrease in binding when a sulfur was replaced by one methylene unit. This result indicated the sulfur atom directly contributed to binding of E. coli ribosomes. Further modifications of the dithioacetal portion of the molecule resulted in the preparation of a compound in which the entire mono-oxodithioacetal side chain was replaced by a hydrogen atom (18f). This analogue was completely inactive. Conversely, replacement of the mono-oxodithioacetal side chain by an S-deoxo-S-benzyl group (18e) resulted in a derivative which displayed binding comparable to S-deoxosparsomycin. The experimental

data suggested hydrophobic groups extending into the region occupied by the $-CH_2-S-CH_3$ chain of sparsomycin were tolerated.

Another structural parameter examined by Lee and Vince was the sulfoxide moiety of sparsomycin. A comparison of the K_i values derived from E. coli ribosomes for D sparsomycin (18) and DL-S-deoxosparsomycin (18a) showed the calculated value for D-deoxosparsomycin was equivalent to the 0.5 μM value of sparsomycin. This led to the conclusion that the sulfoxide oxygen did not significantly contribute to binding. Moreover, the S-deoxo-S-propyl analogue 18b and the analogue 18g which lacked both sulfur atoms displayed equivalent binding affinities indicating that relatively little binding was attributed to the sulfoxide sulfur. Other experiments demonstrated, however, that the sulfoxide moiety significantly contributed to sparsomycin's ability to inhibit the puromycin reaction. When sparsomycin was incubated with the ribosomes prior to the addition of puromycin, an increased inhibition of the puromycin reaction was noted. Conversely, preincubation of the S-deoxosparsomycin analogue 18a with ribosomes did not affect the level of inhibition of the puromycin reaction. Lee and Vince suggested that the initial reversible sparsomycin-ribosome complex (SR) changed its conformation. The conformational change, which may have involved the formation of an irreversible complex (SR_{ir}), enabled sparsomycin to bind more tightly to the ribosomes than S-deoxosparsomycin (Equation I).



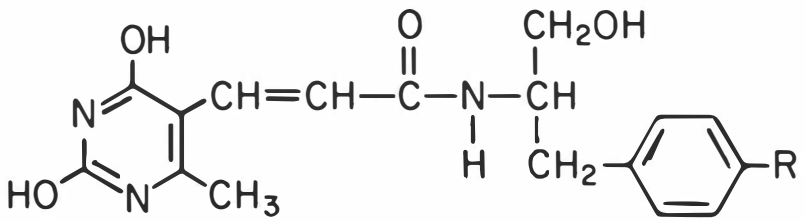
EQUATION I

Since the K_i values reported by Vince and Lee represented the dissociation constant of the initial reversible complex, the conclusion that the sulfoxide moiety of sparsomycin was not involved in the initial binding to the ribosome was valid. The results suggest, however, that a sulfoxide moiety, or equivalent substitution, may enable the sparsomycin molecule to undergo additional reaction at the binding site which results in irreversible inhibition of the puromycin reaction.

The final structural feature of the sparsomycin molecule examined by the investigators preparing analogues was the uracil ring. Dubois, et al.(49) replaced the uracil ring by numerous heterocyclic rings. None of the heterocyclic analogues displayed antitumor activity when assessed in systems using P388 lymphocytic or L-1210 lymphoid leukemia. The data suggested that the uracil ring was required for optimal activity.

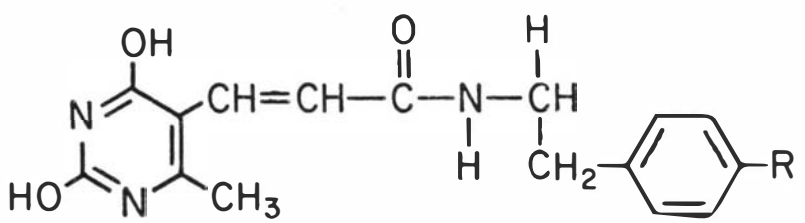
Overall, the small number of sparsomycin analogues previously prepared and evaluated limited definitive statements concerning the specific functional groups required for antitumor activity. In order to expand and clarify the structure-activity relationships for sparsomycin, the syntheses of four series of novel analogues of sparsomycin were proposed (Figure IX and X). The compounds of Series I and II were designed to elucidate the effect on activity of replacing the mono-oxodithioacetal side chain of sparsomycin with hydrophobic groups. To more clearly define the interaction,

FIGURE IX. PROPOSED COMPOUNDS OF SERIES I AND II



R = H
CH_3
OCH_3
Br

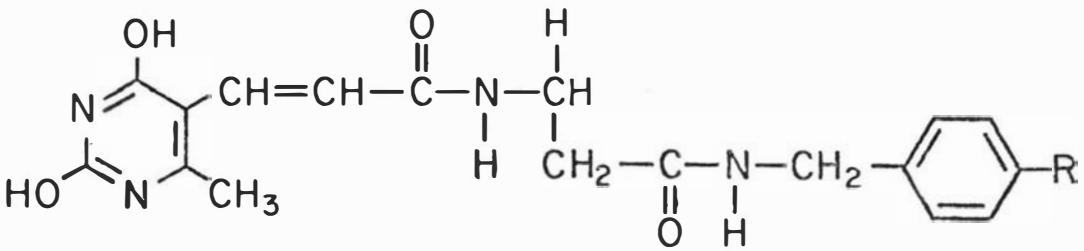
SERIES I



R = H
CH_3
OCH_3
Br

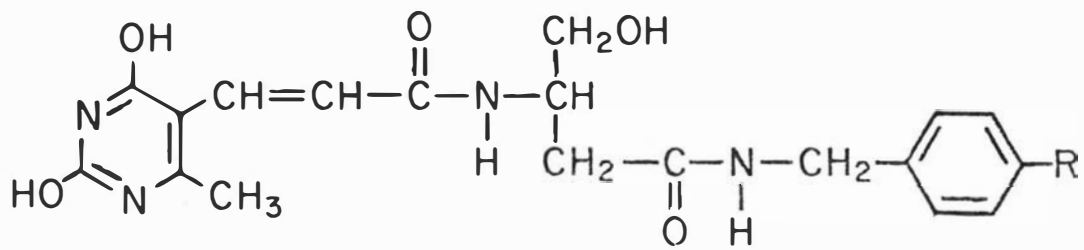
SERIES II

FIGURE X. PROPOSED COMPOUNDS OF SERIES III AND IV



R = H
CH_3
OCH_3
Br
3, 4 diCl

SERIES III



R = H
CH_3
OCH_3
Br
3, 4 diCl

SERIES IV

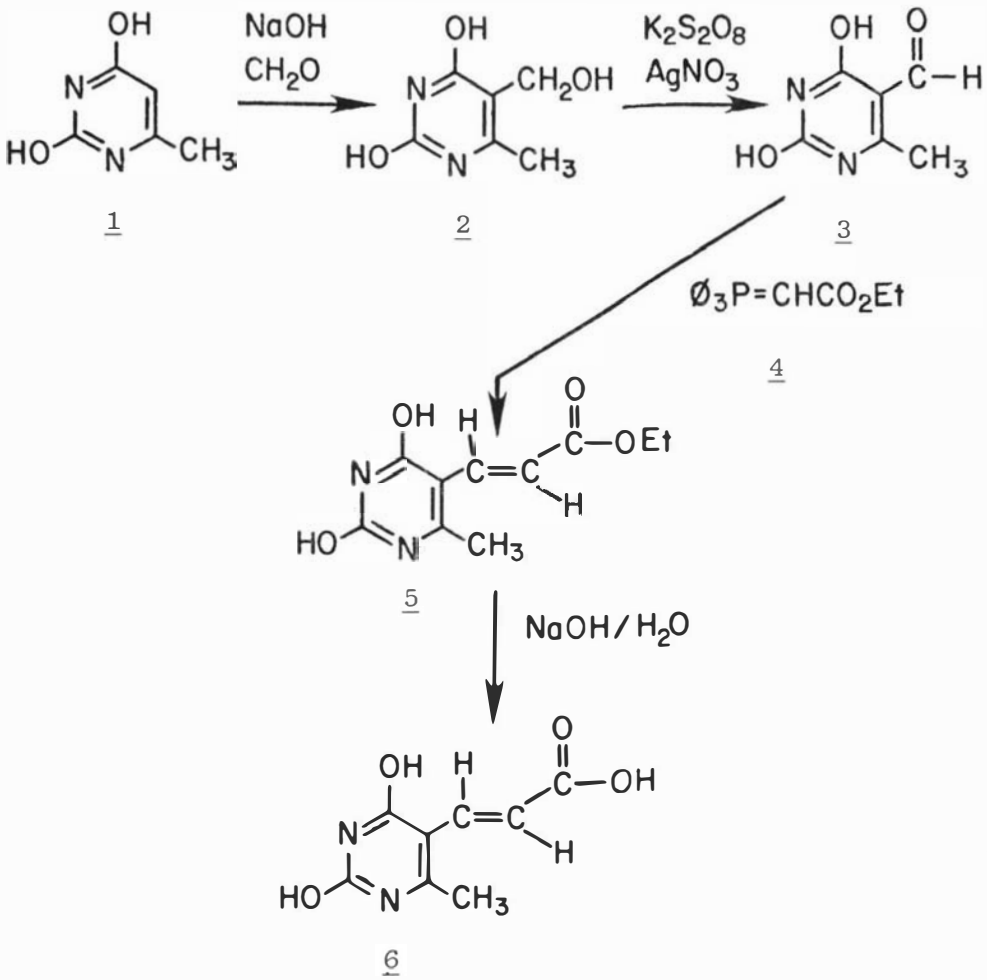
substitutions on the phenyl ring were made which varied the electronic and hydrophobic characteristics of the phenyl side chain. To further analyze the requirements for activity, the two series were designed to include (Series I) or exclude (Series II), the hydroxymethyl functional group found in sparsomycin. The compounds of Series III and IV were proposed to investigate the potential interaction of an amide oxygen in contrast to the sulfoxide oxygen of sparsomycin. Analogous to Series I and II, a hydrophobic phenyl ring with diverse substituents was included to elaborate the structure-activity relationships of sparsomycin. As in Series I and II, the feature distinguishing between Series III and IV was the inclusion or exclusion of the hydroxymethyl moiety.

Upon completion of the synthetic phase of this project, pharmacological studies using bone marrow cells, P388 lymphoma cells, and P815 mastocytoma cells will be performed to assess the ability of sparsomycin and the proposed analogues to inhibit protein synthesis, DNA synthesis, and cell growth.

III. DISCUSSION OF SYNTHESIS

Analogous to the approach for the total synthesis of sparsomycin, the structure of the target sparsomycin analogues was viewed as consisting of two fragments which could be coupled in the final step of a convergent synthesis. The synthesis of each target analogue utilized the same fragment A, the β-uracilacrylic acid 6. The second fragment, the amino moiety of the analogues, introduced the portion of the molecule which made the analogues unique. All of the amino fragments were prepared from racemic starting materials, which after coupling with acid 6, led to racemic final products.

The preparation of the acid 6 (Scheme V) was well documented in the literature (4,5,45-47) and began with 5-hydroxymethyl-6-methyluracil (2) prepared by reacting 6-methyluracil (1) with formaldehyde and aqueous sodium hydroxide. The alcohol 2 was oxidized to the aldehyde 3 with potassium persulfate and a catalytic amount of silver nitrate. In a Wittig reaction, the aldehyde 3 was condensed with carbethoxymethylenetriphenylphosphorane (4), which was synthesized by the method of Isler, et al. (53), using triphenylphosphine and ethyl bromoacetate. The product of the Wittig reaction, ester 5, contained a trans double bond as determined by the characteristic coupling constant of the vinylic



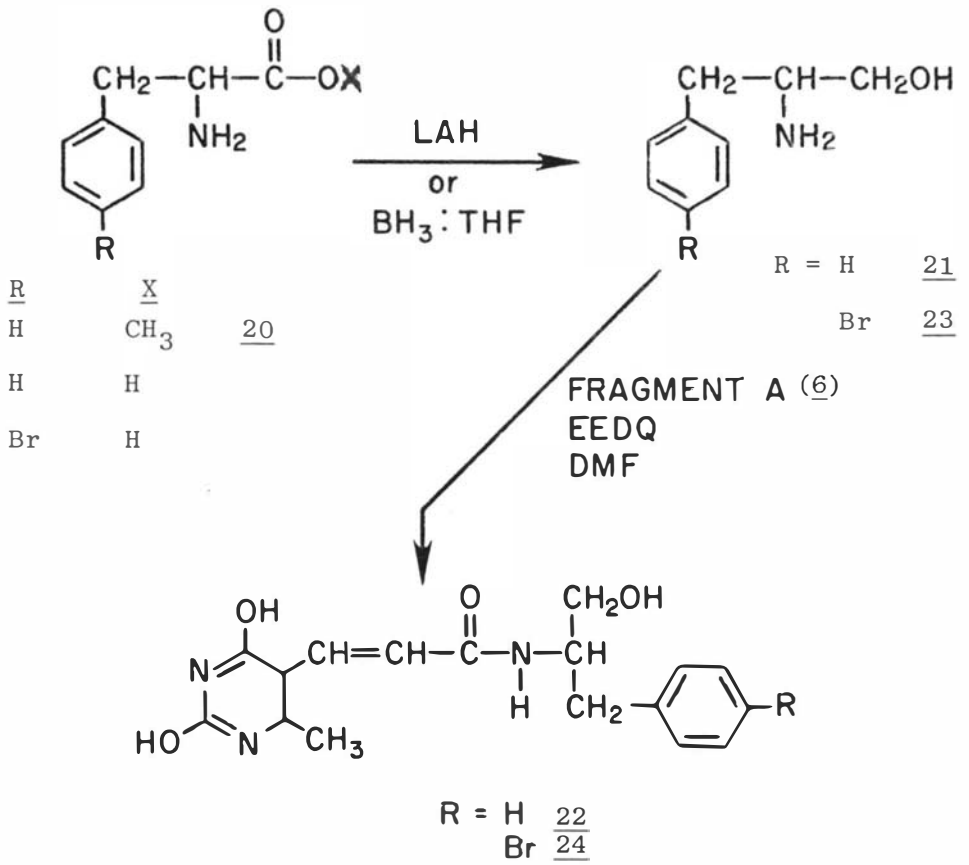
SYNTHESIS OF FRAGMENT A

SCHEME V

protons in the NMR, J = 16Hz (54). Alkaline hydrolysis of the ester 5 in dioxane, methanol, and water, followed by acidification, gave the acid 6 in quantitative yield.

In the final step of the synthesis of each novel compound, the acid 6 was coupled with an amino fragment via an amide bond. N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (55) was the reagent chosen to effect the coupling. The mechanism for the coupling reaction using EEDQ involved the formation of a mixed carbonic anhydride of the ethoxycarbonyl segment of EEDQ with the acid 6. The anhydride was subsequently attacked by the nucleophilic amine of the amino fragment forming an amide bond. Prior to the synthesis of the target analogues, the feasibility of the coupling reaction was tested in a model system. Using the method of Lin and Dubois (51), 2-aminoheptane was reacted with acid 6 to form compound 19. Since the reaction was successful in affording a good yield of a readily obtainable final product, EEDQ was chosen as the coupling reagent for the synthesis of all of the final compounds.

In the first series of analogues, 22, 24, 28, and 35, the amino fragments of the analogues were derived from substituted phenylalanine compounds. To prepare the amino fragment for compound 22 (Scheme VI), phenylalanine was esterified by the procedure of Dubois, et al. (49), affording the methyl ester 20. Using the method of Birkofer and Erlenbach (56), the ester 20 was reduced with lithium aluminum hydride to give the desired amino-alcohol 21. The fi-



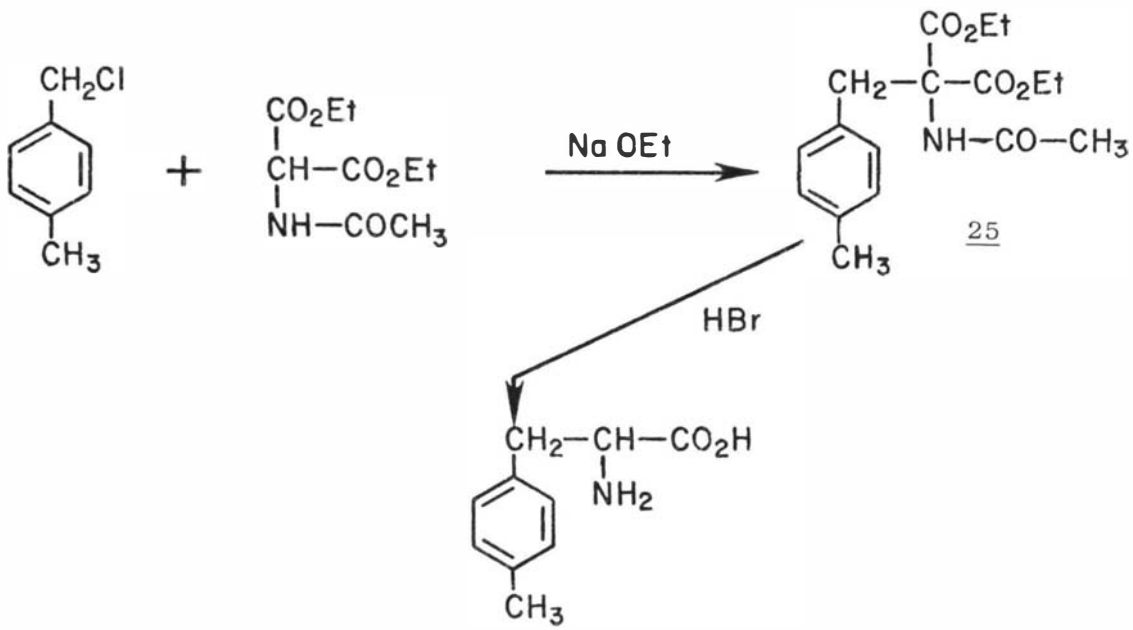
SYNTHESIS OF SERIES I

SCHEME VI

nal step in the synthesis of compound 22 was the coupling of amino-alcohol 21 with acid 6 using EEDQ.

The second compound of Series I, the bromo substitued analogue 24, was prepared by the method of Anhoury, et al. (57), which shortened the synthetic approach by one step. In this synthesis (Scheme VI), 4-bromophenylalanine was directly reduced with borane in tetrahydrofuran (58,59) to afford the amino-alcohol 23. As in the synthesis of analogue 22, the amino-alcohol 23 was coupled with acid 6 using EEDQ to afford the desired bromo substituted analogue 24.

The phenylalanine derivatives needed for the synthesis of the methyl (28) and methoxy (35) substituted analogues of Series I were not commercially available, which necessitated the inclusion of additional synthetic steps in the preparation of these compounds. The synthesis of the methyl substituted amine fragment for compound 28 (Schemes VII and VIII) was performed using the procedure of Albertson and Archer (60). The synthesis began with the reaction of 4-methylbenzyl chloride and diethyl acetamidomalonate in sodium ethoxide to give the diester-amide 25. The amide and both ester functionalities of 25 were hydrolyzed with 48% hydrobromic acid to give 4-methylphenylalanine 26. Nystrom and Brown (61) reported that amino acids could be reduced with lithium aluminum hydride to form amino alcohols. Using the procedure of Lee and Vince (50), the amino acid 26 was directly reduced with lithium aluminum hydride. The product of the reduction, the amino-alcohol 27, was coupled with acid 6 using EEDQ to afford the methyl-substituted product 28.

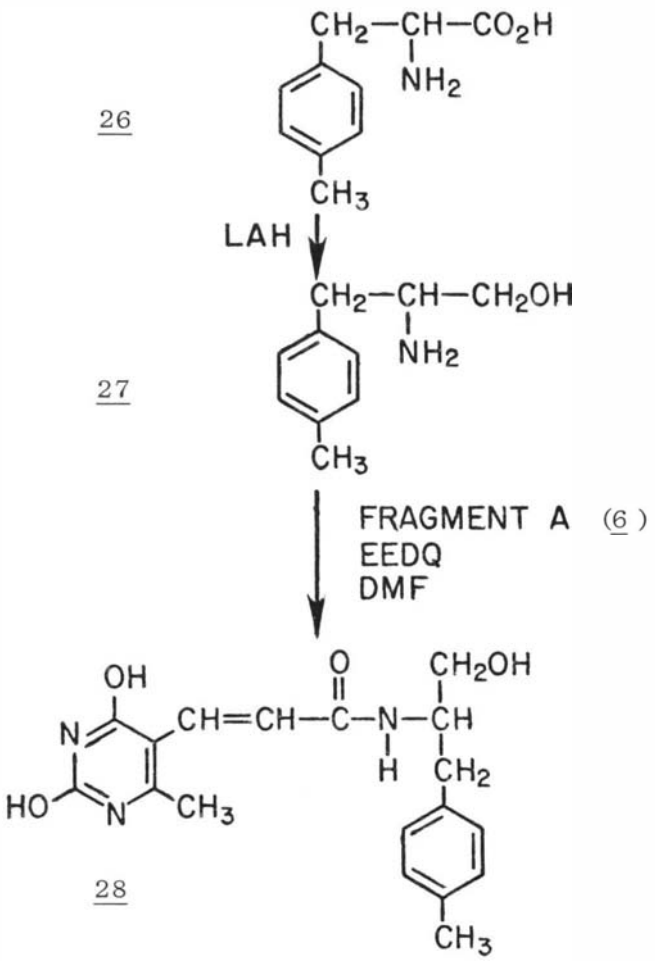


25

26

SYNTHESIS OF SERIES I

SCHEME VII

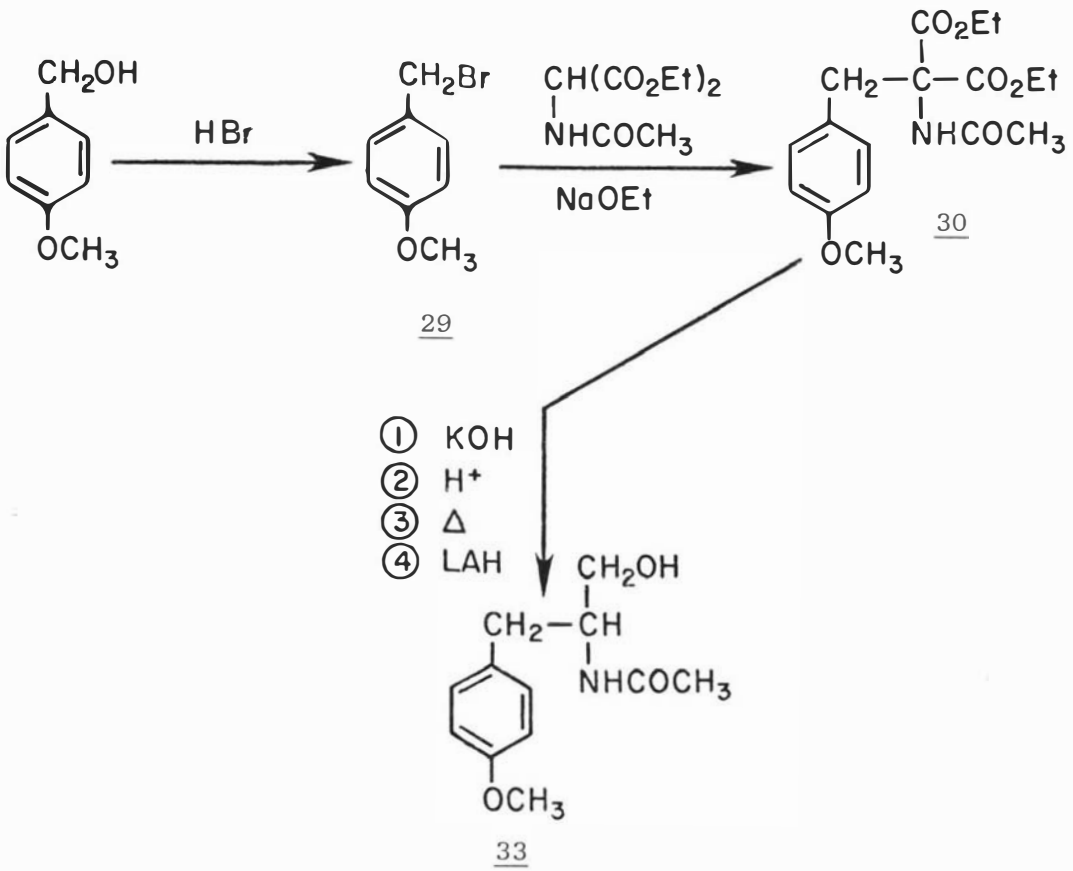


SYNTHESIS OF SERIES I

SCHEME VIII

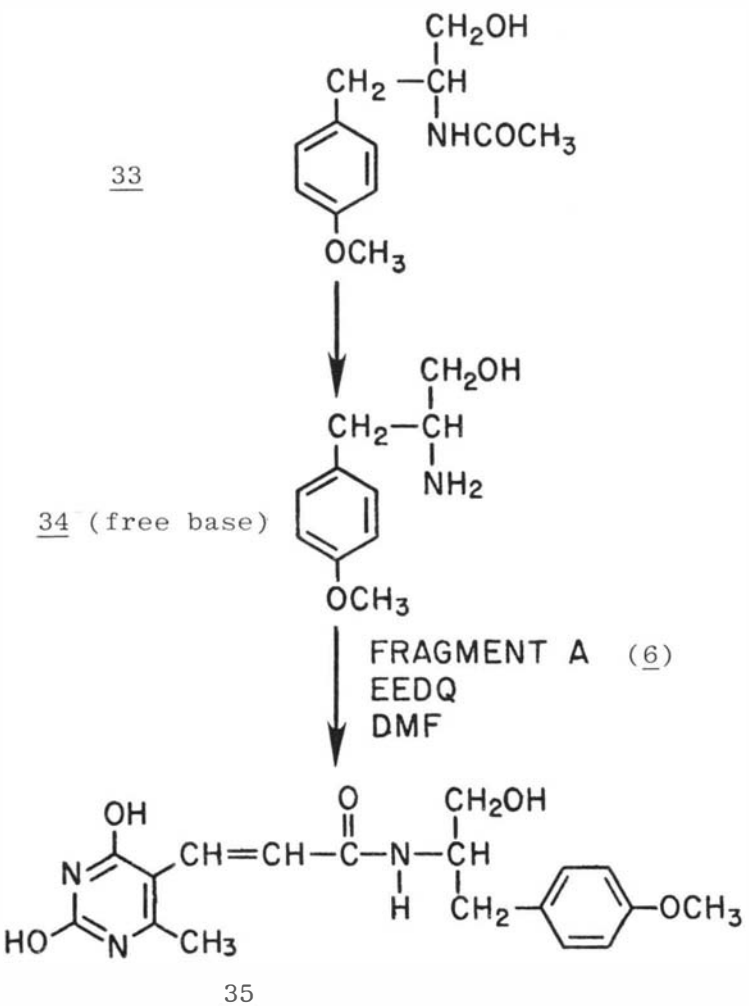
The synthesis of the methoxy-substituted compound 35 (Schemes IX and X) began by treating 4-methoxybenzyl alcohol with 48% hydrobromic acid to afford 4-methoxybenzyl bromide (29). Using the method of Yamamoto, et al.(62), 4-methoxybenzyl bromide was reacted with diethyl acetamidomalonate in sodium ethoxide to give the diethylester-amide compound 30. To avoid possible cleavage of the ether bond of 30, a sequence of reactions employing base instead of hydrobromic acid as in the synthesis of compound 28, was used to obtain the amino-alcohol 34. The sequence began with the procedure of Berlinguet (63) in which compound 30 was treated with a 0.3 N potassium hydroxide solution followed by acidification with hydrochloric acid to afford the mono-acid 31. Heating 31 under reduced pressure effectively decarboxylated the mono-acid 31 producing the ester 32. Reduction of the ester 32 with lithium aluminum hydride by the procedure of Berliguet (63) gave the alcohol 33. Hydrolysis of the amide functionality of 33 was effected with 2 N hydrochloric acid to produce the amino-alcohol hydrochloride 34, which was converted to the free base for coupling by treating 34 with base. The free base of 34 was coupled with the acid 6 using EEDQ to afford the methoxy-substituted compound 35.

The compounds of Series II, 36, 37, 38, and 39, were easily synthesized by coupling the commercially-available phenethylamines with the acid 6 using EEDQ (Scheme XI). Coupling phenethylamine, 2-(4-tolyl)-ethylamine, 4-methoxyphenethylamine, and 4-bromophenethylamine with the acid 6



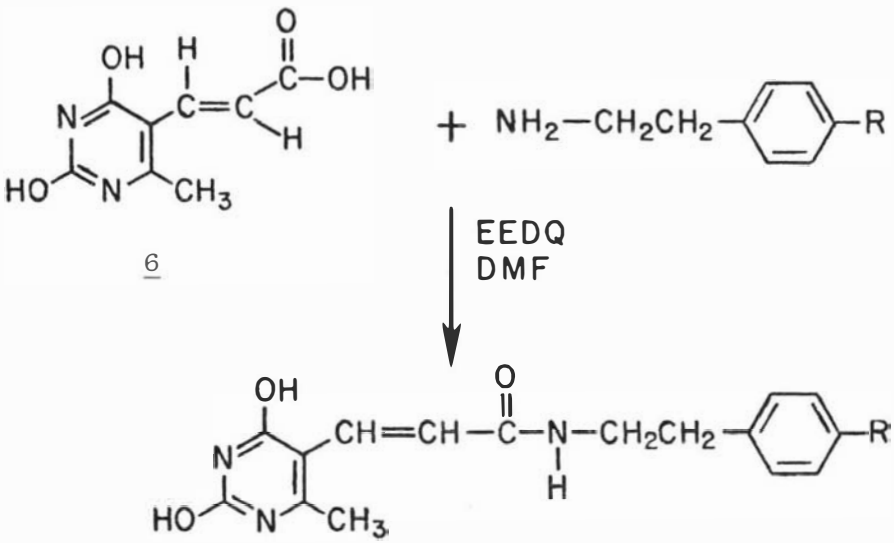
SYNTHESIS OF SERIES I

SCHEME IX



SYNTHESIS OF SERIES I

SCHEME X



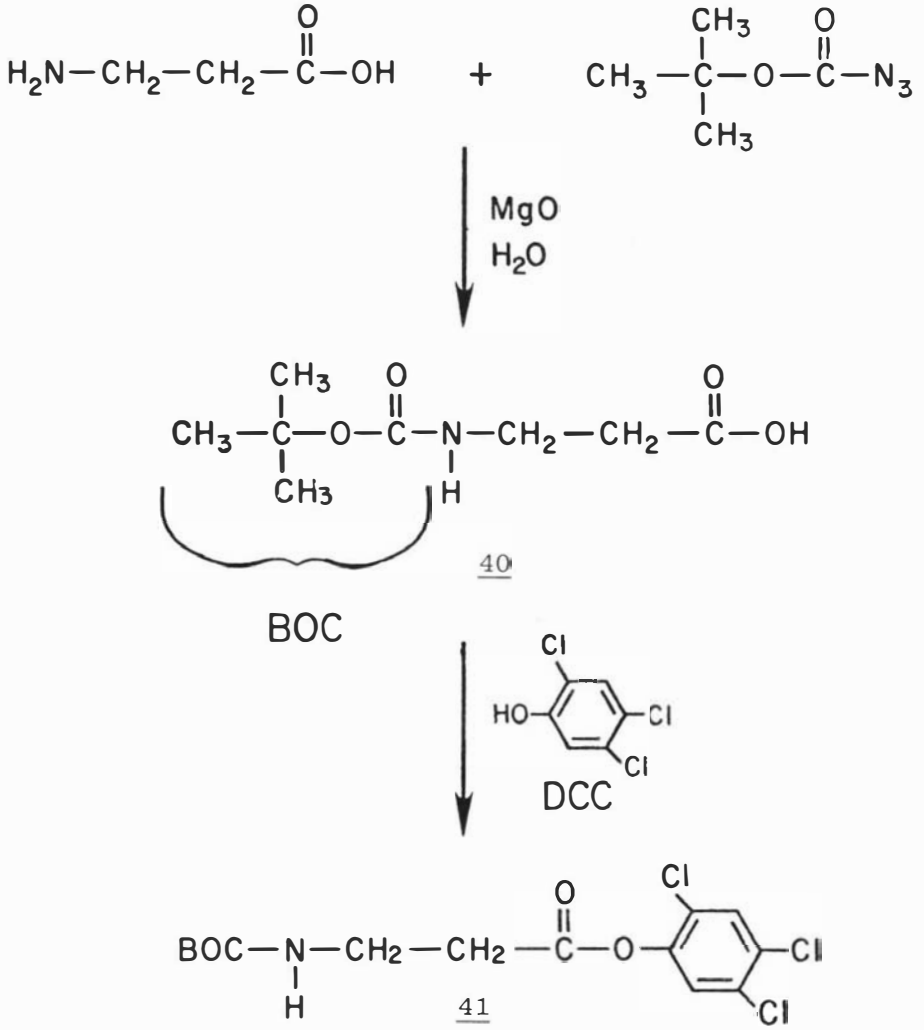
R = H 36
CH3 37
OCH3 38
Br 39

SYNTHESIS OF SERIES II

SCHEME XI

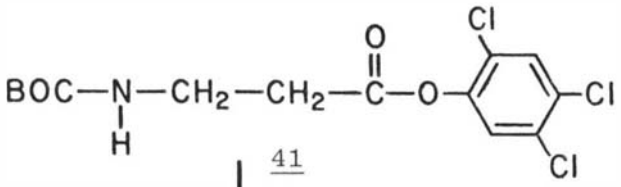
gave compounds 36, 37, 38, and 39, respectively.

Each of the five compounds of Series III, 44, 46, 48, 50, and 52, was prepared using the same procedure (Schemes XII and XIII). The amino fragment of these compounds was derived from substituted β-alanine compounds. The synthesis began by protecting the amine of β-alanine with the tert-butoxycarbonyl (BOC) protecting group. The tert-butoxycarbonyl group was chosen as it was stable to alkali and rapidly cleaved by mildly acidic conditions (64). To form BOC-β-alanine (40), β-alanine was reacted according to the method of Bently, et al. (65) with tert-butylazidoformate in a modified Schötten-Baumann reaction. To enhance the reactivity of the protected β-alanine 40, the active ester 41 was synthesized using the procedure of Pless and Boissonnas (66) in which 40 was reacted with 2,4,5-trichlorophenol in the presence of dicyclohexylcarbodiimide (67). The trichlorophenyl ester 41 was reacted with benzylamine, 4-methylbenzylamine, 4-methoxybenzylamine, 4-bromobenzylamine, and 3,4-dichlorobenzylamine to give the intermediates 42, 45a, 47a, 49a, and 51a, respectively. Each of these compounds was treated with trifluoroacetic acid to effect the facile removal of the tert-butyloxycarbonyl protecting group to form the trifluoroacetate salts 43, 45b, 47b, 49b, and 51b, respectively. During the cleavage by acid, the evolution of gas was noted. The cleavage of tert-butyloxycarbonyl protecting groups proceeds through the formation of tert-butyl cations, which are converted to isobutylene by the elimination of a proton. The loss of the tert-butyl cation from the BOC-derivative

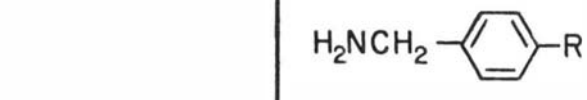


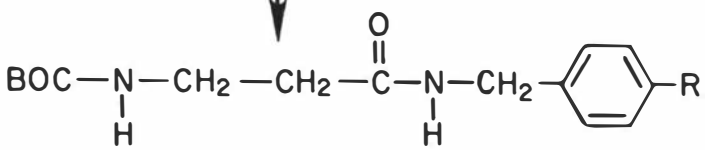
SYNTHESIS OF SERIES III

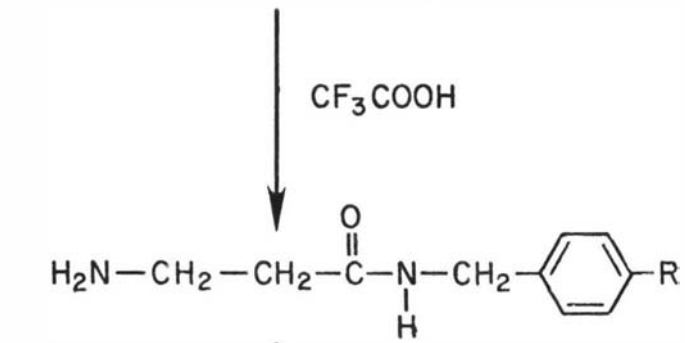
SCHEME XII



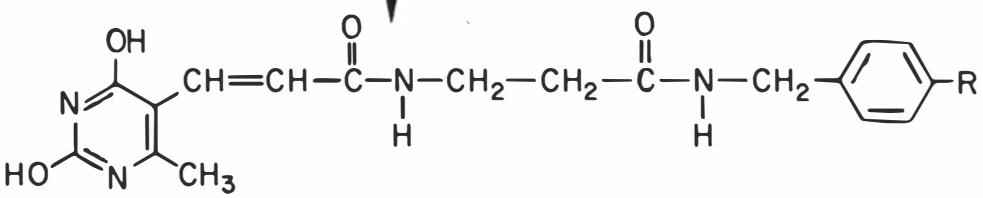
41







FRAGMENT A (6)
EEDQ
DMF



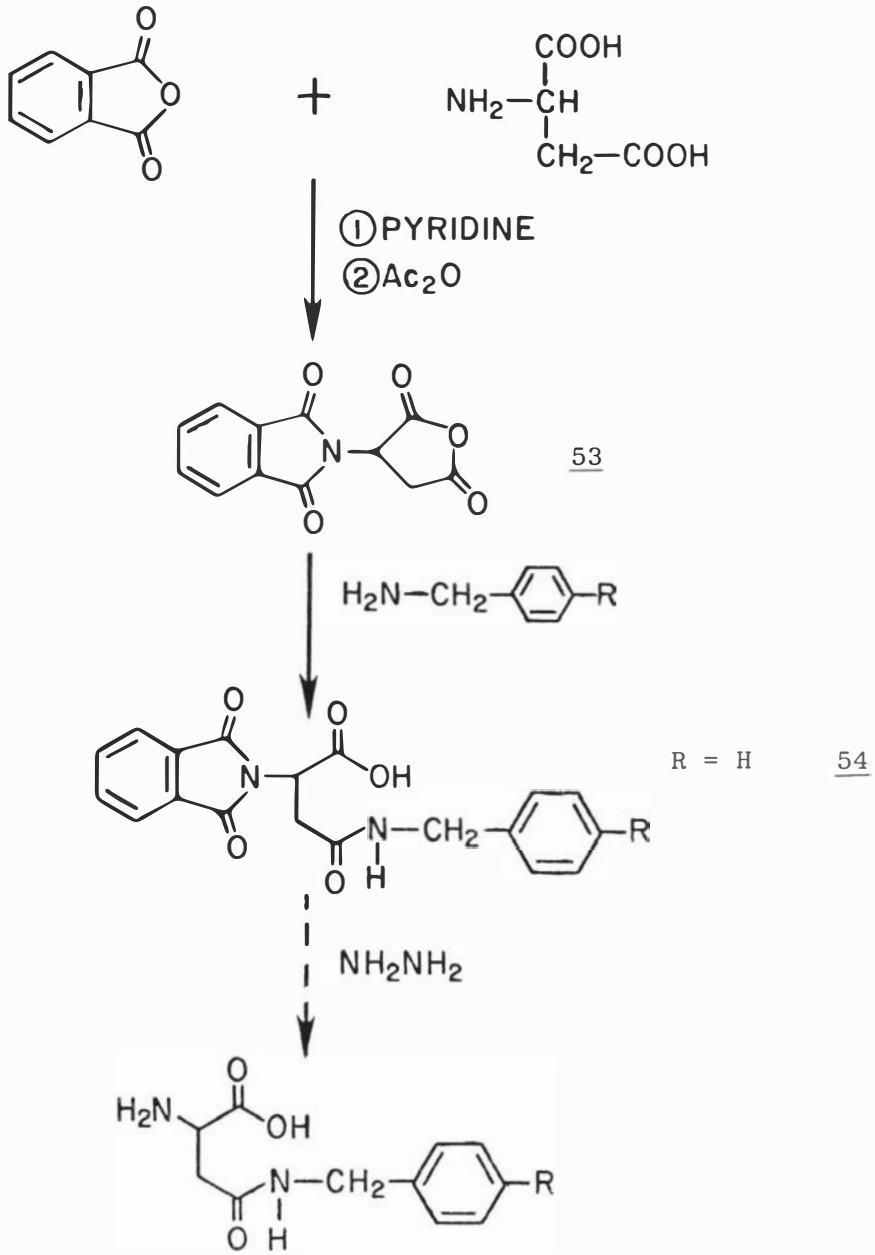
R = H 44
CH_3 46
OCH_3 48
Br 50
3,4 di Cl 52

SYNTHESIS OF SERIES III

SCHEME XIII

affords an N-carboxy intermediate which undergoes decarboxylation to give the trifluoroacetate salt of the amino acid derivative (64). The free bases of the amines were obtained by treatment of the trifluoroacetate salts with sodium hydroxide, followed by ethyl acetate extraction. The free bases were coupled with acid 6 using EEDQ to afford the compounds 44, 46, 48, 50, and 52.

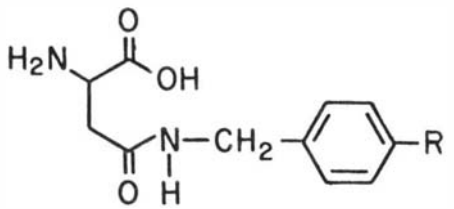
The preparation of the compounds of Series IV, 55, 56, 57, 58, and 59, was approached using two synthetic routes (Schemes XIV - XVII). Both approaches involved the use of aspartic acid which served as the precursor for the amine fragment. In the first attempt to synthesize the compounds of Series IV (Schemes XIV and XV), aspartic acid was reacted with phthalic anhydride according to the methods of Tannenbaum (68) and of Kidd and King (69,70). The product of this reaction was isolated in a crude state and dehydrated in acetic anhydride to give N^2-phthalyl-DL-aspartic anhydride (53). The next step of the sequence was to selectively open the anhydride ring with a substituted benzylamine to form the β-benzylamide-α-acid 54. Several reports in the literature (68,71) indicated the selective opening could be achieved by controlling the solvent used in the reaction. Tannenbaum (68) reported that under anhydrous conditions, the nucleophile would attack the β-carbonyl, whereas, under protic solvent conditions, such as ethanol or water, the nucleophile would attack the α-carbonyl. In the case of the Series IV compounds, the β-amide was required exclusively. As a conse-



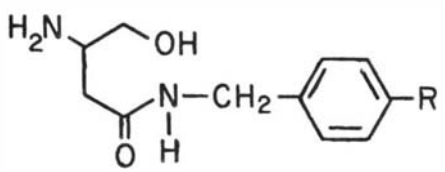
R = H
CH_3
OCH_3
Br
3, 4 di Cl

SYNTHESIS OF SERIES IV

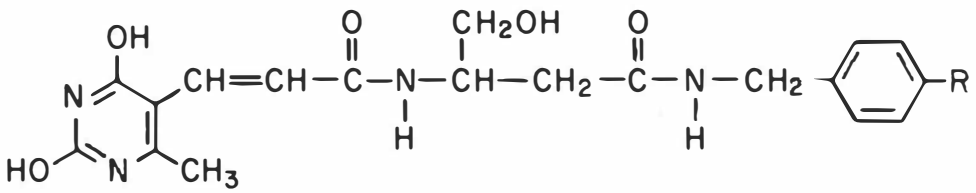
SCHEME XIV



① CH_3OH, $SOCl_2$
② $LiBH_4$



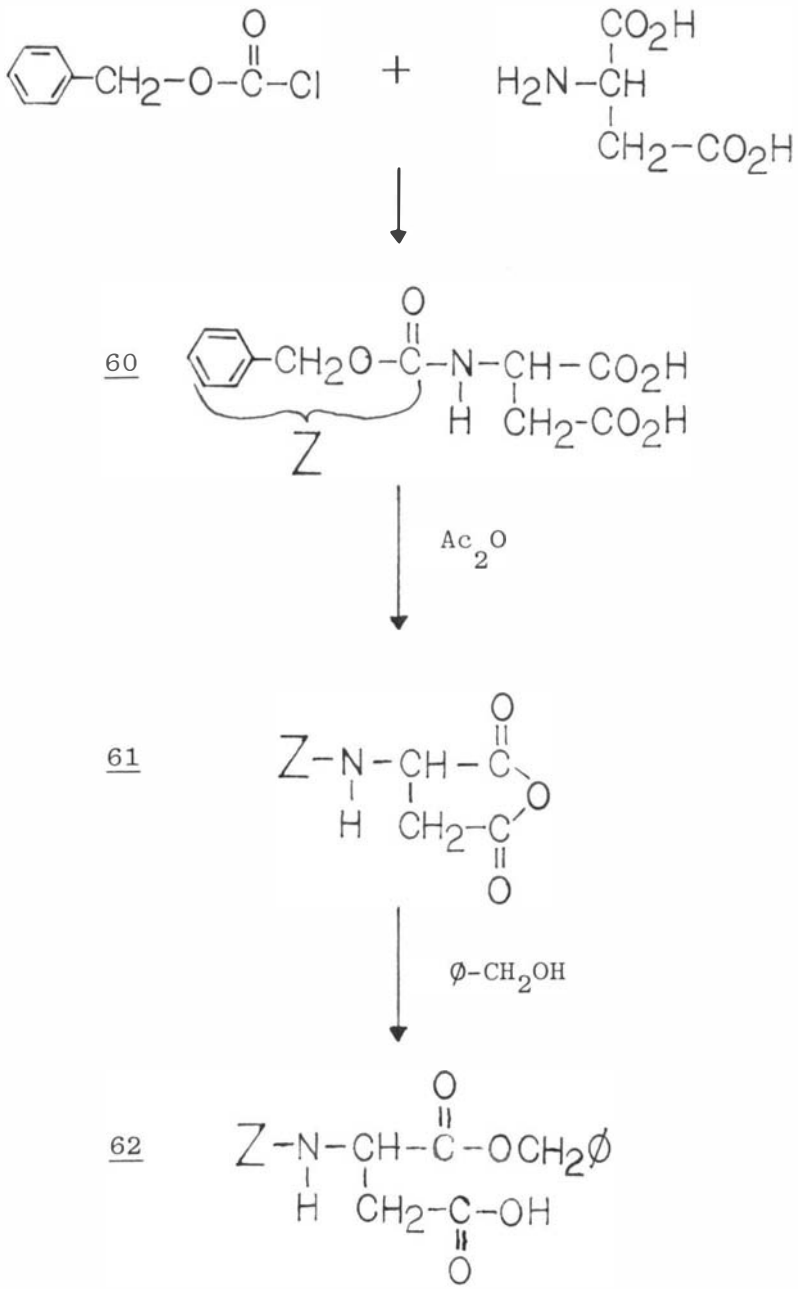
FRAGMENT A (6)
EEDQ
DMF



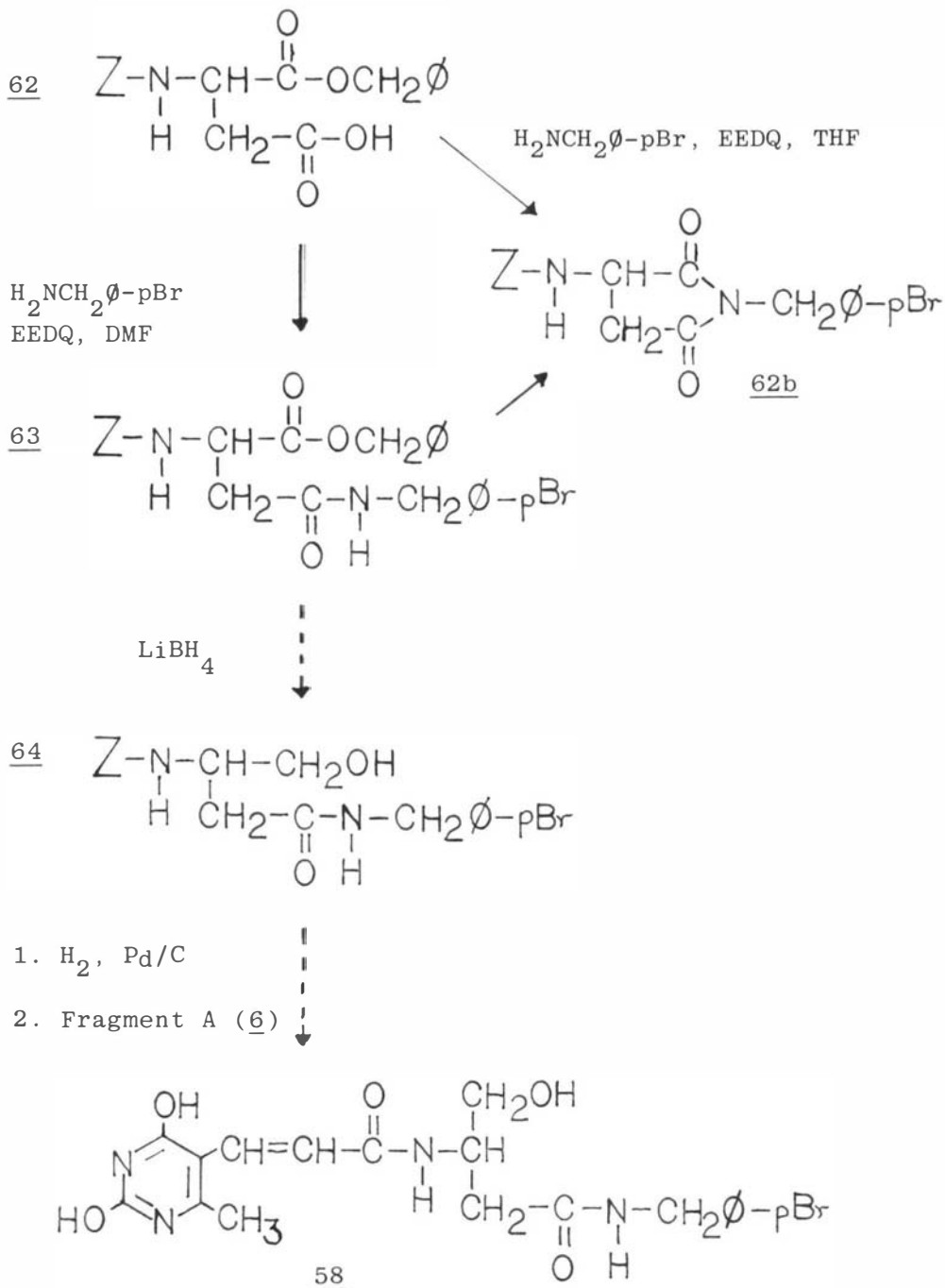
R =	H	55
	CH_3	56
	OCH_3	57
	Br	58
	3,4 diCl	59

SYNTHESIS OF SERIES IV

SCHEME XV


CH2-O-C-Cl
O
+
CO2H
H2N-CH
CH2-CO2H
60
CH2O-C-N-CH-CO2H
O
H CH2-CO2H
Z
Ac2O
61
Z-N-CH-C
H CH2-C
O
O
O
Ø-CH2OH
62
Z-N-CH-C-OCH2Ø
O
H CH2-C-OH
O

SCHEME XVI


62
H2NCH2Ø-pBr, EEDQ, THF
H2NCH2Ø-pBr
EEDQ, DMF
62b
63
LiBH4
64
1. H2, Pd/C
2. Fragment A (6)
58

SCHEME XVII

quence, anhydrous conditions were utilized in the procedure to eliminate α-amide formation as suggested by the literature. In an attempt to prove unequivocably that the β-amide had been obtained exclusively, the procedure was repeated using ethanol, instead of anhydrous ether, to produce the α-amide for comparison. The products of both procedures were identical according to the melting points and spectroscopic analysis, including IR, NMR, and carbon 13 NMR. The problem arose, therefore, of identifying which isomer had been exclusively isolated. As a result, this route was abandoned and the proposed reactions including removal of the phthalyl protecting group with hydrazine, reduction of the ester with lithium borohydride, and coupling of the amino-alcohol with the acid 6, were not completed as depicted in Schemes XIV and XV.

The second approach to the Series IV compounds (Schemes XVI and XVII), also began with aspartic acid. The first step involved the protection of the amino functionality of aspartic acid with the benzyloxycarbonyl (Z) protecting group. The benzyloxycarbonyl derivative of aspartic acid was readily obtainable, stable, and easily converted to the deblocked amino acid by a variety of methods (64). To obtain N-carbobenzoxy-DL-aspartic acid (60), the method of Bergmann and Zervas (72) was followed. Aspartic acid and benzylchloroformate were mixed in the presence of magnesium oxide and water to afford the desired product 60. By dehydrating 60 with acetic anhydride, also by the procedure

of Bergmann and Zervas, the anhydride 61 was obtained. The next step was to selectively open the anhydride with benzyl alcohol to obtain the α-benzyl ester. Once again, the problem existed of identifying unequivocably that the α-ester could be obtained exclusively. Bergmann, et al. (72,73), reported only the α-isomer was obtained when N-benzyloxycarbonyl-L-aspartic anhydride was treated with benzyl alcohol in a sealed tube. LeQuesne and Young (74,75) suggested the reaction of the anhydride with amino-compounds could give both α and β derivatives. In their work, LeQuesne and Young reported they were able to separate the two derivatives by repeated fractional extraction with aqueous sodium carbonate. Bryant, et al. (76), agreed that α and β isomers were both possible products of the reaction of amino-compounds with the anhydride and provided a new synthesis of the respective isomers by selective partial hydrolysis of the dibenzyl ester. Marks and Neuberger (77) later reported they were able to separate the α and β isomers by ion-exchange chromatography.

In the synthesis of the Series IV compound 58, the method of Bergmann, et al. (73), was used in which N-benzyloxycarbonyl-DL-aspartic anhydride 61 was treated with benzyl alcohol. To prove whether or not the α-ester (62) had been obtained exclusively, the β-ester (62a) was synthesized by another route, for comparison with the proposed α-ester (62). The procedure of Berger and Katchalski (78), which involved the selective partial hydrolysis of the dibenzyl ester, was

used to prepare the β-ester (62a). The two esters (62 and 62a) obtained from the different routes were compared by determining the melting points, NMR spectra, and dissociation constants (pKa values) of the compounds (Table II). The results indicated that the α and β esters had been isolated exclusively from the respective routes of synthesis, as the observed values for the three parameters studied were distinctly different. Moreover, the observed pKa values were comparable to the literature values reported by Berger and Katchalski (78). Since the results indicated that the α-ester (62) was isolated exclusively by the method of Bergmann, et al. (72), further separation as performed by LeQuesne and Young (74,75) or Marks and Neuberger (77), was unwarranted.

To continue the synthesis of compound 58, the α-ester 62 was treated with 4-bromobenzylamine and EEDQ in dimethylformamide according to the procedure of Lin and Dubois (51). The product which was isolated from this reaction depended upon the solvent used. When dimethylformamide was used, the desired product, 63, was obtained. When tetrahydrofuran was used, however, cyclization of the aspartic acid moiety occurred, to form the imide 62b.

The next step in the synthetic approach was the reduction of the benzyl ester of 63 to the alcohol. This was attempted by treating the amide-ester 63 with lithium borohydride. Once again, instead of the desired amide alcohol 64, the imide 62b was obtained. Further attempts to reduce

TABLE II: COMPARISION OF THE α AND β-BENZYL ESTERS OF N-BENZYLOXYCARBONYL-DL-ASPARTIC ACID

	α-ESTER (62)	β-ESTER (62a)
Observed melting point	93°-96°	82°-92°
Literature melting point	104.5°	---
NMR-splitting pattern for the 4 benzyl protons at 5.1 ppm.	Doublet	Singlet
Observed pKa	6.7	6.2
Literature pKa	6.6	6.1

the ester of 63 were not pursued, and the proposed reactions, including the removal of the benzyloxycarbonyl protecting group, and coupling of the amino-alcohol 65 with the acid 6, were not completed as depicted in Schemes XVI and XVII.

Since neither of the approaches discussed were successfully completed, the proposed compounds of Series IV were not synthesized.

IV. DISCUSSION OF PHARMACOLOGICAL RESULTS

In the early pharmacological evaluation of sparsomycin, experiments were performed to measure the effect of sparsomycin on DNA, RNA, and protein synthesis in E. coli cells (9-11). The initial studies and subsequent, more detailed investigations, indicated sparsomycin interacted primarily by inhibiting protein synthesis at the ribosomal level. The target compounds synthesized for this project were experimentally examined to investigate their effects on protein synthesis, cell growth, and DNA synthesis in bone marrow cells, P388 lymphocytic leukemia cells, and P815 mastocytoma cells.

To measure the effect on the cell's ability to synthesize protein, the compounds were incubated with normal cells and tumor cells in the presence of L-[^{75}Se]selenomethionine (VI.C.6.c.). The protein synthesis in the cells was monitored by the incorporation of L-[^{75}Se]-selenomethionine into the cells. Although the procedures followed for this project which employed [^{75}Se]-selenomethionine did not differentiate between the actual incorporation of [^{75}Se]-selenomethionine into proteins and the total uptake into the cells, Thomas, et al. (100), have shown that [^{75}Se]-selenomethionine is incorporated into thyroid tumor cells in quantities which correspond to the rate of protein synthesis. Moreover, Awwad, et al. (101,102), have shown

that [^{75}Se]-selenomethionine was readily incorporated into rat tissue proteins and human plasma proteins following a single intravenous administration of the radioactive amino acid analogue to rat or human subjects, respectively. Awwad, et al., further stated that the majority of the radioactivity was due to that which was newly incorporated into proteins. Based upon these findings, the incorporation of [^{75}Se]-selenomethionine into the cells, as quantitated by the assays in this project, was used as an indirect measurement of protein synthesis.

Prior to quantitating the activity of the target compounds in the protein synthesis assay, time-course experiments were conducted to ascertain the optimal time for harvesting the cells (VI.C.6.b.(1)). The time-course study for the P388 cells shown in Figure XI indicates there was a linear incorporation of L-[^{75}Se]-selenomethionine over a twenty-four hour incubation period. Similarly, the time course study for the bone marrow cells shown in Figure XII also displays a linear incorporation of the isotope during the twenty-four hour study. Based upon the findings of the time-course experiment, the cells were incubated for twenty-four hours prior to harvesting in all of the subsequent protein synthesis studies.

The target compounds were tested in the protein synthesis assay using P388 lymphocytic leukemia cells (VI.C.6.b.(3)), P815 mastocytoma cells (VI.C.6.b.(4)), and bone marrow cells (V.C.6.b.(2)). Tables III and IV show the effects of

FIGURE XI. CHARACTERIZATION OF THE PROTEIN SYNTHESIS ASSAY: TIME COURSE FOR INCORPORATION OF [^{75}Se]-SELENOMETHIONINE INTO P388 CELLS.

A typical protein synthesis curve showing the incorporation of L-[^{75}Se]-selenomethionine into P388 cells. The points in the figure represent CPM/6 X 10^5 cells after incubation with the pulse label for 1, 3, 6, 10, 18, 21, and 24 hours. Each point represents the mean and standard error for six replicate wells as described in section VI.C.6.b.(1).

FIGURE XI: CHARACTERIZATION OF THE PROTEIN SYNTHESIS ASSAY: TIME COURSE FOR INCORPORATION OF [^{75}Se]-SELENOMETHIONINE INTO P388 CELLS

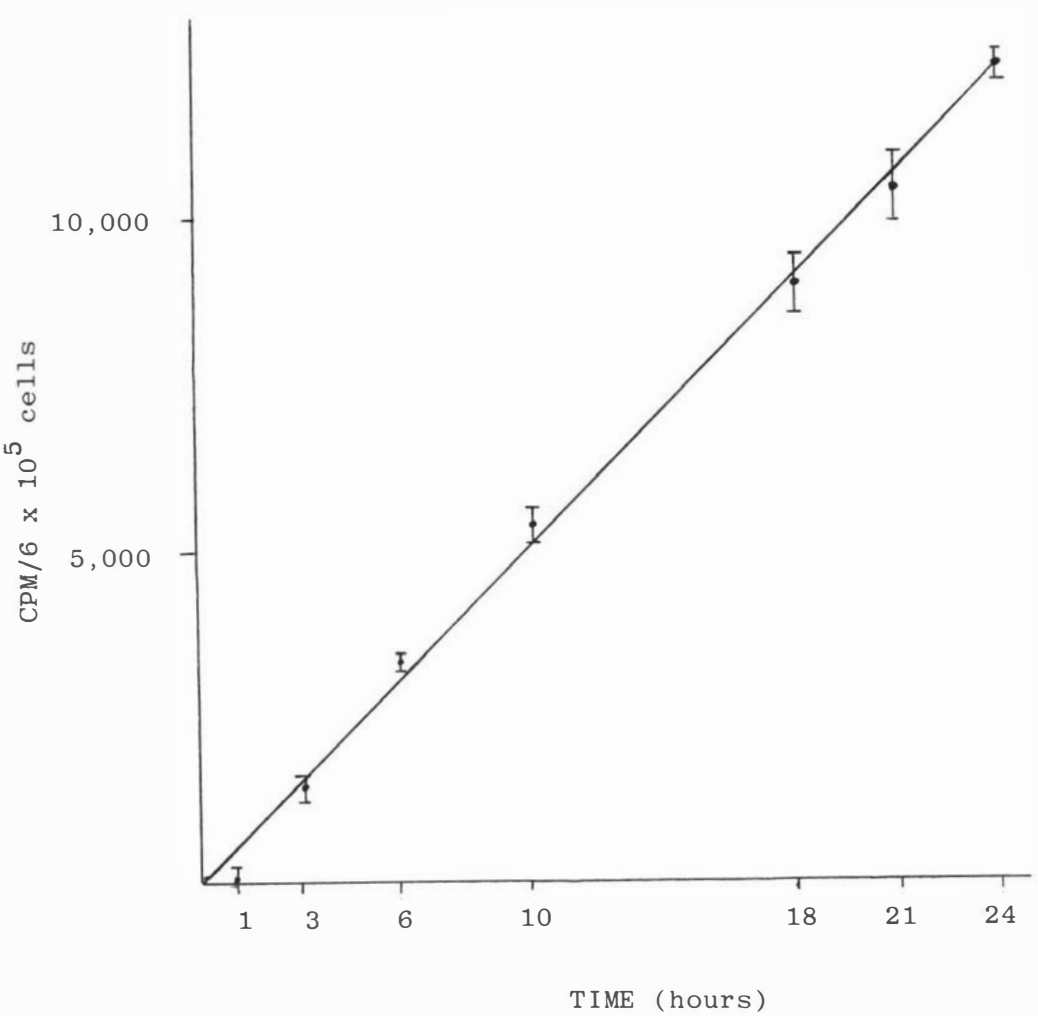


FIGURE XII. CHARACTERIZATION OF THE PROTEIN SYNTHESIS ASSAY: TIME COURSE FOR INCORPORATION OF [^{75}Se]-SELENOMETHIONINE INTO BONE MARROW CELLS

A typical protein synthesis curve showing the incorporation of L-[^{75}Se]-selenomethionine into bone marrow cells. The points in the figure represent CPM/6 X 10^5 cells after incubation with the pulse label for 1, 3, 14, 18, and 24 hours. Each point represents the mean and standard error for six replicate wells as described in section VI.C.6.b.(1).

FIGURE XII: CHARACTERIZATION OF THE PROTEIN SYNTHESIS ASSAY: TIME COURSE FOR INCORPORATION OF [^{75}Se]-SELENOMETHIONINE INTO BONE MARROW CELLS.

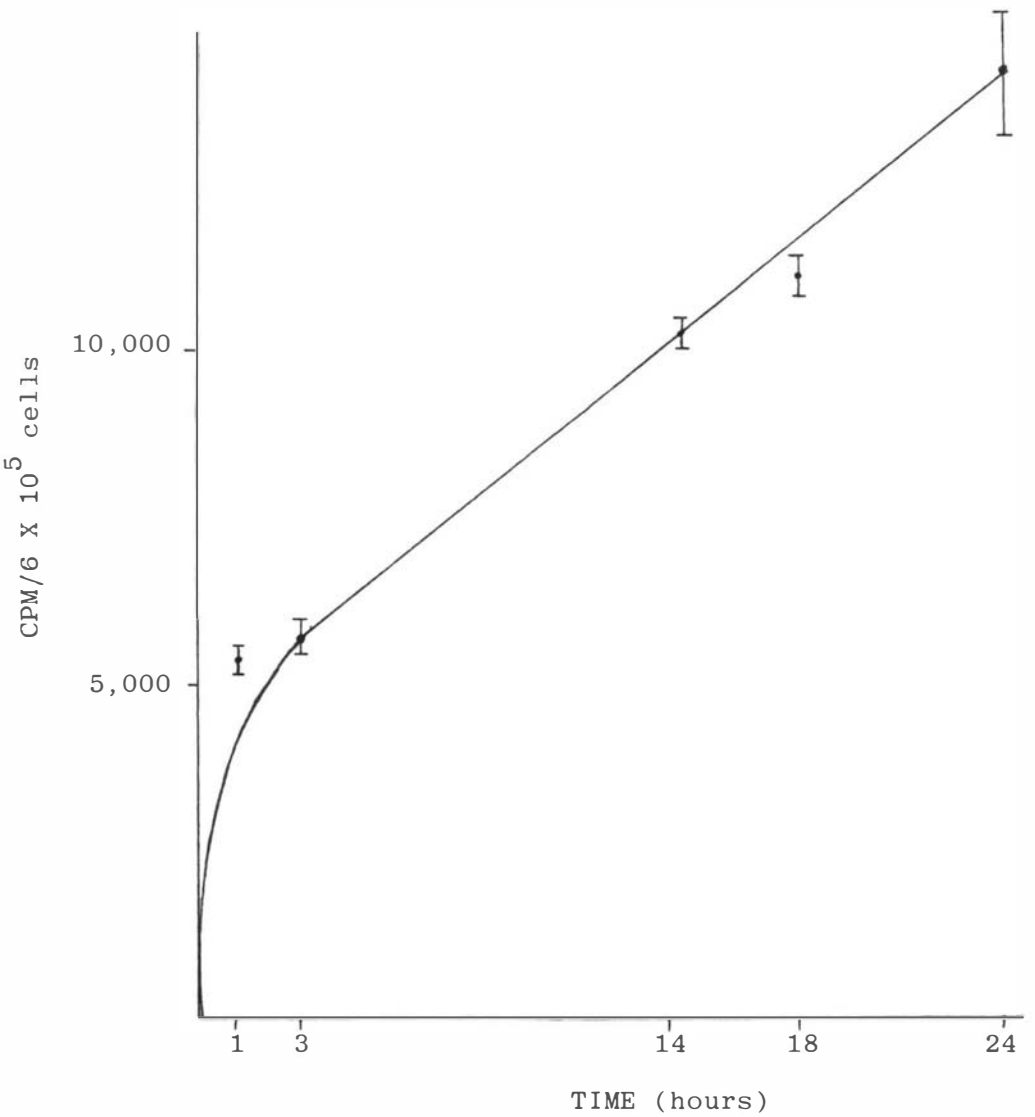


TABLE III: INCORPORATION OF [^{75}Se]-SELENOMETHIONINE INTO P388 CELLS

COMPOUND[a]	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
CONTROL	13580 ± 1168	14149 ± 492	13278 ± 523
22	13282 ± 750	12805 ± 362	12421 ± 125
24	7792 ± 459[c]	7449 ± 969[c]	7651 ± 121[c]
28	12956 ± 649	12917 ± 830	21351 ± 289
36	13584 ± 938	12950 ± 146	13129 ± 532
37	13801 ± 447	14664 ± 750	15395 ± 315
38	14364 ± 243	13706 ± 570	14233 ± 110
39	11416 ± 568	9029 ± 509[c]	9696 ± 339[c]
48	13664 ± 20	12527 ± 150	14537 ± 495
50	12078 ± 318	8465 ± 446[c]	11471 ± 252[c]
19	11439 ± 254[c]	11304 ± 392[c]	11830 ± 436
35	15292 ± 393	14448 ± 985	17802 ± 268
1% ETHANOL	15282 ± 220	17370 ± 308	18494 ± 285
PUROMYCIN	6085 ± 101[c]	3965 ± 121[c]	4867 ± 324[c]
VEHICLE[b]		14479 ± 488	13400 ± 65

The numbers represent the mean ± standard error for six replicate wells containing 6 X 10^5 cells as described in section VI.C.6.b.(3).

[a]All compounds were tested at 10^{-3}M except 35 which was tested at 10^{-4}M in 1% ethanol.

[b]Distilled water was used as the vehicle.

[c]$p<0.05$ as compared to controls. (See Section VI.C.7).

TABLE IV: INHIBITION OF [^{75}Se]-SELENOMETHIONINE INCORPORATION IN P388 CELLS

COMPOUND[a]	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	AVERAGE
22	2	9	6	6
24	43	47	42	44
28	5	9	7	7
36	0	8	1	3
37	0	0	0	0
38	0	3	0	1
39	16	36	27	26
48	0	11	0	3
50	11	40	14	22
19	16	20	11	16
35	0	0	0	0
1% ETHANOL	0	0	0	0
PUROMYCIN	55	72	63	63
VEHICLE[b]	-	0	0	

The numbers represent the percent inhibition for six replicate wells containing 6 X 10^5 P388 cells as described in section VI.C.6.b.(3).

[a]All compounds were tested at 10^{-3}M except 35 which was tested at 10^{-4}M in 1% ethanol.

[b]Distilled water was used as the vehicle.

the compounds on the incorporation of [^{75}Se]-selenomethionine into P388 tumor cells. The results of the three experiments indicate the unsubstituted compouds, 22 and 36, the methoxy-substituted analogues, 35, 38, and 48, and the methyl substituted analogues, 28 and 37, were all inactive. Conversely, the analogues containing bromine, 24, 39, and 50, were the most active inhibitors of the incorporation of the pulse label. The average inhibition by the most active compound, 24, was within twenty percent of the inhibition of the control drug, puromycin.

To more fully characterize the activity of the compounds, the protein synthesis experiments were repeated using P815 mastocytoma cells. The data in Table V indicate the P815 cells were more sensitive to the target compounds than the P388 cells (Tables III and IV). In the assay with the P815 cells, compounds 24, 39 and 50 were again the most active compounds. In this investigation, however, compound 24 was as active as the control inhibitor, puromycin. The different response to the target compounds exhibited by the P388 and P815 cells may be due to a fundamental difference in the two types of tumor cells. The P815 mastocytoma is a mast-cell neoplasm which arose in DBA/2 male mice whose skin had been selectively exposed to 3-methylcholanthrene (79). Although the P388 tumor was also induced by treatment of DBA/2 mice with 3-methylcholanthrene, this tumor is a lymphoid neoplasm (80). The different responses to the target compounds may reflect the difference in the

TABLE V: INHIBITION OF [^{75}Se]-SELENOMETHIONINE INCORPORATION IN P815 CELLS

COMPOUND[a]	MEAN CPM ± STANDARD ERROR	% INHIBITION
CONTROL	23647 ± 920	
22	11421 ± 146[c]	52
24	1514 ± 349[c]	94
28	8361 ± 704[c]	65
36	23163 ± 373	2
37	21035 ± 694[c]	11
38	24147 ± 299	0
39	2511 ± 748[c]	89
48	28442 ± 447	0
50	7400 ± 365[c]	69
19	16303 ± 488[c]	31
35	28407 ± 272	0
1% ETHANOL	25217 ± 413	0
PUROMYCIN	405 ± 82[c]	98
VEHICLE[b]	27046 ± 537	0

The numbers represent the mean ± standard error and the percent inhibition for four replicate wells containg 6 X 10^5 P815 cells as described in section VI.C.6.b.(4).

[a]All compounds were tested at 10^{-3}M except 35 which was tested at 10^{-4}M in 1% ethanol.

[b]Distilled water was used as the vehicle.

[c]$p<0.05$ as compared to controls.

abilities of the two cellular types to synthesize protein or take up the [^{75}Se]-selenomethionine.

Further clarification of the inhibitory activity of the compounds was obtained from experiments conducted to ascertain the potential interference with normal cellular protein synthesis. To quantitate these effects, the protein synthesis assay was repeated using bone marrow cells. The results shown in Table VI suggest all of the compounds except 35 inhibited the incorporation of [^{75}Se]-selenomethionine into bone marrow cells. Once again, the bromo-containing analogues, 24, 39, and 50, were among the most active inhibitors. In this assay, however, analogues 28, 38, and 19, although structurally dissimilar, were also active inhibitors. As in the protein synthesis study with tumor cells, the methoxy-substituted compounds 48 and 35 were inactive.

Compounds 24, 39, and 50 were consistently active inhibitors of [^{75}Se]-selenomethionine incorporation into P815, P388, and bone marrow cells. In order to establish a more accurate description of the activity of these analogues, a relationship was sought between the concentration of the test compounds and the inhibition of [^{75}Se]-selenomethionine incorporation. The results of the experiments which were conducted are tabulated in Tables VII, VIII, and IX. The data failed to display a clearly defined dose-response relationship in P388 cells or bone marrow cells. As a result, further experimentation was warranted in order to quantitate

TABLE VI: INHIBITION OF [^{75}Se]-SELENOMETHIONINE INCORPORATION IN BONE MARROW CELLS

COMPOUND[a]	MEAN CPM ± STANDARD ERROR	% INHIBITION
CONTROL	8203 ± 219	
22	5401 ± 153[b]	34
24	2920 ± 66[b]	64
28	4891 ± 136[b]	40
36	5375 ± 138[b]	34
37	5311 ± 176[b]	35
38	4614 ± 77[b]	44
39	4744 ± 153[b]	42
48	7276 ± 363	11
50	5837 ± 240[b]	29
19	4570 ± 341[b]	44
35	6808 ± 99[b]	0
1% ETHANOL	6526 ± 268[b]	20
PUROMYCIN	3218 ± 100[b]	61

The numbers represent the mean ± standard error and the percent inhibition for six replicate wells containing 6 X 10^5 bone marrow cells as described in section VI.C.6.b.(2).

[a]All compounds were tested at 10^{-3} M except 35 which was tested at 10^{-4}M in 1% ethanol.

[b]$p<0.05$ as compared to controls.

TABLE VII: RELATIONSHIP BETWEEN DOSE AND [^{75}Se]-SELENOMETHIONINE INCORPORATION IN P388 CELLS

COMPOUND	CONCENTRATION	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
CONTROL	6×10^5 cells/mL	21907 ± 477	21332 ± 483	20127 ± 257
PUROMYCIN	10^{-3} M	9875 ± 194	1593 ± 181	8287 ± 542
	5×10^{-4} M	10353 ± 160	---	11101 ± 470
	10^{-4} M	9705 ± 310	8039 ± 549	10031 ± 848
	10^{-5} M	18035 ± 588	15724 ± 508	17843 ± 545
24	10^{-3} M	15996 ± 754	9059 ± 186	11779 ± 657
	5×10^{-4} M	15850 ± 127	11522 ± 637	15346 ± 533
	10^{-5} M	16725 ± 270	19133 ± 542	20795 ± 3000
SPARSOMYCIN	10^{-3} M	11290 ± 113	8742 ± 236	10295 ± 302
	10^{-4} M	10737 ± 197	8417 ± 232	9856 ± 390
	10^{-5} M	12095 ± 483	11986 ± 313	11999 ± 215
	10^{-6} M	18623 ± 1373	18055 ± 905	17148 ± 284
	10^{-7} M	20099 ± 971	---	17866 ± 377

The numbers represent the mean ± standard error for triplicate wells containing 6×10^5 P388 cells as described in section VI.C.6.b.(3).

TABLE VIII: RELATIONSHIP BETWEEN DOSE AND INHIBITION OF [^{75}Se]-SELENOMETHIONINE INCORPORATION IN P388 CELLS

COMPOUND	CONCENTRATION	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	AVERAGE
CONTROL	6×10^5 cells/mL				
PUROMYCIN	10^{-3} M	55	93	59	69
	5×10^{-4} M	53	--	45	49
	10^{-4} M	56	62	50	56
	10^{-5} M	18	26	11	18
24	10^{-3} M	27	56	41	41
	5×10^{-4} M	28	46	24	33
	10^{-5} M	24	10	3	12
SPARSOMYCIN	10^{-3} M	48	59	49	52
	10^{-4} M	51	60	51	54
	10^{-5} M	45	44	40	43
	10^{-6} M	15	15	15	15
	10^{-7} M	8	--	11	10

The numbers represent the percent inhibition for triplicate wells containing 6×10^5 P388 cells as described in section VI.C.6.b.(3).

TABLE IX: RELATIONSHIP BETWEEN DOSE AND INHIBITION OF [^{75}Se]-SELENOMETHIONINE INCORPORATION IN BONE MARROW CELLS

COMPOUND	CONCENTRATION	MEAN CPM ± STANDARD ERROR	% INHIBITION
CONTROL	6 X 10^5 cells/mL	8855 ± 158	
PUROMYCIN	10^{-3} M	8077 ± 1570	9
	5 X 10^{-4} M	6449 ± 334	27
	10^{-4} M	6481 ± 777	27
	10^{-5} M	7562 ± 61	15
24	10^{-3} M	6666 ± 500	25
	5 X 10^{-4} M	7574 ± 168	14
	10^{-4} M	8761 ± 534	1
	10^{-5} M	7981 ± 514	10
SPARSOMYCIN	10^{-3} M	6260 ± 186	29
	10^{-4} M	6265 ± 612	29
	10^{-5} M	8181 ± 85	8
	10^{-6} M	8858 ± 313	0
	10^{-7} M	8797 ± 1500	0

The numbers represent the mean ± standard error and the percent inhibition for triplicate wells containing 6 X 10^5 bone marrow cells as described in section VI.C.6.b.(2).

the relationship between dose and response to the test compounds. The investigation of a dose-response relationship was continued by conducting several cell growth experiments with P815 mastocytoma cells. As seen in Figure XIII, P815 mastocytoma cells grew exponentially over a 48 hour time period (VI.C.6.c.(1). The three most active compounds in the protein synthesis studies in P815 cells, 24, 39, and 50, and the control drugs, puromycin and sparsomycin, were tested to determine their effect on the cell growth of P815 mastocytoma cells. Using the results of the time-course experiment in Figure XIII and the data in Table X, the optimal time for enumerating cell populations during the experiment was chosen to be twenty-four hours. The results of the cell growth studies in P815 cells were tabulated in Tables XI, XII, XIII, XIV, and XV. These data displayed a dose-response relationship andwere used to determine approximate ED_{50} values by linear regression analysis. The accuracy of the values was limited by the number of data points collected for the linear regression analysis. As a consequence, the values for compounds 39 and 50 were not as reliable as the ED_{50} values for compound 24. The following ED_{50} values were obtained: (See page 81).

FIGURE XIII: CHARACTERIZATION OF THE P815 CELL GROWTH ASSAY: TIME COURSE FOR P815 CELL GROWTH

A typical growth curve for P815 cells in CDMEM is shown. The points represent the log cells/mL X 10^{-5} after incubation for 9, 24, 33, and 48 hours. Each point represents the mean and standard error for four replicate wells as described in section VI.C.6.c.(1).

FIGURE XIII: CHARACTERIZATION OF THE P815 CELL GROWTH ASSAY: TIME COURSE FOR P815 CELL GROWTH

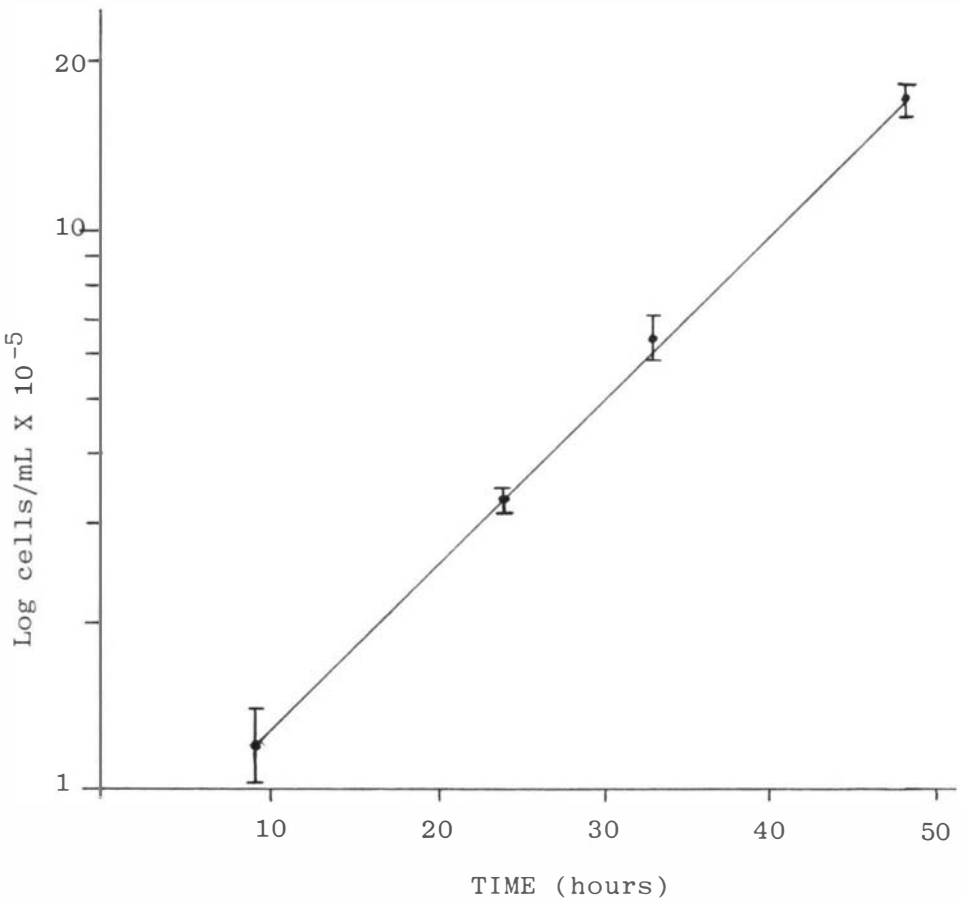


TABLE X: TIME COURSE STUDY OF THE EFFECT OF PUROMYCIN AND COMPOUND 24 ON P815 CELL GROWTH

COMPOUND	9 HOURS	24 HOURS	33 HOURS	48 HOURS
CONTROL	1.3 ± 0.2	3.6 ± 0.1	6.9 ± 0.3	19 ± 1
VEHICLE[a]	1.4 ± 0.2	3.3 ± 0.4	---	---
PUROMYCIN 1×10^{-3} M	0.9 ± 0.1	0	---	---
24 1×10^{-3} M	0.75 ± 0.1	0	---	---
24 5×10^{-4} M	0.71 0.02	0	---	---
24 1×10^{-4} M	0.77 0.05	0	---	---

The numbers represent the concentration of cells/mL X 10^{-5} ± standard error for four replicate wells of P815 cells as described in section VI.C.6.c.(1).

[a] Distilled water was used as the vehicle.

TABLE XI: RELATIONSHIP BETWEEN THE CONCENTRATION OF COMPOUND 24 AND P815 CELL GROWTH.

	CELLS/mL	% INHIBITION	CELLS/mL	% INHIBITION	CELLS/mL	% INHIBITION
CONTROL	9.1 ± 0.5		17-19 ± 2		14 ± 1	
VEHICLE[a]			19 ± 1		14 ± 1	
24 1×10^{-4} M	0	100	0	100		
24 5×10^{-5} M			0	100		
24 1×10^{-5} M	3.1 ± 0.2	62	7.8 ± 0.2	59	5.6 ± 0.3	60
24 7.5×10^{-6} M					5.5 ± 0.2	60
24 5×10^{-6} M			9.7 ± 0.1	43	8.0 ± 0.4	43
24 2.5×10^{-6} M					10 ± 3	29
24 1×10^{-6} M	6.9 ± 0.6	16	14 ± 2	18	11 ± 3	21
24 5×10^{-6} M			16 ± 4	6		
24 1×10^{-7} M	8.4 ± 0.6	0				
24 1×10^{-8} M	8.8 ± 0.6	0				

The numbers represent the concentration of cells/mL X 10^{-5} ± standard error and the percent inhibition for four replicate wells of P815 cells which were incubated for 24 hours in the presence of varying concentrations of 24 as described in section VI.C.6.c.(2).

[a]Distilled water was used as the vehicle.

TABLE XII: RELATIONSHIP BETWEEN THE CONCENTRATION OF PUROMYCIN AND P815 CELL GROWTH

		CELLS/ML	% INHIBITION	CELLS/mL	% INHIBITION
CONTROL		19 ± 2		14 ± 1	
VEHICLE[a]				14 ± 1	
PUROMYCIN	$1x10^{-4}M$	0	100		
PUROMYCIN	$5x10^{-5}M$	0	100		
PUROMYCIN	$1x10^{-5}M$	0	100	0	100
PUROMYCIN	$7.5x10^{-6}M$			0	100
PUROMYCIN	$5x10^{-6}M$	0.8 ± 0.3	95	0	100
PUROMYCIN	$2.5x10^{-6}M$			11 ± 7	21
PUROMYCIN	$1x10^{-6}M$	17 ± 0.5	0	14 ± 4	0
PUROMYCIN	$5x10^{-7}M$	16 ± 1	6		

The numbers represent the concentration of cells/mL X 10^{-5} ± standard error and the percent inhibition for four replicate wells of P815 cells which were incubated 24 hours in the presence of varying concentrations of puromycin as described in section VI.C.6.c.(2).

[a] Distilled water was used as the vehicle.

TABLE XIII: RELATIONSHIP BETWEEN THE CONCENTRATION OF SPARSOMYCIN AND P815 CELL GROWTH

	CELLS/mL	% INHIBITION
CONTROL	14 ± 7	
VEHICLE[a]	14 ± 7	
SPARSOMYCIN 1×10^{-5} M	0	100
SPARSOMYCIN 7.5×10^{-6} M	0	100
SPARSOMYCIN 5×10^{-6} M	0	100
SPARSOMYCIN 2.5×10^{-6} M	3.9 ± 0.2	72
SPARSOMYCIN 1×10^{-6} M	5.7 ± 0.4	59

The numbers represent the concentration of cells/mL X 10^{-5} ± standard error and the percent inhibition for four replicate wells of P815 cells which were incubated 24 hours in the presence of varying concentrations of sparsomycin as described in section VI.C.6.c.(2).

[a] Distilled water was used as the vehicle.

TABLE XIV: RELATIONSHIP BETWEEN THE CONCENTRATION OF COMPOUND 39 AND P815 CELL GROWTH

	CELLS/mL	% INHIBITION
CONTROL	13 ± 1	
VEHICLE[a]	12 ± 1	
39 10^{-4} M	4.4 ± 0.2	66
39 10^{-5} M	4.8 ± 0.4	63
39 10^{-6} M	8.0 ± 0.9	38

The numbers represent the concentration of cells/mL X 10^{-5} ± standard error and the percent inhibition for four replicate wells of P815 cells which were incubated 24 hours in the presence of varying concentrations of compound 39 as described in section VI.C.6.c.(2).

[a] Distilled water was used as the vehicle.

TABLE XV: RELATIONSHIP BETWEEN THE CONCENTRATION OF COMPOUND 50 AND P815 CELL GROWTH

	CELLS/mL	% INHIBITION
CONTROL	13 ± 1	
VEHICLE[a]	12 ± 1	
50 10^{-4} M	5.1 ± 0.3	60
50 10^{-5} M	7.4 ± 0.1	42
50 10^{-6} M	9.1 ± 0.1	30

The numbers represent the concentration of cells/mL X 10^{-5} ± standard error and the percent inhibition for four replicate wells of P815 cells which were incubated 24 hours in the presence of varying concentration of compound 50 as described in section VI.C.6.c.(2).

[a] Distilled water was used as the vehicle.

TABLE	COMPOUND	APPROXIMATE ED_{50}
XI	24	8.9×10^{-6} M
XII	PUROMYCIN	2.6×10^{-6} M
XIII	SPARSOMYCIN	2.2×10^{-7} M
XIV	39	5.8×10^{-6} M
XV	50	6.0×10^{-5} M

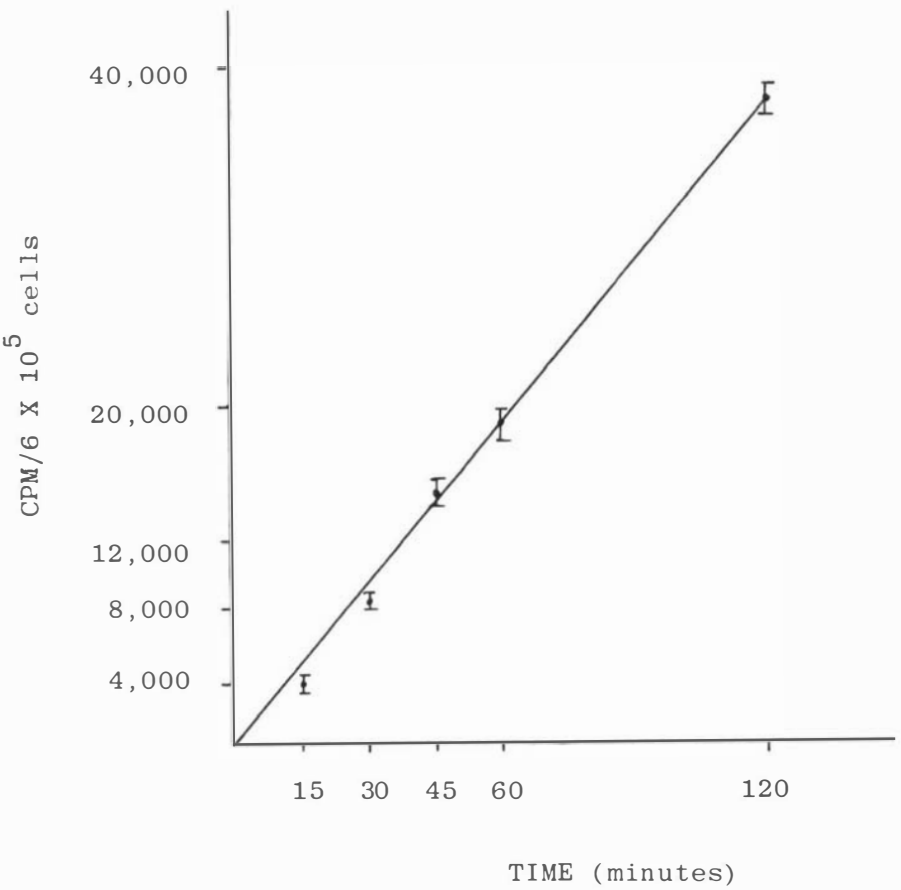
The results show the compounds 24, 39, and 50 were significantly active compounds and have ED_{50} values comparable to the control drug, puromycin.

Since three of the fourteen target compounds inhibited protein synthesis as reflected in the incorporation of [^{75}Se]-selenomethionine and cell growth in concentrations approaching the control drugs, studies were conducted to detect any effect the compounds may have on DNA synthesis. To measure the effect on the cell's ability to synthesize DNA, the compounds were incubated with normal cells and tumor cells in the presence of 5-^{125}I-iodo-2-deoxyuridine (^{125}IUdR) (V.C.6.a). The DNA synthesis was monitored by the incorporation of ^{125}IUdR into the cells. As in the protein synthesis studies, a preliminary time-course experiment was conducted to ascertain the optimal time for harvesting the cells. Analogous to the findings of White, et al. (81), Figure XIV shows there was a linear incorporation of ^{125}IUdR over a two-hour incubation period. Based on this information, the cells were incubated two hours prior to harvesting in

FIGURE XIV: CHARACTERIZATION OF THE DNA SYNTHESIS ASSAY: TIME COURSE FOR INCORPORATION OF ^{125}IUdR INTO BONE MARROW CELLS

A typical DNA synthesis curve showing the incorporation of ^{125}IUdR into bone marrow cells. The points in the figure represent CPM/6 X 10^5 cells after incubation with the pulse label for 15, 30, 45, 60, and 120 minutes. Each point represents the mean and the standard error for triplicate wells as described in section VI.C.6.a.(1).

FIGURE XIV: CHARACTERIZATION OF THE DNA SYNTHESIS ASSAY: TIME COURSE FOR INCORPORATION OF ^{125}IUdR INTO BONE MARROW CELLS.



all subsequent studies on DNA synthesis.

The target compounds were tested in the DNA synthesis assay using bone marrow cells (VI.C.6.a.(2)) and P388 tumor cells (VI.C.6.a.(3)). Table XVI shows the results with the bone marrow cells and indicates only one compound, compound 24, inhibited the incorporation of ^{125}IUdR. This inhibition was much less than the control inhibitor, AraC, and was not statistically significant at $p<0.05$ when compared to the control cells. Table XVII displays the results with P388 cells and, once again, shows that compound 24 was the most active inhibitor, but the inhibition was, although statistically significant, very small when compared to the control inhibitor, AraC. The data suggested the target compounds have little or no effect on DNA synthesis as measured by the incorporation of ^{125}IUdR into cells or the effect cannot be detected within the two hour time frame of the assay.

TABLE XVI: INHIBITION OF ^{125}IUdR INCORPORATION IN BONE MARROW CELLS

COMPOUND[a]	MEAN CPM ± STANDARD ERROR	% INHIBITION
CONTROL	20863 ± 662	
22	27961 ± 352	0
24	17672 ± 1359	15
28	25508 ± 418	0
36	22900 ± 1273	0
37	25556 ± 461	0
38	23391 ± 565	0
39	20978 ± 1004	0
19	28074 ± 662	0
AraC	148[b]	99

The numbers represent the mean ± standard error and the percent inhibition for six replicate wells containing 6 X 10^5 bone marrow cells as described in section VI.C.6.a.(2).

[a] All compounds were tested at 10^{-3} M.

[b] $p<0.05$ as compared to controls.

TABLE XVII: INHIBITION OF ^{125}IUdR INCORPORATION IN P388 CELLS

COMPOUND[a]	MEAN CPM ± STANDARD ERROR	% INHIBITION
CONTROL	21592 ± 1196	
22	20605 ± 655	5
24	14272 ± 468[b]	34
28	22838 ± 2938	0
36	19633 ± 1176	9
37	23089 ± 955	0
38	21163 ± 783	2
39	17960 ± 638	17
19	19326 ± 369	10
AraC	242[b]	99

The numbers represent the mean ± standard error and the percent inhibition for six replicate wells containing 6 X 10^5 P388 cells as described in section VI.C.6.b.(3).

[a] All compounds were tested at 10^{-3} M.

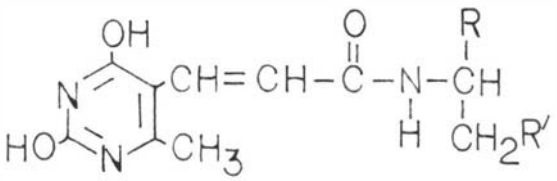
[b] $p<0.05$ as compared to controls.

V. CONCLUSIONS

The target compounds were analyzed in three assays, the protein synthesis assay, the DNA synthesis assay, and the cell growth assay using P388 cells, P815 cells, or bone marrow cells. In the DNA and protein synthesis assays, the incorporation of the pulse labels ^{125}IUdR and [^{75}Se]-selenomethionine, respectively, were quantitated. These measurements were used as indirect measurements of DNA and protein synthesis. Analytical inspection of the data summarized in Table XVIII, revealed three of the fourteen target compounds, 24, 39, and 50, were significantly active inhibitors of [^{75}Se]-selenomethionine incorporation in bone marrow cells, P388 lymphoma cells, and P815 mastocytoma cells at $p<0.05$. Furthermore, these three compounds inhibited P815 cell growth with ED_{50} values near those of the control drugs, puromycin and sparsomycin. Closer examination of the pharmacological data in an attempt to establish a structure-activity relationship, disclosed compounds 35, 38, and 48, were usually the least active analogues. Compounds 19, 22, 28, 36, and 37 were moderately active in most of the experiments. Several conclusions can be drawn relating the structure of the analogues to their biological effectiveness in P815, P388, and bone marrow cells.

TABLE XVIII: SUMMARY OF THE RESULTS FOR THE PROTEIN SYNTHESIS AND CELL GROWTH ASSAYS.

The data which appears in Table XVIII summarizes the pharmacological results for the following target compounds which were synthesized.



	R	R'	X	COMPOUND
Series I	$-CH_2OH$	$-C_6H_4-X$	H	22
	$-CH_2OH$	$-C_6H_4-X$	4-Br	24
	$-CH_2OH$	$-C_6H_4-X$	$4-CH_3$	28
	$-CH_2OH$	$-C_6H_4-X$	$4-OCH_3$	35
Series II	-H	$-C_6H_4-X$	H	36
	-H	$-C_6H_4-X$	$4-CH_3$	37
	-H	$-C_6H_4-X$	$4-OCH_3$	38
	-H	$-C_6H_4-X$	4-Br	39
Series III	-H	$-CO-NH-C_6H_4-X$	H	44
	-H	$-CO-NH-C_6H_4-X$	$4-CH_3$	46
	-H	$-CO-NH-C_6H_4-X$	$4-OCH_3$	48
	-H	$-CO-NH-C_6H_4-X$	4-Br	50
	-H	$-CO-NH-C_6H_3-X_2$	3,4 diCl	52

... Continued

TABLE XVIII: SUMMARY OF THE RESULTS FOR THE PROTEIN SYNTHESIS AND CELL GROWTH ASSAYS (Continued)

COMPOUND	P388[a]	P815[a]	BM[a]	ED_{50} FROM P815 CELL GROWTH ASSAY
22	6	52	34	
24	44	94	64	8.9×10^{-6} M
28	7	65	40	
35	0	0	0	
36	3	2	34	
37	0	11	35	
38	1	0	44	
39	26	89	42	5.8×10^{-6} M
44[b]	--	--	--	
46[b]	--	--	--	
48	3	0	11	
50	22	69	29	6.0×10^{-5} M
52[b]	--	--	--	

[a] The numbers represent the percent inhibition of [^{75}Se]-selenomethionine incorporation in the protein synthesis assays.

[b] These compounds were insoluble at the required concentrations in water or ethanol, which precluded testing.

First, the results indicate the removal of the hydroxymethyl functional group as seen in sparsomycin has a varying effect on activity. In the P815 cells, removal of the hydroxymethyl group decreased the ability of the target compounds to inhibit [^{75}Se]-selenomethionine incorporation. In support of this, the Series I compounds 22 and 28 were active inhibitors, whereas, the Series II analogues 36 and 37 were inactive. In the P388 cells and the bone marrow cells, inclusion or exclusion of the hydroxymethyl functional group only effected the bromophenyl- and methoxyphenyl-substituted analogues' ability to inhibit [^{75}Se]-selenomethionine incorporation. In the case of the bromophenyl analogues, inclusion of the hydroxymethyl group resulted in an increased inhibition of [^{75}Se]-selenomethionine incorporation in the P388 and bone marrow cells (24 versus 39). Conversely, the addition of the hydroxymethyl group to the methoxyphenyl-substituted compound 38 eliminated the inhibitory activity in the bone marrow cells, as shown by the inactivity of compound 35. In contrast to the results with P815, P388, and bone marrow cells in the protein synthesis assays, the results of the P815 cell growth studies indicated the presence or absence of the hydroxymethyl functional group had no effect on activity as evidenced in the comparison of the similar ED_{50} values for compound 24 and 39.

Additional information for expansion of the structure activity relationship for the target compounds evolves from the data on the Series II compounds in the bone marrow

protein synthesis assay. In these experiments, the variation of substituents on the phenyl ring did not change the ability of the compounds to inhibit [^{75}Se]-selenomethionine incorporation. All four of the Series II compounds, 36-39, had approximately the same activity.

In Series III, the only significantly active compound was 50 in the protein synthesis and cell growth studies. This compound was not as active as 24 or 39 which suggests the removal of the hydroxymethyl functional group and substitution of the mono-oxodithioacetal side chain of sparsomycin with a substituted benzyl amide moiety is not beneficial for activity.

Continuing the analysis of the data from the protein synthesis assays revealed that the bromophenyl-substituted analogues consistently imparted the greatest inhibitory activity. Furthermore, the methoxyphenyl substituted analogues were inactive in most of the studies. The methylphenyl analogues were slightly more active than the unsubstituted phenyl compounds, and overall, the methylphenyl and unsubstituted phenyl analogues fell in between the bromophenyl- and methoxyphenyl-substituted compounds. The activity may correspond to the lipophilic and electronic characteristics of the substituents on the phenyl moiety of the analogues. Table XIX list the σ and π values for the four substituents investigated. In conclusion, it appears that the bromophenyl substituent of high lipophilicy and electron withdrawing character is optimal for inhibitory activity and

TABLE XIX: SUBSTITUENT CONSTANTS (82)

SUBSTITUENT	σ PARA	π (PARA)
bromo	0.23	1.19
methyl	-0.17	0.60
hydrogen	0	0
methoxy	-0.27	-0.03

conversely, the methoxyphenyl substituent of hydrophilic and electron donating character is least desirable. These results substantiate the supposition of Lee and Vince (50) that hydrophobic groups extending into the region occupied by the $-CH_2-S-CH_3$ chain of sparsomycin were tolerated, and in the case of several compounds (Series I: 22 versus 24 Series II: 36 versus 39) actually enhanced activity.

VI. EXPERIMENTAL

A. General

Melting points were determined on a Thomas-Hoover melting point apparatus and are reported as uncorrected values. Infrared spectra were obtained from a Beckman AccuLab 8 spectrophotometer using samples of the compounds as a solution in the indicated solvent or neat. The strong, sharp band of polystyrene film at 1601.4 cm^{-1} was used as a reference marker for all infrared spectra. Nuclear magnetic resonance (NMR) spectra were measured on a Pekin-Elmer Model R-24 spectrometer. Chemical shifts of the compounds in the indicated solvent are denoted in units of parts per million (δ), downfield or upfield from a known reference peak. When trifluoracetic acid was used as the solvent, the trifluoroacetic acid proton at 11.3 ppm was used as the reference. In all other solvents, the shifts are reported downfield from tetramethylsilane which was added as an internal standard. Ultraviolet spectra were generated from a Beckman Model 25 spectrophotometer using samples in a solution of the indicated solvent.

Reaction solvents were stored over the appropriate size molecular sieves (83). Dimethyformamide was distilled over barium oxide prior to storage over molecular sieves.

Tetrahydrofuran was reflexed over cuprous chloride, distilled, and finally distilled again over lithium aluminum hydride prior to storage over molecular sieves. Ethanol was made super dry by distilling over magnesium and a catalytic amount of iodine prior to storage over molecular sieves (84). Removal of solvents under reduced pressure was done with a Buchler Flash Evaporator at water aspirator pressure. Drying was accomplished under a vacuum of less than one mm Hg over the indicated solvent.

The reactions leading to the isolation of the Series III compounds were followed by thin layer chromatography on silica gel plates (Analtech, Inc, Newark, DE). The plates were developed in a solvent system containing chloroform, isopropyl ether, and acetic acid (6:3:1). The target compounds of each series were also analyzed by thin layer chromatography using chloroform and methanol (8:2).

All compounds are denoted by underlined Arabic numerals. In addition to spectroscopic characterization, all new compounds were identified by elemental analysis (Atlantic Microlab., Atlanta, Georgia).

B. Compounds Synthesized

2,4-Dihydroxy-5-hydroxymethyl-6-methylpyrimidine (2). The method of Ottenheijm, vanNispen, and Sinnige (46) was followed. Sodium hydroxide (6.4 g, 0.16 mole) was dissolved in water (60 mL), cooled to room temperature, and added to 2,4-dihydroxy-6-methylpyrimidine (10.0 g, 80 mmol) in an open 100-mL round-bottomed flask, equipped with a magnetic stirrer. Thirty-seven percent formaldehyde solution (19.2 g, 0.24 mole) was added and stirred at room temperature for three hours. The white precipitate was filtered and dissolved in boiling water (50-60 mL). One equivalent of glacial acetic acid (4.8 g, 80 mmol) was slowly added. The solution was cooled in the refrigerator overnight. The precipitate was filtered, and dried under reduced pressure (toluene, 111°) to afford 9.9 g (80%) of 2 as a white solid, mp 300-310° (dec); literature (85) mp 305-310° (dec). IR (Nujol): 3380 cm^{-1} (broad, O-H); 1700 (medium, C=O). NMR (Me_2SO-d_6/D_2O): δ 2.5 (s, 2, CH_2); 2.1 (s, 3, CH_3).

(2,4-Dihydroxy-6-methyl-5-pyrimidinyl) carboxaldehyde (3). The aldehyde 3 was prepared via the procedure of Brossmer and Ziegler (86). Potassium persulfate (10.8 g, 40 mmol) was dissolved in water (150 mL) in a 250-mL Erlenmeyer flask equipped with a magnetic stirrer. The previously described hydroxymethylpyrimidine 2 (6.24 g, 40 mmol) was added and heated to 40°. A small amount of un-

dissolved 2 was filtered, and silver nitrate (0.1 g, 0.6 mmol) was added to the filtrate. The filtrate solution was stirred at 40° for thirty minutes (oil bath, 40°), followed by cooling in the refrigerator overnight. The precipitate was collected by filtration, recrystallized from water, and dried under reduced pressure (toluene, 111°) to afford 4.0 g (65%) of 3 as a white solid, mp above 200° (dec); literature (5) mp above 200° (dec). IR (Nujol): 1740 cm^{-1} (strong, C=O). NMR (DMF, d-7): δ 9.7 (s, 1, CHO); 2.2 (s, 3, CH_3). UV max (H_2O): 232 nm (ε = 6300); 280 (ε = 9000); UV min (H_2O): 250 (ε = 3200).

Carbethoxymethyltriphenylphosphonium Bromide (4a). The method described by Isler, et al. (53) was used. Triphenylphosphine (156.8 g, 0.6 mole) and benzene (730 mL) were placed in a 2000-mL round-bottomed flask equipped with a mechanical stirrer, thermometer, and 100-mL pressure compensating dropping funnel with a drying tube. To the solution, ethyl bromacetate (100 g, 0.6 mole) was added dropwise over a thirty-minute period. The temperature rose from room temperature to 35-40°. After stirring overnight, the phosphonium bromide was collected by filtration, washed with benzene and hexane, and dried under reduced pressure for several hours to give 257 g (100%) of 4a as a white solid, mp 162°; literature (53) mp 158°.

Carbethoxymethylenetriphenylphosphorane (4). The procedure of Isler, et al. (53) was used. To a solution of carbethoxymethyltriphenylphosphonium bromide 4a (96.0 g,

0.22 mole) in water (about 700-800 mL), a five percent solution of sodium hydroxide was added dropwise with stirring until a pH of 8.7 was obtained. The resulting precipitate was collected by filtration, washed with water, and recrystallized from ethyl acetate/hexane to give 60.0 g (86%) of 4 as a white solid, mp 116-118°; literature (53) mp 116-117°. NMR ($CDCl_3$): δ 7.55 (m, 15, Ar-H); 4.0 (q, 2, CH_2); 2.9 (s, 1, P=CH); 1.0 (t, 3, CH_3).

(E)-Methyl 3-(2,4-Dihydroxy-6-methyl-5-pyrimidinyl) propenoate (5). The method described by Wiley and MacKeller (5), was followed. A mixture of 3 (3.08 g, 20 mmol), carbethoxymethylenetriphenylphosphorane (4) (14.0 g, 40 mmol), in dimethylformamide (60 mL) was placed in a 250-mL round-bottomed flask and heated at reflux for six hours (condenser, drying tube, oil bath). About 30 mL of the dimethylformamide was removed under reduced pressure, and water (30 mL) was added. The white solid was collected by filtration, recrystallized from ethylene glycol monomethyl ether, and dried (toluene, 111°), to give 1.86 g (42%) of 5 as a white solid, mp 298-300° (dec); literature (5) mp 299-302° (dec). IR (Nujol): 1750 cm^{-1} (strong, C=O); 1680 (strong, C=C). NMR (Me_2SO, d-6): δ 11.3 (s, 2, O-H, exchangeable); 7.2 (d of d, 2, trans CH=CH, J=16 Hz); 4.2 (q, 2, CH_2); 2.3 (s, 3, pyrimidine-CH_3); 1.2 (t, 3, CH_3). UV max (EtOH): 303 nm (ε = 21,000).

(E)-3-(2,4-Dihydroxy-6-methyl-5-pyrimidinyl)propenoic Acid (6). The procedure of Ottenheijm, et al. (46) was followed. A solution of 5 (1.0 g, 4.5 mmol) in a 1:1:1 (v/v) mixture of dioxane, methanol, and water (50 mL) containing sodium hydroxide (0.54 g, 13.5 mmol) was warmed on the steam bath for two hours. The solution was cooled to room temperature and the dioxane and methanol were removed under reduced pressure. The remaining solution was acidified with a five percent solution of hydrochloric acid and fluffy white crystals precipitated. The precipitate was collected by filtration, recrystallized from water, and dried (toluene, 111°), to give 0.87 g (99%) of 6 as a white solid, mp 267-270° (dec); literature (5) mp 265° (dec). IR (Nujol): 3480 cm^{-1} (broad, O-H); 1740 (strong, C=O). NMR (Me_2SO, d-6): δ 11.4 (s, 1, COOH); 7.25 (d of d, 2, trans CH=CH, J=16 Hz); 2.38 (s, 3, CH_3). UV max (H_2O): 294 nm (ε = 11,500).

(E)-(±)-N-(2-Heptanyl)-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (19). The method of Lin and Dubois (51) was modified. A mixture of 6 (1.0 g, 5 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.56 g, 6 mmol) in dry dimethylformamide (32 mL) was magnetically stirred at 35° (oil bath, 35°) until solution formed. Subsequently, 2-aminoheptane (0.58 g, 5 mmol) in dimethylformamide (5 mL) was added, followed by water (4.2 mL). The solution was stirred for four hours at 35°. The precipitate was collected by filtration, recrystallized

from ethanol, and dried under reduced pressure (toluene, 111°) to afford 1.11 g (78%) of 19 as a white solid, mp 194-195° (dec). IR (Nujol): 3400 cm^{-1} (medium, N-H): 1710 (strong, C=O), 1650 (strong, C=C). NMR (Me_2SO, d-6, heated to effect solution): δ 7.1 (d of d, 2, trans CH=CH, J=16 Hz); 5.5 (s, 3, 2 O-H, N-H, exchangeable); 2.3 (s, 3, CH_3); 0.8-1.4 (m, 15, heptane C-H).

Anal. Calculated for $C_{15}H_{23}N_3O_3 \cdot H_2O$: C, 57.88; H, 8.04; N, 13.50. Found: C, 57.84; H, 8.10; N, 13.50.

Methyl 2-Amino-3-phenylpropanoate Hydrocholoride (20). The procedure of Dubois, et al. (49) was used. Thionyl chloride (3.95 mL, 55 mmol) was added dropwise to fifteen mL of methanol at -25° (dry ice, 2-propanol) in a 50-mL round-bottomed flask. Subsequently, phenylalanine (8.3 g, 50 mmol) was added in small portions, while the temperature was maintained below -5°. The mixture was warmed to room temperature, then heated at 45° for four hours (oil bath, 45°), and finally stirred (magnetic) at room temperature overnight. After addition of benzene (2-5 mL), the solvent was removed under reduced pressure. The residue was triturated with ether to give 8.9 g (99%) of 20 as a white solid which was recrystallized from ethanol/ether and dried under reduced pressure (toluene, 111°), mp 158-160°; literature (87) 158°. IR (Nujol): 3400 cm^{-1} (broad, N-H); 1730 (strong, C=O); 1600 (medium, C=C). NMR (Me_2SO, d-6): δ 9.0 (s, 3, $NH_2 \cdot HCl$); 7.3 (s, 5, Ar-H); 4.25 (t, 1, CH); 3.75 (s, 3, OCH_3); 3.3 (m, 2, CH_2).

2-Amino-3-phenyl-1-propanol (21). The method of Birkofer and Erlenbach (56) was used. After flame-drying the system under a stream of nitrogen, lithium aluminum hydride (2.3 g, 60 mmol) was suspended in anhydrous ether (150 mL) in a 500-mL three-necked, round-bottomed flask equipped with a magnetic stirrer, reflux condenser, and one glass stopper. To the reaction mixture was added 20 (4.5 g, 21 mmol) suspended in 100 mL of ether. The reaction mixture was stirred overnight. The excess lithium aluminum hydride was decomposed with a solution of ammonium chloride (neutralized to pH 7). The white precipitate was collected by filtration, and the two layers of filtrate were separated. The aqueous layer was extracted with ether (3 x 25 mL). All of the ether extracts were combined, washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate, and concentrated to dryness under reduced pressure to give 0.9 g (24%) of 21 as a yellow, waxy solid. A small amount was converted to the hydrochloride salt by the procedure of Matuszak and Matuszak (6) to give a white solid, mp 131-134°; literature (88), mp 156°; (89) mp 130-133°. IR (Nujol): 3350 cm^{-1} (broad, O-H, N-H). NMR ($CDCl_3$): δ 7.25 (s, 5, Ar-H); 2.6-3.7 (m, 5, CH_2CHCH_2); 2.1 (s, 3, NH_2, OH).

(E)-(±)-N-(1-Hydroxy-3-phenyl-2-propyl)-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (22). The procedure of Lin and Dubois (51) was modified. A mixture of 6 (1.0 g, 5 mmol) and N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (1.56 g, 6 mmol) in dry dimethylformamide (35 mL)

was magnetically stirred at 35° (oil bath, 35°) until solution was effected. A solution of 2-amino-3-phenyl-1-propanol (0.76 g, 5 mmol) in dimethylformamide (5 mL) was added, followed by water (4 mL). The solution was stirred at 35° for six hours. The precipitate was collected by filtration to afford 1.63 g (99%) of 22 as a white solid. Recrystallization from ethanol gave 22, mp 191-191.5°. IR (Nujol): 3350 cm^{-1} (broad, N-H); 3100 (broad, O-H); 1710 (strong, C=O); 1650 (strong, C=C). NMR (Me_2SO, d-6): 6.7-7.5 (m, 7, Ar-H, trans CH=CH); 4.7 (s, 4, N-H, 3 O-H, exchangeable); 2.5-3.5 (m, 5, CH_2CHCH_2); 2.3 (s, 3, CH_3).

Anal. Calculated for $C_{17}H_{19}N_3O_4 \cdot H_2O$: C, 58.78; H, 6.05; N, 12.10. Found: C, 58.73; H, 6.11; N, 12.06.

2-Amino-3-(4-bromophenyl)propanol (23). The procedure of Anhoury, et al. (57) was followed. A 100-mL three-necked, round-bottomed flask equipped with a nitrogen inlet adapter, pressure compensating dropping funnel with a septum, reflux condenser with a nitrogen outlet adapter, and magnetic stirring bar, was assembled and flame dried while flushing the system with nitrogen. Under a positive nitrogen pressure, the reaction flask was charged with 4-bromophenylalanine (1.0 g, 4 mmol) and flushed with nitrogen for one minute. Dry tetrahydrofuran (25 mL) was added. To this solution was added dropwise, with stirring, 1.0 M borane-tetrahydrofuran solution (5.7 mL). The solution was stirred at room temperature for 15 hours, after which

additional 1.0 M borane-tetrahydrofuran solution (5.7 mL) was added. The reaction was stirred for another fifteen hours. The excess borane was destroyed by adding ethanol (about 60 mL) until evolution of hydrogen gas ceased. The solution was acidified with 3 N hydrocholoric acid to pH 1. After adding 2-propanol (10 mL) to the flask, the water was azeotropically removed by distillation under reduced pressure, leaving a white solid which was recrystallized from ethanol/ether to give 0.88 g (82%) of 23-HCl. A solution of 23-HCl (0.87 g, 3 mmol) in water (minimum amount) was treated with a five percent solution of sodium hydroxide to a pH of 12-14. After adding 2-propanol (10 mL), the water was azeotropically removed by distillation under reduced pressure. The residue was dissolved in ether (50 mL), washed with water (25 mL), and saturated sodium chloride solution, dried over anhydrous sodium sulfate, and the ether removed under reduced pressure. The resulting yellow oil crystallized by drying under reduced pressure, to give 0.56 g (75%) of 23 as a pale yellow solid, mp 85-87°; literature (90) mp 93-94°. IR (Nujol): 3400 cm^{-1} (broad, O-H, N-H). NMR ($CDCl_3$): δ 7.2 (d of d, 4, Ar-H); 2.5-3.7 (m, 5, CH_2CHCH_2); 2.1 (s, 3, O-H, NH_2, exchangeable).

(E)-(±)-N-[1-Hydroxy-3-(4-bromophenyl)-2-propyl]-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (24). The method of Lin and Dubois (51) was modified. A mixture of 6 (0.382 g, 2 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-

dihydroquinoline (0.556 g, 2.2 mmol) in dry dimethylformamide (10 mL) was magnetically stirred at 35° (oil bath, 35°) until solution was effected. A solution of 23 (0.45 g, 2 mmol) in dimethylformamide (5 mL) was added. After the addition of water (1 mL), the solution was stirred for three days, during which time a white precipitate appeared. The precipitate was collected by filtration and washed with ether to give 0.45 g (57%). Recrystallization from absolute ethanol afforded 24 as a white solid, mp 188-189.5°. IR (Nujol): 3300 cm^{-1} (weak, N-H); 3050 (broad, O-H); 1710 (medium, amide C=O); 1650 (medium, C=C). NMR (Me_2SO, d-6): δ 6.7-7.5 (m, 6, Ar-H, CH=CH); 5.0 (s, 4, 3 O-H, N-H, exchangeable) 2.5-3.3 (m, 5, CH_2CHCH_2); 2.2 (s, 3, CH_3).

Anal. Calculated for $C_{17}H_{18}BrN_3O_4 \cdot 1.25\ H_2O$: C, 47.40; H, 4.50; N, 9.75. Found: C, 47.37; H, 4.82; N, 9.73.

Diethyl 2-Acetamido-2-(4-methylbenzyl) malonate (25). The procedure of Albertson and Archer (60) was followed. Sodium metal (1.15 g, 50 mmol) was rinsed in methanol, then washed in a benzene-methanol solution, and weighed in a flask containing petroleum ether. The sodium was transferred to a flame-dried, 250 mL, three-necked, round-bottomed flask, equipped with a mechanical stirrer, condenser and pressure-equalizing funnel, containing absolute ethanol (100 mL). Diethyl acetamidomalonate (10.9 g, 50 mmol) was suspended in absolute ethanol (20-30 mL) and added to the solution. The mixture was heated to reflux and 4-methylbenzyl chloride (9.1 g, 65 mmol) was added dropwise. This solution was

heated at reflux for two hours. After cooling to room temperature, the solution was concentrated under reduced pressure, and water (25 mL) and chloroform (25 mL) were added. The aqueous layer was extracted with chloroform (3 x 100 mL), dried over sodium sulfate, and concentrated under reduced pressure to give a yellow, oily residue which crystallized after standing overnight. The white solid was recrystallized from ethanol to give 8.2 g (51%) of 25, mp 111-112°. IR ($CHCl_3$): 3400 cm^{-1} (weak, N-H); 1740 (strong, ester C=O); 1680 (strong, amide C=O). NMR ($CDCl_3$): δ 7.05 (m, 4, Ar-H); 6.6 (1, s, NH); 4.3 (q, 4, 2 OCH_2); 3.65 (s, 2, Ar-CH_2); 2.34 (s, 3, Ar-CH_3); 2.05 (s, 3, CH_3C=O); 1.35 (t, 6, C-CH_3).

Anal. Calculated for $C_{17}H_{23}NO_5$: C, 63,55; H, 7.16; N, 4.36. Found: C, 63.67; H, 7.21; N, 4.35.

2-Amino-3-(4-tolyl) propionic Acid (26). The method of Albertson and Archer (60) was followed. A mixture of 25 (11.0 g, 30 mmol) and hydrobromic acid (48%, 28 mL), in a 100-mL round-bottomed flask equipped with a reflux condenser and a magnetic stirrer, was heated at reflux for 7.5 hours. The solution was decolorized with charcoal and filtered. The filtrate was treated with concentrated ammonium hydroxide to pH 6 and placed in the refrigerator overnight. The white solid was filtered and dried under reduced pressure (toluene, 111°) to afford 6.0 g (98%) of 26, mp 207-209° (dec); literature (91), 211-218°. IR (Nujol): 3100 cm^{-1} (broad, O-H); 1620 (strong, C=O); 1590

(strong, C=C). NMR (TFA): δ 7.2 (s, 4, Ar-H); 4.7 (m, 1, CH): 3.5 (d, 2, CH_2); 2.4 (s, 3, Ar-CH_3).

4-(2-Amino-3-hydroxypropyl)toluene (27). The procedure of Lee and Vince (50) was used. A 100-mL three-necked, round-bottomed flask, equipped with a condenser and nitrogen inlet and outlet valves, was flame-dried under a stream of nitrogen. A mixture of lithium aluminum hydride (1.0 g, 26 mmol) and dry tetrahydrofuran (75 mL) was heated at reflux for two hours under nitrogen. The mixture was cooled in an ice bath and 26 (2.0 g, 11 mmol) was added in small portions. The mixture was heated at reflux for twelve hours under nitrogen. The excess lithium aluminum hydride was decomposed by adding in succession, water (2 mL), aqueous sodium hydroxide (10%, 20 mL), and water (5 mL). The mixture was filtered, and the precipitate was washed with tetrahydrofuran. The filtrate was concentrated under reduced pressure to leave a yellow oil which was dissolved in chloroform (50 mL). The chloroform layer was washed with aqueous sodium hydroxide (5%, 25 mL), water (25 mL), and saturated aqueous sodium chloride solution (25 mL). The chloroform extract was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give 1.23 g (67%) of 27 as a pale yellow oil. A small amount was converted to the hydrochloride salt by the procedure of Matuszak and Matuszak (92) to give a white solid, which was recrystallized from ethanol/ether, mp 173-175°. IR (Nujol): 3350 cm^{-1} (strong, O-H, N-H). NMR (D_2O): 7.35 (s, 4, Ar-H); 3.8 (m, 3, O-CH_2-CH);

3.0 (d, 2, CH_2-Ar); 2.4 (s, 3, CH_3).

Anal. Calculated for $C_{10}H_{16}ClNO$: C, 59.56; H, 8.00; N, 6.94. Found: C, 59.61; H, 8.00; N, 6.94.

(E)(±)-N-[1-Hydroxy-3-(4-methylphenyl)-2-propyl]-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (28). The method of Lin and Dubois (51) was modified. A mixture of 6 (0.22 g, 1.1 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.33 g, 1.3 mmol) in dry dimethylformamide (10 mL) was magnetically stirred at 35° (oil bath, 35°) until solution formed. Subsequently, 4-(2-amino-3-hydroxypropyl)-toluene 27 (0.18 g, 1.1 mmol) in dimethylformamide (5 mL) was added, followed by water (1 mL). The solution was stirred for five hours at 35°. The precipitate was collected by filtration and recrystallized from ethanol/ether to give 0.24 g (62%) of 28 as a white solid, mp 187-187.5°. IR (Nujol): 3320 cm^{-1} (medium, N-H); 3150 (broad, O-H); 1710 (strong, amide C=O); 1655 (strong, C=C). NMR (TFA): δ 6.2-7.8 (m, 10, Ar-H, CH=CH, 3 O-H, N-H); 3.7-4.1 (m, 3, O-CH_2-CH-); 2.7 (2, m, Ar-CH_2); 2.3 (s, 3, pyrimidine-CH_3; 2.1 (s, 3, Ar-CH_3).

Anal. Calculated for $C_{18}H_{21}N_3O_4 \cdot H_2O$: C, 59.83; H, 6.37; N, 11.63. Found: C, 59.80; H, 6.44; N, 11.63.

4-Methoxybenzyl Bromide (29). The procedures of Birch, et al. (93), and Fujo, et al. (94) were used. Ice-cooled hydrobromic acid (48%, 15 mL) was added to ice-cooled 4-methoxybenzyl alcohol (8.6 g, 62 mmol), and the mixture was vigorously stirred (magnetic) in a 50-mL round-bottomed

flask for thirty minutes to separate a colorless heavy oil. The oil was extracted with benzene and washed successively with water, saturated aqueous sodium bicarbonate, and saturated sodium chloride. The combined benzene extracts were dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give an oil which was distilled *in vacuo* to afford 7.3 g (58%) of *29* as a colorless liquid, bp 88^{o}/1 mm; literature (95) bp 131^{o}/19 mm. IR ($CHCl_3$): 1610 and 1590 cm^{-1} (strong, C=C); 1240 (broad, Ar-O-C); 740 (strong, CH_2-Br). NMR ($CDCl_3$): δ 7.0 (d of d, 4, Ar-H): 4.4 (s, 2, CH_2); 3.7 (s, 3, OCH_3).

Ethyl 2-Acetamido-2-carbethoxy-3-(4-methoxyphenyl) propanoate (*30*). The method of Yamamoto, *et al.* (62) was used. A sodium ethoxide solution was prepared in a 250-mL three-necked, round-bottomed flask equipped with a mechanical stirrer, condenser, and pressure compensating dropping funnel. Sodium metal (0.83 g, 36 mmol) was rinsed in ethanol, and benzene, weighed in a flask containing petroleum ether, and transferred to the 250-mL flask containing super dry ethanol (100 mL)(84). To the flask was added diethyl acetamidomalonate (8.5 g, 39.2 mmol). The solution was cooled in an ice bath while 4-methoxybenzyl bromide (*29*) (7.3 g 36.3 mmol) was slowly added dropwise. After stirring for five hours at room temperature, water (375 mL) was added to precipitate *30* as a white solid, which was collected by filtration, and dried to give 10.37 g (85%) of *30*, mp $93-95^{o}$; literature (62) mp $96-98^{o}$. IR ($CHCl_3$): 3400 cm^{-1} (medium, N-H); 1740 (strong, ester C=O); 1640 (strong,

amide (C=O); 1610 (strong, C=C); 1250 (strong, Ar-O-C). NMR ($CDCl_3$): δ 6.9 (m, 4, Ar-H); 4.3 (q, 4, 2 CH_2O); 3.8 (s, 3, O-CH_3); 3.6 (s, 2, CH_2Ar); 2.05 (s, 3, CH_3-C=O); 1.3 (6, t, 2 CH_3-C).

2-Acetamido-2-carbethoxy-3-(4-methoxyphenyl) propanoic Acid (31). The procedure of Berlinguet (63) was followed. To a 500-mL round-bottomed flask containing 0.3 N alcoholic potassium hydroxide (250 mL) was added 30 (22.8 g, 68 mmol). The mixture was agitated with a magnetic stirrer overnight. The solution was filtered, and the filtrate was concentrated under reduced pressure while heating on a steam bath. Cold water (400 mL) was added to the flask and 31 dissolved leaving the unreacted propionic ester as a solid which was collected by filtration. The filtrate was acidified with concentrated hydrochloric acid to pH 1. The heavy oil which resulted crystallized in the cold. The white precipitate was collected by filtration and dried to give 13.2 g (63%) of 31, mp 139-140°; literature (63) mp 132-133°. IR (Nujol); 3300 cm^{-1} (medium, N-H); 1720 (strong, ester C=O); 1600 (strong, amide C=O); 1220 (medium, Ar-O-C). NMR (Me_2SO, d-6): δ 7.8 (s, 2, N-H, COOH); 7.0 (s, 4, Ar-H); 4.2 (q, 2, C-CH_2-O); 3.8 (s, 3, OCH_3); 3.5 (s, 2, CH_2); 2.0 (s, 3, CH_3C=O); 1.2 (t, 3, C-CH_3).

Ethyl 2-Acetamido-3-(4-methoxyphenyl) propanoate (32). The method of Berlinguet (63) was used. In a flame-dried, 100-mL round-bottomed flask connected to a vacuum pump was placed 31 (8.0 g, 26 mmol). The flask

was immersed in an oil bath, and the temperature was raised to 150°. The compound began to vigorously decarboxylate at 120°. The flask was left in the oil bath at 150° for fifteen minutes. The resultant yellow oil was crystallized and recrystallized from petroleum ether (bp 60-80°) to give 6.6 g (96%) of 32 as a white solid, mp 65-65.5°; literature (63) mp 72-73°. IR ($CHCl_3$): 3400 cm^{-1} (medium, N-H); 1220 (strong, Ar-O-C). NMR ($CDCl_3$): δ 7.0 (d of d, 4, Ar-H); 6.2 (s, 1, N-H); 4.9 (t, 1, CH); 4.2 (q, 2, $C-CH_2-O$); 3.75 (s, 3, OCH_3); 3.0 (d, 2, CH_2-Ar); 2.0 (s, 3, $CH_3-C=O$); 1.2 (t, 3, $C-CH_3$).

2-Acetamido-3-(4-methoxyphenyl)propanol (33). The method of Berlinguet (63) was used. A magnetically stirred solution of 32 (6.55 g, 25 mmol) in absolute ether (250-300 mL) was cooled in an ice bath while a suspension of lithium aluminum hydride (1.3 g, 34 mmol) in absolute ether (75 mL) was slowly added dropwise. The mixture was stirred for two hours at room temperature. The excess lithium aluminum hydride was decomposed with small lumps of ice (about seven grams) until evolution of hydrogen gas ceased. The mixture was heated and filtered. The precipitate was washed twice with boiling ethanol, and the combined filtrates were evaporated under reduced pressure. The resultant oil was crystallized by drying under reduced pressure and washing with hot ether to give 4.9 g (89%) of 33 as a white solid, mp 98-104°, literature (63) 110-111°.

IR (Nujol): 3300 cm^{-1} (weak, N-H); 3200 (broad, O-H); 1640 (strong, C=O); 1250 (strong, Ar-O-C). NMR ($CDCl_3$): δ 7.0 (d of d, 4, Ar-H); 4.1 (m, 1, CH); 3.75 (s, 3, OCH_3); 3.55 (m, 2, CH_2); 2.8 (d, 2, CH_2-Ar); 1.95 (s, 3, CH_3-C=O).

2-Amino-3-(4-methoxyphenyl) propanol Hydrochloride (34). The procedure of Berlinguet (63) was used. A mixture of 33 (4.88 g, 22 mmol) and 2 N hydrochloric acid (100 mL) was placed in a 250-mL round-bottomed flask and heated at reflux for three hours. The solution was then concentrated under reduced pressure. The resultant residue was dissolved in water, and purified with activated charcoal. After filtering over Celite, the clear solution was again evaporated under reduced pressure, and the residue was crystallized from ethanol/ether to give 2.78 g (58%) of 34 as a white solid, mp 190-194°; literature (63) mp 195-197°. IR (Nujol): 3390 cm^{-1} (medium, N-H, O-H); 1260 (strong, Ar-O-C). NMR (D_2O): δ 7.2 (d of d, 4, Ar-H); 3.8-4.0 (m, 6, CH-CH_2 and OCH_3); 3.0 (d, 2, CH_2O).

(E)-(±)-N-[1-Hydroxy-3-(4-methoxyphenyl)-2-propyl]-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (35). The method of Lin and Dubois (51) was modified. A mixture of 6 (0.74 g, 3.8 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.1 g, 4.4 mmol) in dry dimethylformamide (20 mL) was magnetically stirred at 35° (oil bath, 35°) until solution was effected. A solution of 34 (1.0 g, 4.6 mmol) in water (minimum amount) was treated with a five

percent aqueous solution of sodium hydroxide to pH 14. The water was removed by azeotropic distillation with 2-propanol under reduced pressure to afford 34 (free base) as a yellow oil which crystallized in cold ether. A solution of 34 (free base) (0.68 g, 3.8 mmol) in dimethylformamide (8 mL) was added to the reaction mixture. After the addition of water (3 mL), the solution was stirred for three days at 35°. The dimethylformamide was removed under reduced pressure, and the resultant yellow residue was crystallized with absolute ether to afford 0.99 g (73%) of 35 as a yellow solid. Recrystallization from ethanol afforded 35 as a white solid, mp 179-181°. IR (Nujol): 3320 cm^{-1} (medium, N-H); 3100 (broad, O-H); 1710 (strong, C=O); 1650 (strong, C=C); 1250 (medium, Ar-O-C). NMR (Me_2SO, d-6): δ 6.7-8.0 (m, 10, Ar-H, CH=CH, N-H, 3 O-H); 4.0 (m, 1, CH); 3.7 (s, 3, OCH_3); 2.8-3.5 (m, 4, 2 CH_2): 2.5 (s, 3, CH_3).

Anal. Calculated for $C_{18}H_{21}N_3O_5 \cdot \frac{1}{2}H_2O$: C, 58.32; H, 5.98; N, 11.34. Found: C, 58.24; H, 5.76; N, 11.42.

(E)-(±)-N-(2-Phenylethyl)-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (36). The procedure of Lin and Dubois (51) was modified. A mixture of 6 (2.0 g, 10 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (2.97 g, 12 mmol) in dry dimethylformamide (50 mL) was magnetically stirred at 35° (oil bath, 35°) until solution was effected. To the mixture described above phenethylamine (1.21 g, 10 mmol) was added. The solution was stirred

at 35° for twenty-four hours. The precipitate was collected by filtration and washed with ether to give 1.8 g (59%) of crude product. Recrystallization from water afforded 36 as a white solid, mp 218° (dec). IR (Nujol): 3100 cm^{-1} (broad, N-H); 1710 (strong, amide C=O); 1650 (strong, C=C) NMR (TFA): δ 6.6-7.6 (m, 7, Ar-H, CH=CH); 5.5-6.5 (s, 3, 2 O-H, N-H); 3.2 (m, 2, CH_2-C=O); 2.8 (m, 2, CH_2-Ar); 2.2 (s, 3, CH_3).

Anal. Calculated for $C_{16}H_{17}N_3O_3 \cdot H_2O$: C, 60.50; H, 6.04; N, 13.24. Found: C, 60.49; H, 6.07; N, 13.20.

(E)-(±)-N-[2-(4-Methylphenyl)-ethyl]-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (37). The method of Lin and Dubois (51) was modified. A mixture of 6 (1.0 g, 5 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.56 g, 6 mmol) in dry dimethylformamide (25 mL) was magnetically stirred at 35° (oil bath, 35°) until solution was effected. To the mixture described above, 2-(4-tolyl)-ethylamine (0.68 g, 5 mmol) was added. The solution was stirred at 35° for twenty-four hours. The precipitate was collected by filtration and washed with anhydrous ether to give 1.3 g (83%) of 37 as a white solid. Recrystallization from water afforded 37, mp 214.5-215.5° (dec) with evolution of gas. IR (Nujol): 3100 cm^{-1} (broad, N-H); 1710 (strong, C=O); 1650 (strong, C=C). NMR (TFA): δ 6.8-7.7 (m, 6, Ar-H, CH=CH); 3.3 (q, 2, CH_2C=O); 2.8 (t, 2, CH_2-Ar); 2.3 (s, 3, CH_3-pyrimidine); 2.05 (s, 3, CH_3-Ar).

Anal. Calculated for $C_{17}H_{19}N_3O_3 \cdot H_2O$: C, 61.62; H, 6.39; N, 12.68. Found: C, 61.53; H, 6.40; N, 12.67.

(E)-(±)-N-[2-(4-Methoxyphenyl)-ethyl]-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (38). The procedure of Lin and Dubois (51) was modified. A mixture of 6 (0.9 g, 4.6 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.36 g, 5.5 mmol) in dry dimethylformamide (20 mL) was magnetically stirred at 35° (oil bath, 35°) until solution was effected. To the mixture described above 4-methoxyphenethylamine (0.7 g, 4.6 mmol) was added. The solution was stirred at 35° for twenty-four hours. The precipitate was collected by filtration and washed with anhydrous ether to give 1.1 g (75%) of 38 as a white solid. Recrystallization from water afforded 38, mp 200.5-201.5° (dec) with evolution of gas. IR (Nujol): 3100 cm^{-1} (broad, N-H); 1720 (strong, C=O); 1650 (strong, C=C); 1250 (strong, Ar-O-C). NMR (TFA): δ 6.8-7.7 (m, 6, Ar-H, CH=CH); 3.7 (s, 3, OCH_3); 3.2 (q, 2, $CH_2C=O$); 2.8 (t. 2, CH_2Ar); 2.25 (s, 3, CH_3-pyrimidine).

Anal. Calculated for $C_{17}H_{19}N_3O_4 \cdot H_2O$: C, 58.78; H, 6.09; N, 12.10. Found: C, 58.74; H, 6.11; N, 12.07.

(E)-(±)-N-[2-(4-Bromophenyl)-ethyl]-3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamide (39). The procedure of Lin and Dubois (51) was modified. A mixture of 6 (0.8 g, 4.1 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.23 g, 5 mmol) in dry dimethylformamide (20 mL) was magnetically stirred at 35° (oil bath, 35°) until solution was effected. To the mixture described above 4-bromophenethylamine (0.82 g, 4.1 mmol) was added. The

solution was stirred at 35° for twenty-four hours. The precipitate was collected by filtration and washed with anhydrous ether to give 1.4 g (92%) of 39 as a white solid. Recrystallization from water afforded 39, mp 198-199.5° (dec) with evolution of gas. IR (Nujol): 3100 cm^{-1} (broad N-H); 1710 (strong, C=O); 1650 (strong, C=C). NMR (TFA): δ 6.8-7.2 (m, 6, Ar-H, CH=CH); 3.2 (q, 2, CH_2C=O); 2.8 (t, 2, CH_2-Ar); 2.25 (s, 3, CH_3-pyrimidine).

Anal. Calculated for $C_{16}H_{16}BrN_3O_3 \cdot H_2O$: C, 48.50; H, 4.58; N, 10.60. Found: C, 48.50; H, 4.52; N, 10.55.

3-(tert-Butyloxycarbonylamino)propanoic Acid (40). The procedure of Bentley, et al. (65) was followed. Magnesium oxide (9.0 g, 0.224 mole), 3-aminopropanoic acid (10 g, 0.112 mole), tert-butyloxyazidoformate (34.9 mL, 0.246 mole), water (145 mL), and dioxane (335 mL) were stirred in a 1000-mL, round-bottomed flask at 40-45° for twenty-four hours. The dioxane and excess azide were removed under reduced pressure. Water (450 mL) was added to the flask, and the solution was filtered. The filtrate was acidified with saturated aqueous citric acid solution to pH 3. The aqueous portion was extracted with ethyl acetate (3 X 200 mL). The combined ethyl acetate portions were washed with saturated sodium chloride solution (50 mL), dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give 40 as a white solid. Recrystallization from ethyl acetate/petroleum ether (bp 60-90°) gave 16.4 g (77%) of 40, mp 71.5-73°; literature

(65) 73-74°. IR (Nujol): 3420 cm^{-1} (strong, N-H); 1700 (strong, C=O). NMR ($CDCl_3$): δ 11.1 (s, 1, COOH); 5.3 (s, 1, N-H); 3.5 (q, 2, $N-CH_2-C$); 2.7 (t, 2, $CH_2-C=O$); 1.5 (s, 9, 3 CH_3).

2,4,5-Trichlorophenyl 3-(tert-Butyloxycarbonylamino) propanoate (41). The procedure of Pless and Boissonnas (66) was followed. 3-(tert-Butyloxycarbonylamino)-propanoic acid (40) (15 g, 79 mmol), 2,4,5-trichlorophenol (17.1 g, 87 mmol), and ethyl acetate (260 mL) were magnetically stirred in a 500-mL, round-bottomed flask until solution was effected. Dicyclohexylcarbodiimide (16.3 g, 79 mmol) was added. After stirring for three hours, the reaction mixture was filtered. The filtrate was evaporated to dryness to give 41 as a dark red solid. Recrystallization from benzene/petroleum ether (bp 60-90°) afforded 19.0 g (65%) of 41 as a light pink solid, mp 90-92°; literature (65) 93-94°. IR (Nujol): 3340 cm^{-1} (strong, N-H); 1770 (strong, ester C=O); 1680 (strong, carbamate C=O). NMR ($CDCl_3$): δ 7.5 (2, d, Ar-H); 5.0 (1, s, N-H); 3.6 (q, 2, $N-CH_2-C$); 2.85 (t, 2, $CH_2-C=O$); 1.5 (s, 9, 3 CH_3).

(±)-N-Benzyl-3-(tert-butyloxycarbonylamino) propanamide (42). A solution of benzylamine (0.65 g, 6 mmol) in methylene chloride (10 ML) was added to a 50-mL, round-bottomed flask containing 41 (2.0 g, 5.4 mmol). The solution was stirred for four hours at room temperature. The methylene chloride solution was washed with 1 N potassium bisulfate (10 mL), sodium hydroxide solution (5%, 10 mL), and satu-

rated aqueous sodium chloride solution (10 mL). The methylene chloride portion was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give 42 as a white solid. Recrystallization from ethyl acetate/petroleum ether (bp 60-90°) afforded 1.2 g (80%) of 42, mp 116-118°. IR (Nujol): 3300 cm^{-1} (strong, N-H); 1690 (strong, carbamate C=O); 1650 (strong, amide C=O). NMR (Me_2SO, d-6): δ 8.2 (s, 1, carbamate N-H); 7.25 (s, 5, Ar-H); 6.7 (s, 1, NH-C=O); 4.25 (d, 2, CH_2-Ar); 3.2 (m, 2, C-CH_2-N); 2.3 (t, 2, CH_2-C=O); 1.3 (s, 9, 3 CH_3).

Anal. Calcualted for $C_{15}H_{22}N_2O_3$: C, 64.73; H, 7.97; N, 10.07. Found: C, 64.86; H, 7.95; N, 10.05.

3-Amino-N-benzylpropanamide Trifluoroacetate (43). The protected amine 42 (1.0 g, 3 mmol) was dissolved in trifluoroacetic acid (4 mL) in a 25-mL, round-bottomed flask. The solution was stirred for thirty minutes at room temperature. The trifluoroacetic acid was removed under reduced pressure to give 0.84 g (80%) of 43 as a white solid, mp 65-67°. IR (Nujol): 3300 cm^{-1} (medium, N-H); 1700 (strong, acid C=O); 1650 (strong, amide C=O); NMR (D_2O): δ 7.5 (s, 5, Ar-H); 4.5 (s, 2, CH_2-Ar); 3.3 (t, 2, C-CH_2-N); 2.8 (t, 2, CH_2-C=O).

Anal. Calculated for $C_{12}H_{15}F_3N_2O_3$: C, 49.32; H, 5.14; N, 9.59. Found: C, 49.28; H, 5.18; N, 9.57.

(E)-(±)-N-Benzyl-3-[3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamido] propanamide (44). The method of Lin

and Dubois (51) was modified. A solution of 43 (0.76 g, 2.6 mmol) in water (minimum amount) was treated with an aqueous solution of sodium hydroxide (5%) to pH 14. The aqueous layer was extracted with ethyl acetate (3 X 15 ML). The combined ethyl acetate extracts were washed with saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give 43 (free base) as a clear oil. A solution of 43 (free base) (0.4 g, 2.2 mmol) in dimethylformamide (4 mL) was added to a solution of 6 (0.43 g, 2.2 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.65 g, 2.6 mmol) in dry dimethylformamide (8 mL). The solution was magnetically stirred for twenty-four hours at 35°. The dimethylformamide was removed under reduced pressure. The resultant yellow residue was crystallized with anhydrous ether to give 0.56 g (72%) of 44 as a yellow solid. Recrystallization from water afforded 44 as a white solid, mp 232.5-233.5°. IR(Nujol): 3320 cm^{-1} (medium, N-H); 1710 (strong, C=O); 1660 (strong, C=C). NMR (TFA): δ 8.1 (s, 3, N-H); 7.1-7.7 (m, 7, Ar-H, CH=CH); 4.4 (s, 2, CH_2-Ar); 3.6 (m, 2, C-CH_2-N); 2.9 (m, 2, CH_2-C=O); 2.3 (s, 3, CH_3).

Anal. Calculated for $C_{18}H_{20}N_4O_4.H_2O$: C, 59.23; H, 5.80; N, 15.35. Found: C, 59.61; H, 5.80; N, 15.43.

(±)-N-(4-Methylbenzyl)-3-(tert-butyloxycarbonylamino) propanamide (45a). A solution of 4-methylbenzylamine (0.73 g, 6 mmol) in methylene chloride (10 mL) was added to a 50 mL, round-bottomed flask containing 41 (2.0 g, 5.4 mmol). The solution was stirred for four hours at room temperature. The methylene chloride solution was washed with 1 N potassium bisulfate (10 mL), sodium hydroxide solution (5%, 10 mL), and saturated aqueous sodium chloride solution (10 mL). The methylene chloride portion was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give 45a as a white solid. Recrystallization from ethyl acetate/petroleum ether (bp 60-90°) afforded 1.4 g (86%) of 45a, mp 124-125.5°. IR (Nujol): 3310 cm^{-1} (strong, N-H); 1680 (strong, carbamate C=O); 1640 (strong, amide C=O). NMR (Me_2SO, d-6): δ 8.5 (s, 1 carbamate N-H); 7.3 (s, 4, Ar-H); 6.8 (s, 1, NH-C=O); 4.3 (d, 2, CH_2-Ar); 3.2 (m, 2, C-CH_2-N); 2.3 (m, 5, CH_3-Ar, CH_2-C=O); 1.4 (s, 9, 3 CH_3).

Anal. Calculated for $C_{16}H_{24}N_2O_3$: C, 65.73; H, 8.27; N, 9.58. Found: C, 65.64; H, 8.27; N, 9.53.

(E)-(±)-N-(4-Methylbenzyl)-3-[3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamido] propanamide (46). The method of Lin and Dubois (51) was modified. The protected amine 45a (1.36g, 4.6 mmol) was dissolved in trifluoroacetic acid (5 mL) in a 25-mL, round-bottomed flask. The solution was stirred for thirty minutes at room temperature. The

trifluoroacetic acid was removed under reduced pressure to give 1.29 g. (92%) of 45b, mp 83.5-84.5°. IR (Nujol): 3300 cm^{-1} (strong, N-H); 1700 (strong, acid C=O); 1650 strong, amide C=O). NMR (D_2O): δ 7.3 (s, 4, Ar-H); 4.4 (s, 2, CH_2-Ar); 3.35 (t, 2, C-CH_2-N); 2.75 (t, 2, CH_2-C=O), 2.35 (s, 3, CH_3). A solution of 45b (1.29 g, 4.2 mmol) and water (minimum amount) was treated with an aqueous solution of sodium hydroxide (5%) to pH 14. The aqueous layer was extracted with ethyl acetate (3 X 20 mL). The combined ethyl acetate extracts were washed with saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give 45b (free base) as a white solid. A solution of 45b (free base) (0.69 g, 3.5 mmol) in dimethylformamide (5 mL) was added to a solution of 6 (0.68 g, 3.5 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.0 g, 4.2 mmol) in dry dimethylformamide (10 mL). The solution was magnetically stirred for twenty-four hours at 35°. The dimethylformamide was removed under reduced pressure. The resultant yellow residue was crystallized with anhydrous ether to give 0.8 g (60%) of 46 as a pale yellow solid. The yellow solid was washed with boiling water to afford 46 as a white solid, mp 262-265°. IR (Nujol): 3300 cm^{-1} (weak, N-H); 1710 (strong, C=O); 1650 (strong, C=C). NMR (TFA): δ 6.8-8.0 (m, 10, Ar-H, CH=CH, 4 N-H); 4.3 (m, 2, CH_2-Ar); 3.6 (m, 2, C-CH_2-N); 2.8 (m, 2, CH_2C=O); 2.3 (s, 3, pyrimidine-CH_3); 2.1 (s, 3, Ar-CH_3).

Anal. Calculated for $C_{19}H_{22}N_4O_4$: C, 61.61; H, 5.99; N, 15.12. Found: C, 61.57; H, 5.99; N, 15.08.

(±)-N-(4-Methoxybenzyl)-3-(tert-butyloxycarbonylamino) propanamide (47a). A solution of 4-methoxybenzylamine (0.82 g, 6 mmol) in methylene chloride (10 mL) was added to a 50-mL, round-bottomed flask containing 41 (2.0 g, 5.4 mmol). The solution was stirred for four hours at room temperature. The methylene chloride solution was washed with 1 N potassium bisulfate (10 mL), sodium hydroxide solution (5%,10 mL), and saturated aqueous sodium chloride solution (10 mL). The methylene chloride portion was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give 47a as a white solid. Recrystallization from ethyl acetate/petroleum ether (bp 60-90°) afforded 1.1 g (67%) of 47a, mp 104-105°. IR (Nujol): 3300 cm^{-1} (strong, N-H); 1680 (strong, carbamate C=O); 1640 (strong, amide C=O). NMR (Me_2SO, d-6): δ 8.2 (s, 1, carbamate N-H); 6.95 (d of d, 4, Ar-H); 6.55 (s, 1, NH-C=O); 4.25 (d, 2, CH_2-Ar); 3.65 (s, 3, O-CH_3); 3.1 (q, 2, C-CH_2-N); 2.25 (t, 2, CH_2-C=O); 1.25 (s, 9, 3 CH_3).

Anal. Calculated for $C_{16}H_{24}N_2O_4$: C, 62.31; H, 7.84; N, 9.08. Found: C, 62.41; H, 7.88; N, 9.00.

(E)-(±)-N-(4-Methoxybenzyl)-3-[3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamido] propanamide (48). The method of Lin and Dubois (51) was modified. The protected amine 47a (1.0 g, 3.2 mmol) was dissolved in trifluoroacetic acid (4 mL) in a 25-mL, round-bottomed flask.

The solution was stirred for thirty minutes at room temperature. The trifluoroacetic acid was removed under reduced pressure to give 1.0 g (97%) of 47b, mp 89.5-91°. IR (Nujol); 3260 cm^{-1} (strong, N-H); 1680 (strong, acid C=O); 1640 (strong, amide C=O). NMR (D_2O): δ 7.2 (d of d, 4, Ar-H); 4.4 (s, 2, CH_2-Ar); 3.9 (s, 3, O-CH_3); 3.3 (m, 2, C-CH_2-N); 2.7 (m, 2, CH_2-C=O). A solution of 47b (0.9 g, 2.8 mmol) and water (minimum amount) was treated with an aqueous solution of sodium hydroxide (5%) to pH 14. The aqueous layer was extracted with ethyl acetate (3 X 20 mL). The combined ethyl acetate extracts were washed with saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give 47b (free base) as a white solid. A solution of 47b (free base) (0.3 g, 1.4 mmol) in dimethylformamide (5 mL) was added to a solution of 6 (0.28 g, 1.4 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.43 g, 1.7 mmol) in dry dimethylformamide (5 mL). The solution was magnetically stirred for twenty-four hours at 35°. The dimethylformamide was removed under reduced pressure. The resultant residue was crystallized with anhydrous ether to give 0.3 g (71%) of 48 as a pale yellow solid. Recrystallization from water afforded 48 as a white solid, mp 187-188°. IR (Nujol): 3280 cm^{-1} (weak, N-H); 1700 (strong, C=O); 1650 (strong, C=C). NMR (TFA): δ 6.8-7.8 (m, 10, Ar-H, CH=CH, 4, N-H); 4.4 (s, 2, CH_2-Ar): 3.9 (s, 3, O-CH_3); 3.55 (m, 2, C-CH_2-

N); 2.9 (t, 2, CH_2-C=O); 2.45 (s, 3, CH_3).

Anal. Calculated for $C_{19}H_{22}N_4O_5 \cdot H_2O$: C, 56.45; H, 5.94; N, 13.86. Found: C, 56.43; H, 6.00; N, 13.85.

(±)-N-(4-Bromobenzyl)-3-(tert-butyloxycarbonylamino) propanamide (49a). A solution of 4-bromobenzylamine hydrochloride (2.0 g, 9 mmol) and water (100 mL) was treated with an aqueous sodium hydroxide solution (10%) to pH 12-14. The aqueous solution was extracted with methylene chloride (3 X 50 mL). The combined methylene chloride extracts were washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give 4-bromobenzylamine as an opaque liquid. A solution of 4-bromobenzylamine (1.6 g, 8 mmol) in methylene chloride (10 mL) was added to a 50-mL, round-bottomed flask containing 41 (2.0 g, 5.4 mmol). The solution was stirred for four hours at room temperature. The methylene chloride solution was washed with 1 N potassium bisulfate (10 mL), sodium hydroxide solution (5%, 10 mL), and saturated aqueous sodium chloride solution (10 mL). The methylene chloride portion was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give 49a as a white solid. Recrystallization from ethyl acetate/petroleum ether (bp 60-90°) afforded 1.1 g (57%)of 49a, mp 122.5-124.5°. IR (Nujol): 3300 cm^{-1} (strong, N-H); 1680 (strong, carbamate C=O); 1640 (strong, amide C=O). NMR (Me_2SO, d-6): δ 8.4 (s, 1, carbamate N-H); 7.4 (d of d, 4, Ar-H); 6.7 (s, 1,

NH-C=O); 4.3 (d, 2, CH_2-Ar); 3.1 (m, 2, C-CH_2-N); 2.3 (t, 2, CH_2-C=O); 1.35 (s, 9, 3 CH_3).

Anal. Calculated for $C_{15}H_{21}BrN_2O_3$: C, 50.43; H, 5.93; N, 7.84. Found: C, 50.34; H, 5.97; N, 7.81.

(E)-(±)-N-(4-Bromobenzyl)-3-[3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl-2-propenamido] propanamide (50). The method of Lin and Dubois (51) was modified. The protected amine 49a (1.0 g, 2.8 mmol) was dissolved in trifluoroacetic acid (5 mL) in a 25-mL, round-bottomed flask. The solution was stirred for thirty minutes at room temperature. The trifluoroacetic acid was removed under reduced pressure to give 1.0 g (96%) of 49b, mp 106.5-108°. IR (Nujol): 3300 cm^{-1} (medium, N-H); 1690 (strong, acid C=O); 1650 (strong, amide C=O). NMR (D_2O); δ 7.5 (d of d, 4, Ar-H); 4.5 (s, 2, CH_2-Ar); 3.4 (t, 2, C-CH_2-N); 2.8 (t, 2, CH_2-C=O). A solution of 49b (0.95 g, 2.6 mmol) and water (minimum amount) was treated with an aqueous solution of sodium hydroxide (5%) to pH 14. The aqueous layer was extracted with ethyl acetate (3 X 20 mL). The combined ethyl acetate extracts were washed with saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give 49b (free base) as a white solid. A solution of 49b (free base) (0.61 g, 2.4 mmol) in dimethylformamide (5 mL) was added to a solution of 6 (0.46 g, 2.4 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.71 g, 2.9 mmol) in dimethylformamide (8 mL). The solution was magnetically stirred for twenty-

four hours at 35°. The dimethylformamide was removed under reduced pressure. The resultant yellow residue was crystallized with anhydrous ether to give 0.59 g (56%) of 50 as a pale yellow solid. Recrystallization from water afforded 50 as a white solid, mp 191-192°. IR (Nujol): 3320 cm^{-1} (medium, N-H); 1710 (strong, C=O); 1640 (strong, C=C). NMR (TFA): δ 6.7-7.7 (m, 10, Ar-H, CH=CH, 4 N-H); 4.4 (d, 2, CH_2-Ar); 3.6 (q, 2, C-CH_2-N); 3.0 (t, 2, CH_2-C=O); 2.5 (s, 3, CH_3).

Anal. Calculated for $C_{18}H_{19}BrN_4O_4 \cdot H_2O$: C, 47.70; H, 4.67; N, 12.36. Found: C, 47.69; H, 4.67; N, 12.38.

(±)-N-(3,4-Dichlorobenzyl)-3-(tert-butyloxycarbonylamino)propanamide (51a). A solution of 3,4-dichlorobenzylamine (1.06 g, 6 mmol) in methylene chloride (10 mL) was added to a 50-mL, round-bottomed flask containing 41 (2.0 g, 5.4 mmol). The solution was stirred for four hours at room temperature. The methylene chloride solution was washed with 1 N potassium bisulfate (10 mL), sodium hydroxide solution (5%, 10 mL), and saturated aqueous sodium chloride solution (10 mL). The methylene chloride portion was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to give 51a as a white solid. Recrystallization from ethyl acetate/petroleum ether (bp 60-90°) afforded 1.3 g (69%) of 51a, mp 124-128°. IR (Nujol): 3320 cm^{-1} (strong, N-H); 1680 (strong, carbamate C=O); 1640 (strong, amide C=O). NMR (Me_2SO, d-6):

δ 8.6 (s, 1, carbamate N-H); 7.5 (m, 3, Ar-H); 6.8 (s, 1, NH-C=O); 4.4 (d, 2, CH_2-Ar); 3.2 (q, 2, C-CH_2-N); 2.4 (t, 2, CH_2-C=O); 1.4 (s, 9, 3 CH_3).

Anal. Calculated for $C_{15}H_{20}Cl_2N_2O_3$: C, 51.88; H, 5.81; N, 8.07. Found: C, 51.84; H, 5.84; N, 8.04.

(E)-(±)-N-(3,4-Dichlorobenzyl)-3-[3-(2,4-dihydroxy-6-methyl-5-pyrimidinyl)-2-propenamido] propanamide (52). The method of Lin and Dubois (51) was modified. The protected amine 51a (1.18 g, 3.4 mmol) was dissolved in trifluoroacetic acid (5 mL) in a 25-mL, round-bottomed flask. The solution was stirred for thirty minutes at room temperature. The trifluoroacetic acid was removed under reduced pressure to give 1.03 g (84%) of 51b, mp 71-73°. IR (Nujol): 3300 cm^{-1} (medium, N-H); 1680 (strong, acid C=O); 1650 (strong, amide C=O). NMR (D_2O): δ 7.5 (m, 3, Ar-H); 4.5 (s, 2, CH_2-Ar); 3.5 (t, 2, C-CH_2-N); 2.9 (t, 2, CH_2-C=O). A solution of 51b (0.98 g, 2.7 mmol) and water (minimum amount) was treated with an aqueous solution of sodium hydroxide (5%) to pH 14. The aqueous layer was extracted with ethyl acetate (3 X 20 mL). The combined ethyl acetate extracts were washed with saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give 51b (free base) as a white solid. A solution of 51b (free base) (0.62 g, 2.5 mmol) in dimethylformamide (4 mL) was added to a solution of 6 (0.49 g, 2.5 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.74 g, 3.0 mmol) in dry di-

methylformamide (8 mL). The solution was magnetically stirred for twenty-four hours at 35°. The dimethylformamide was removed under reduced pressure. The resultant yellow residue was crystallized with anhydrous ether to give 0.65 g (61%) of 52 as a pale yellow solid. The yellow solid was washed with boiling water to afford 52 as a white solid, mp 262-264° (dec). IR (Nujol): 3300 cm^{-1} (weak, N-H); 1710 (medium, C=O); 1650 (medium, C=C). NMR (TFA): δ 6.9-8.0 (m, 9, Ar-H, CH=CH, 4 N-H); 4.3 (m, 2, CH_2-Ar); 3.7 (m, 2, C-CH_2-N); 2.8 (m, 2, CH_2-C=O); 2.3 (s, 3, CH_3).

Anal. Calculated for $C_{18}H_{18}Cl_2N_4O_4$: C, 50.84; H, 4.27; N, 13.17. Found: C, 50.80; H, 4.30; N, 13.14.

N^2-Phthalyl-DL-aspartic Anhydride (53). The method of Murphy and Stubbins (71) was used. DL-Aspartic acid (26.6 g, 0.2 mole) and phthalic anhydride (29.6 g, 0.2 mole) in pyridine (400 mL) were heated under reflux for two hours in a 1000-mL, round-bottomed flask. The mixture was cooled to room temperature, and the undissolved solids were removed by filtration. The filtrate was concentrated under reduced pressure to a yellow oil. Acetic anhydride (100 mL) was added to the oil while vigorously agitating. After chilling the mixture at 0° overnight, the precipitate was collected by filtration, washed with anhydrous ether, and recrystallized from 1,4-dioxane to give 31.8 g (65%) of 53, mp 220-222°; literature (71) mp 224-226°. IR (KBr): 1875 cm^{-1} (strong, O=COC=O); 1795 (strong, C=O); 1785

(strong, imide C=0); 1705 (strong, imide C=0). NMR (Me_2SO, d-6): δ 7.95 (s, 4, Ar-H); 5.7 (t, 1, CH); 3.5 (d, 2, CH_2).

N^4-Benzyl-N^2-phthalyl-DL-asparagine (54). The procedure of Murphy and Stubbins (71) was modified. N^2-Phthalyl-DL-aspartic anhydride (53) (5.0 g, 20 mmol) was suspended in 125 mL of anhydrous ether in a 250-mL, three-necked, round-bottomed flask equipped with nitrogen inlet and outlet adapters and a rubber septum. Benzylamine (4.3 g, 40 mmol) was added to the reaction flask via a syringe and the mixture was magnetically stirred at room temperature for twenty-four hours. At the end of the alloted time, the solid material present was collected by filtration and washed with anhydrous ether (2 X 25 mL). The solid was then suspended in water (about 150 mL) and the suspension was acidified with 6 N hydrochloric acid to pH 1. The white solid was collected by filtration, washed with boiling acetonitrile, and dried (toluene, 111°) to give 4.5 g (63%) of 54, mp 213-215°; literature (71) mp 220-222°. IR (Nujol): 3365 cm^{-1} (medium, NH); 1780 (weak, imide C=0); 1710 (strong, imide C=0); 1620 (strong, amide C=0). NMR (TFA): δ 7.8 (s, 4, phthalyl-H); 7.1 (s, 5, Ar-H); 5.7 (t, 1, CH); 4.4 (s, 2, CH_2-Ar); 3.3 (m, 2, CH_2-C=0).

N-Benzyloxycarbonyl-DL-aspartic Acid (60). The procedure of Bergmann and Zervas (72) was modified. DL-Aspartic acid (8.0 g, 60 mmol), magnesium oxide (7.4 g, 0.18 mole), water (100 mL), and diethyl ether (30 mL) were stirred in a 250-mL, round-bottomed flask while cooling in

an ice bath. The suspension was continuously stirred while benzylchloroformate (20.4 g, 0.12 mole) was added dropwise over a thirty minute period. The mixture was stirred at room temperature overnight. Following the allotted reaction time, the ether portion was separated and discarded. The aqueous portion was acidified with concentrated hydrochloric acid to pH 1. The aqueous solution was then extracted with ethyl acetate (3 X 50 mL). The combined ethyl acetate extracts were washed with hydrochloric acid (5%, 25 mL), saturated sodium chloride solution (25 mL), and dried over anhydrous sodium sulfate. The ethyl acetate was evaporated under reduced pressure to leave a white solid which was recrystallized from ethyl acetate/petroleum ether to give 10.2 g (64%) of 60, mp 100-103°; literature (96) mp 114-115°. IR (Nujol): 3310 cm^{-1} (broad, NH); 3100 (broad, OH); 1730 (strong; acid C=O); 1700 (strong, carbamate C=O). NMR (Me_2SO, d-6): δ 7.7 (m, 1, NH); 7.4 (s, 5, Ar-H); 5.1 (s, 2, CH_2-Ar); 4.5 (m, 1, CH-C=O); 2.7 (m, 2, CH_2-C=O).

N-Benzyloxycarbonyl-DL-aspartic Anhydride (61). The method of Bergmann and Zervas (72) was used. Acetic anhydride (16 mL) was added to carbobenzoxy-DL-aspartic acid (60) (5.4 g, 0.02 mole) and heated to boiling. The solution was immediately cooled and treated with anhydrous ether and petroleum ether (bp 30-60°) (30:70; v/v) to induce crystallization. The white solid was collected by filtration, washed with anhydrous ether, and dried to give 4.6 g (91%) of 61. Recrystallization from ethyl acetate/petroleum

ether gave 61 as a white solid, mp 126-128°; literature (96) mp 130°. IR (Nujol): 3380 cm^{-1} (medium, NH); 1780 (strong, anhydride C=O); 1690 (strong, carbamate C=O). NMR (Me_2SO, d-6): δ 8.2 (m, 1, NH); 7.4 (s, 5, Ar-H); 5.1 (s, 2, CH_2Ar); 4.8 (m, 1, CH-C=O); 3.2 (m, 2, CH_2-C=O).

α-Benzyl N-Benzyloxycarbonyl-DL-aspartate (62). The procedure of Bergmann, et al. (73) was followed. A mixture of freshly distilled benzyl alcohol (1.3 g, 12 mmol) and carbobenzoxy-DL-aspartic anhydride (61) (2.0 g, 8 mmol) were heated for 3.5 hours at 100° (oil bath, 100°). The clear, pale yellow syrup which resulted was dissolved in ether, and extracted with sodium bicarbonate (5%, 2 X 50 mL). The combined bicarbonate portions were washed once with ether (25 mL), and acidified with 2 N hydrochloric acid to pH 1. The product crystallized after standing overnight at room temperature. The solid was collected by filtration, and recrystallized from ethyl acetate/petroleum ether, benzene, or toluene, to give 2.5 g (86%) of 62 as a white solid, mp 93-96°; literature (96) mp 104.5°. A potentiometric titration of 40 mg of 62 in 25 mL of water/dioxane (1:2) was carried out at 25° using 0.1 M NaOH to give a pKa of 6.7; literature (78) pKa 6.6. IR (Nujol): 3310 cm^{-1} (medium, NH); 1760 (strong, ester C=O); 1720 (strong, acid C=O); 1695 (strong, carbamate C=O). NMR ($CDCl_3$): δ 9.2 (s, 1, OH); 7.3 (s, 10, Ar-H); 5.8 (m, 1, NH); 5.15 (d, 4, CH_2-Ar); 4.7 (m, 1, CHC=O); 3.0 (m, 2, CH_2C=O).

β-Benzyl N-Benzyloxycarbonyl-DL-aspartate (62a). The procedure of Berger and Katchalski (78) was used. N-Benzyloxycarbonyl-DL-aspartic acid (60) (5.0 g, 19 mmol) and benzyl alcohol (25 mL, 0.36 mole) were added to a boiling solution of p-toluenesulfonic acid (0.3 g, 1.6 mmol) in toluene (30 mL). The solution was heated at reflux for 2.5 hours, during which time, water was removed azeotropically. The solution was cooled to room temperature, shaken for ten minutes with magnesium oxide (0.6 g, 15 mmol) and filtered. The toluene and excess benzyl alcohol were removed from the filtrate under reduced pressure to leave the intermediate, dibenzyl N-benzyloxycarbonyl-DL-aspartate as a viscous yellow oil. A mixture of 2 N sodium hydroxide (2.5 mL) water (12 mL), and dioxane (30 mL) was added to a solution of the crude dibenzyl N-benzyloxycarbonyl-DL-aspartate (2.3 g, 6 mmol) in dioxane (25 mL) and water (10 mL). The solution was stirred at room temperature for twenty-four hours. The pH of the solution was adjusted to 5.5 with 6 N hydrochloric acid and the solvents were evaporated under reduced pressure. The residue was treated with 1 N potassium bicarbonate (5 mL). The mixture was extracted with ether (1 X 10 mL) to remove any unreacted dibenzyl ester. Upon acidification of the aqueous portion with 6 N hydrochloric acid, the product 62a separated as a yellow oil which was extracted with ether (3 X 10 mL). The combined ether extracts were washed with saturated sodium chloride (5 mL), and dried over anhydrous sodium sulfate. The solvent was

removed under reduced pressure to leave 0.42 g (23%) of 62a as a white semi-solid, mp 82-92°. A potentiometric titration of 40 mg of 62a in 25 mL of water/dioxane (1:2) was carried out at 25° using 0.1 M NaOH to give a pKa of 6.2, literature (78) pKa 6.1. IR (Nujol): 3330 cm^{-1} (weak NH); 1730 (medium, ester C=O); 1700 (medium, acid C=O); 1650 (medium, carbamate C=O). NMR ($CDCl_3$): δ 8.1 (s, 2, NH, OH); 7.3 (s, 10, Ar-H); 5.1 (s, 4, CH_2-AR); 4.6 (m, 1, CHC=O); 2.9 (m, 2, CH_2C=O).

N-(4-Bromobenzyl)-3-benzyloxycarbonylamino-2,5-pyrrolidinedione (62b). The method of Lin and Dubois (51) was modified. A mixture of 62 (3.0 g, 8.4 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (2.3 g, 9 mmol) in tetrahydrofuran (15 mL) was magnetically stirred at 35° (oil bath, 35°) until solution was effected. Subsequently, 4-bromobenzylamine (1.6 g, 8.4 mmol) was added. The solution was stirred for forty-eight hours at 35°. The tetrahydrofuran was removed under reduced pressure. The clear residue was dissolved in ether (25 mL), washed with hydrochloric acid (5%, 10 mL), sodium hydroxide solution (5%, 10 mL), saturated aqueous sodium chloride solution (10 mL), and dried over anhydrous sodium sulfate. The ether was evaporated under reduced pressure to give 1.35 g (31%) of 62b as a white solid, mp 164-168°. IR (Nujol): 3280 cm^{-1} (medium, NH); 1700 (strong, carbamate C=O); 1680 (strong, imide C=O). NMR ($CDCl_3$): δ 7.3-8.0 (m, 10, 9Ar-H, NH); 5.2 (s, 2, OCH_2Ar); 4.7 (s, 2, CH_2ArBr); 4.4 (t, 1, CHC=O); 3.0 (t, 2, CH_2C=O).

Anal. Calculated for $C_{19}H_{17}BrN_2O_4$: C, 54.69; H, 4.11; N, 6.71. Found: C, 54.69; H, 4.14; N, 6.68.

Benzyl N^2-Benzyloxycarbonyl-N^4-(4-bromobenzyl)-DL-asparaginate (63). The method of Lin and Dubois (51) was modified. A mixture of 62 (1.0 g., 2.8 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0.76 g, 3 mmol) in dimethylformamide (15 mL) was magnetically stirred at 35° (oil bath, 35°) until solution was effected. Subsequently, 4-bromobenzylamine (0.52 g, 2.8 mmol) was added. The solution was stirred for forty-eight hours at 35°. The dimethylformamide was removed under reduced pressure. The resultant residue was crystallized with anhydrous ether to give 0.65 g (44%) of 63 as a white solid. Recrystallization from chloroform/ether afforded 63, mp 124-126°. IR (Nujol): 3280 cm^{-1} (medium, NH); 1740 (strong, ester C=O); 1690 (strong, carbamate C=O); 1650 (strong, amide C=O). NMR ($CDCl_3$: δ 6.85-7.5 (m, 14, Ar-H); 6.0 (m, 2, NH); 5.2 (d, 4, O-CH_2-Ar); 4.8 (m, 1, CH); 4.3 (d, 2, N-CH_2-Ar); 2.8 (m, 2, CH_2-C=O).

Anal. Calculated for $C_{26}H_{25}BrN_2O_5$: C, 59.44; H, 4.80; N, 5.33. Found: C, 59.18; H, 4.88; N, 5.26.

Attempted synthesis of N-(4-Bromobenzyl)-3-benzyloxycarbonylamino-4-hydroxybutanamide (64). The procedure of Lin and Dubois (51) was used. To a solution of lithium borohydride (0.078 g, 3.6 mmol) in tetrahydrofuran (10 mL) under nitrogen was added 63 (0.9 g, 1.7 mmol) dissolved in tetrahydrofuran (5 mL) while cooling in ice. The mixture was stirred at room temperature for twenty hours. The re-

action vessel was cooled in an ice bath, while 1.5 mL of 6 N hydrochloric acid was added. The solution was stirred for one hour at room temperature. The tetrahydrofuran was removed under reduced pressure. The aqueous layer was extracted with chloroform (3 X 20 mL), washed with saturated sodium chloride solution (10 mL) and dried over anhydrous sodium sulfate. The combined chloroform extracts were evaporated under reduced pressure to leave 0.53 g (75%) of a white solid, mp 166.5-168^{o}. The melting point, NMR, and IR were identical to those of 62b.

C. Pharmacological Materials and Methods

1. Experimental Animals

Male CD-1 and DBA/2 mice were obtained from Charles River Breeding Laboratories, Wilmington, MA. The mice were maintained on Purina Laboratory Chow (Ralston Purina, Inc., Richmond, IN) and tap water *ad libitum* in temperature controlled quarters with a twelve hour light/dark cycle.

2. Preparation of Media

With the exception of water, sodium bicarbonate, and sodium hydroxide, all reagents were obtained from either Gibco (Grand Island, NY) or Flow Laboratories (Rockville, MD). All of the solutions were prepared under sterile conditions. Bacterial contamination was monitored by incubating one mL of media with one mL of 50% Brain-Heart Infusion Broth (Difco, Detroit, MI) for seventy-two hours at 37^{o} and 5% carbon dioxide. If bacterial contamination occurred, as visualized by an increase in the turbidity of the solution, the media was discarded. The directions for the preparation of the sterile media employed in the testing procedures of this project follow.

a. Alpha Modification of Eagle's Minimum Essential Medium (αMEM)

A package containing 10.1 g of the commercially available αMEM powdered media, including L-glutamine and excluding ribosides, deoxyribosides, and sodium bicarbonate, was

mixed with 2.2 g of sodium bicarbonate and enough deionized water to make one liter of media. The solution was sterilized by filtration.

b. Complete Dulbecco's Modified Eagle's Minimum Essential Medium (CDMEM)

The following sterile reageants were mixed with enough deionized water to make five liters of CDMEM:

66.9 g Dulbecco's Modified Eagle's Minimum Essential Medium (#430-2100, with glucose and glutamine, and without sodium bicarbonate and sodium pyruvate)

10 mL glutamine (200 mM)

c. Spinner's Medium

The following reagents were mixed to obtain 500 mL of Spinner's media without amino acids:

5.2 g Spinner's Powdered Medium

20.0 mL Penicillin (5000 U/mL)/Streptomycin (5000 µg/mL)

20.0 mL 1 M Hepes buffer (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid)

1.5 mL 7.4% sodium bicarbonate

5.0 mL MEM vitamins (100X)

The volume was adjusted to 500 mL using deionized water and the pH was adjusted to 7.4 using 10 N sodium hydroxide. The solution was sterilized by filtration using a Nalgene filter unit (size 0.45 micron, Nalge Co., Rochester, NY).

When the media was to be used for incubating P815 or P388 cells, 10% heat-inactivated fetal calf serum (heat-

inactivated for one hour at 56°; for Spinner's media: Lot #009463, North American Biological Institute, Chicago, IL; for CDMEM: Sterile Systems, Logan, Utah) was added to the stock media. All incubations were carried out in a model 3028 incubator (Forma Scientific, Marietta, OH) at 37°, 5% carbon dioxide, 95% air, and 95% humidity.

3. Enumeration of Cells

The concentrations of bone marrow cells and tumor cells were determined with a Coulter Counter (model ZBI, Coulter Electronics, Hialeah, FL) or with a Bright-Line hemacytometer (American Optical, Buffalo, NY). To eliminate erythrocytes, three drops of Zapoglobin II lysing agent (Coulter Diagnostics, Hialeah, FL) were added to all cell suspensions in Isoton-II counting solution (Coulter Diagnostics, Hialeah, FL). For hemacytometer counting, 0.3 mL of approximately 5-10 X 10^5 cells /mL was added to the hemacytometer, and the cells were enumerated using a binocular light microscope (Zeiss, West Germany). Cell viability was checked by the trypan blue exclusion test (97,98) using 0.4% trypan blue solution (0.4 g trypan blue/100 mL 1.5 M saline, Gibco, Grand Island, NY).

4. Tumor Line Maintenance

a. In vivo

The P388 tumor cells in 15% dimethylsulfoxide were frozen in liquid nitrogen and were quick-thawed within 5 to 10 seconds at 37° when readied for use. The cells were cen-

trifuged for ten minutes at 300 X g (International CRU-5000 centrifuge, Damon, IEC Division, Needham, MA) and resuspended in Spinner's media. The P388 tumor line was maintained in DBA/2 male mice by serial intraperitoneal (i.p.) passage of 10^5 cells every seven to ten days.

To harvest the tumor cells, the DBA/2 mice were sacrificed by cervical dislocation. The peritoneal cells were collected aseptically by injecting and withdrawing 4-8 mL of Spinner's media from the peritoneal cavity. The cells were spun at 300 X g for ten minutes. The supernatant was decanted and the cells were resuspended in 1-5 mL of Spinner's media containing 10% heat-inactivated fetal calf serum. The cells were enumerated on a Coulter counter and the concentration of cells was adjusted to 3 X 10^6 cells/mL by dilution with Spinner's media.

b. In vitro

P815 cells, courtesy of Dr. Dolph Adams, Department of Pathology, Duke University Hospital, Durham, North Carolina, were maintained in culture flasks (25 cm^2 T flasks, Corning, Corning, New York) in CDMEM. The cells were passaged twice weekly by adding an aliquot of 1 X 10^5 cells to 7 mL of CDMEM. When needed for the protein synthesis studies, the concentration of cells was adjusted to 3 X 10^6 cells/mL by dilution with CDMEM.

5. Preparation of Substrate, Drug, and Inhibitor Solutions.

a. Control Drugs

Puromycin dihydrochloride (NSC-3055) was obtained from Sigma Chemical Company, St. Louis, MO. Sparsomycin (NSC-5972 9NB) and Ara-C (NSC-63878, 4 amino - 1β-D-arabinofuranosyl-2-(1H)-pyrimidinone) were generously obtained from the Natio-al Cancer Institute, Bethesda, MD. Solutions of these drugs at the concentrations specified in the discussion of the pharmacological results were prepared using the appropriate media for the assay.

b. Radioisotopes

5-^{125}I-iodo-2-deoxyuridine (^{125}IUdR) was obtained from New England Nuclear, Boston, MA, and prepared as a solution of 0.1 μCi ^{125}IUdR in 20 μL of 2 X 10^{-5} M fluorodeoxyuridine (FUdR, Sigma Chemical Company, St. Louis, MO). L-[^{75}Se]-selenomethionine was obtained from Amersham Corporation, Arlington Heights, IL, and was prepared as a solution of 0.2 μCi [^{75}Se]-selenomethionine per 20 μL of the indicated media for the assay being performed.

c. Analogues of Sparsomycin

Compounds 19, 22, 24, 28, 36, 37, 38, 39, 48, and 50 were prepared as 10^{-2} M aqueous stock solutions using distilled water. Dilutions were made using media to obtain the concentrations required for experimentation. Compound 35 was insoluble in water and was prepared as a 10^{-2} M alcoholic solution using absolute ethanol. Dilutions were made with media to decrease the percentage of alcohol present in the cell culture. Compounds 44, 46, and 52 were not soluble in water or ethanol at the required concentra-

tion thereby precluding the testing of these three compounds.

6. Experimental Procedures for the Biological Assays

a. DNA Synthesis Inhibition Assay

(1) Time Course Determination

CD-1 mice were sacrificed by cervical dislocation. The lower half of their bodies was washed with 95% ethanol, the femurs were dissected, and all the tissue surrounding the bone was removed. Both ends (if necessary) of the femur were cut off and the marrow was flushed out into a plastic centrifuge tube using a 25-gauge needle filled with αMEM. The bone marrow suspension was centrifuged at 300 X g for ten minutes. The supernatent was decanted and the cells were resuspended in one mL of αMEM. The cells were counted on a Coulter counter and the concentration of cells adjusted to 3 X 10^6 cells/mL by dilution with αMEM. After preparing the cells, 200 μL of the cell suspension was added to each well of a microtiter plate (Microtest II, Falcon Plastics, Oxnard, CA). In addition to the cells, 20 μL of ^{125}IUdR at a concentration of 0.1 μCi ^{125}IUdR/20 μL of 2.0 X 10^{-5} M FUdR was added to each well. The incubation times were varied so that six wells of cells were harvested at each time point: 15, 30, 45, 60, and 120 minutes. Following incubation, the cells were harvested on a Titertek cell harvester while flushing the wells with normal saline (1.5 M sodium chloride) for complete removal of the cells. The filter paper disks containg the ^{125}IUdR

incorporated into DNA were placed in tubes and radioassayed on a gamma counter (model 7000 or model 300, Beckman Instruments, Fullerton, CA).

(2) Bone Marrow Cells

Bone marrow cells at a concentration of 3 X 10^6 cells/mLαMEM were obtained as outlined in section VI.C.6.a.(1). After preparing the cells, 200 μL of the cell suspension was added to each well of a microtiter plate (Microtest II, Falcon Plastics, Oxnard, CA). In addition to the cells, 20 μL of ^{125}IUdR at a concentration of 0.1 μCi ^{125}IUdR/20 μL of 2.0 X 10^{-5} M FUdR was added to each well. The compounds to be tested were added to the microtiter plate in 20 μL aliquots of 10^{-2} M solutions. As a negative control for DNA synthesis, 20 μL of 10^{-2} M AraC was added to six wells of cells. The final microtiter plate contained:

6 wells of bone marrow cells (BMC) and ^{125}IUdR

6 wells of <u>each</u> test compound + BMC + ^{125}IUdR

6 wells of AraC + BMC + ^{125}IUdR

6 wells of ^{125}IUdR only

The cells were incubated with the drugs on a rocker platform for two hours. After incubation, the cells were harvested on a Titertek cell harvester and radioassayed as described in section IV.C.6.a.(1).

(3) P388 Cells

The assay described in section VI.C.6.a.(2) was repeated using P388 cells harvested from DBA/2 mice with matured P388 tumors according to section VI.C.4.a. αMEM

was substituted for Spinner's media.

b. Protein Synthesis Inhibition Assay

(1) Time Course Determination

P388 cells were harvested from DBA/2 mice with matured P388 tumors according to section VI.C.4.a. The prepared cells were added to a microtiter plate (Microtest II, Falcon Plastics, Oxnard, CA) in aliquots of 200 μL per well. To each well was added 0.2 μCi L-[^{75}Se]-selenomethionine in 20 μL of Spinner's media. Incubation times were varied so that six wells of cells were harvested at each time point: 1, 3, 6, 10, 18, 21, and 24 hours. Following the alloted incubation period, the cells were harvested on a Titertek cell harvester and radioassayed as described in section IV.C.6.a.(1).

The procedure was repeated using bone marrow cells obtained as described in section VI.C.6.a.(1). Incubation times were varied so that three wells of cells were harvested at each time point: 1, 3, 14, 18, and 24 hours.

(2) Bone Marrow Cells

The bone marrow cells were obtained as described in Section VI.C.6.a.(1), using Spinner's media instead of αMEM. After adding 200 μL of the prepared bone marrow cells to each well of a microtiter plate (Microtest II, Falcon Plastics, Oxnard, CA), each of the test compounds was added to four different wells in 20 μL aliquots. In addition, four wells of control cells were prepared by adding 20 μL of Spinner's media to each of four wells containing bone marrow cells.

As a negative control for protein synthesis, 20 μL of 10^{-2} M puromycin was added to four wells of bone marrow cells. The cells were incubated on a rocker platform for 1.5 hours. After the allotted incubation period, 20 μL of 0.2 μCi L-[^{75}Se]-selenomethionine/20 μL of Spinner's media was added to each of the wells. The final microtiter plate contained:

4 wells BMC + [^{75}Se]-selenomethionine

4 wells of <u>each</u> test compound + BMC + [^{75}Se]-selenomethionine

4 wells of puromycin + BMC + [^{75}Se]-selenomethionine

4 wells of [^{75}Se]-selenomethionine only

The microtiter plate was incubated on a rocker platform for twenty-four hours. The cells were harvested on a Titertek cell harvester and radioassayed as described in section VI.C.6.a.(1).

(3) P388 cells

The assay described in section VI.C.6.b.(2) was repeated using P388 cells harvested from DBA/2 mice with matured P388 tumors according to section VI.C.4.a.

(4) P815 cells

The assay described in section VI.C.6.b.(2) was repeated using P815 cells obtained from the cultures described in section IV.C.4.b. CDMEM was used instead of Spinner's media.

c. Cell Growth Studies

(1) Time Course Determination

P815 cells maintained in culture VI.C.4.b.) were added to four different microtiter plates, (Costar , Bellco, Vineland, New Jersey) in aliquots containing 1 X 10^5 P815 cells per well. Each of the test compounds, in aliquots of 20 µL, was added to four different wells on each plate, leaving four wells of cells as controls (20 µL of CDMEM was added to these four wells). The incubation times for each plate were varied to include 9, 24, 33, and 48 hours of incubation. Following incubation, the cells were enumerated using a hemacytometer viewed under a binocular light microscope.

(2) ED_{50} Calculation

P815 cells maintained in culture (VI.C.4.b.) were added to each well of a microtiter plate (Costar, Bellco, Vineland, New Jersey) in 180 µL aliquots containing approximately 2.5 X 10^5 cells/mL. Each of the test compounds, in 20 µL aliquots, was added to four different wells of cells, leaving four wells of cells as controls (20 µL of CDMEM was added to these four wells). After incubating the microtiter plate for twenty-four hours, the cells were enumerated using a hemacytometer viewed under a binocular light microscope. Variations were made in the concentration of the test drugs to determine the ED_{50}.

7. Statistical Analysis

The level of confidence for all experiments was set at ninety-five percent. A one-way analysis of variance

(ANOVA) with a Dunnett's t-test was used to compare a control to more than one experimental group (99). Linear regression analysis was used to determine the slopes of cell growth curves.

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