NUCLEOSIDE ANTIBIOTICS

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Preface

The nucleoside antibiotics represent a diverse group of biological compounds structurally related to the purine and pyrimidine nucleosides and or nucleotides found in the cell. The nucleoside antibiotics have been useful as models for conformational studies, mass spectrometry, nmr, and ORD measurements. They have also been equally important as biochemical tools in cellular reactions, as illustrated in those studies where they have aided in the elucidation of the complex steps involved in reading the genetic message on the ribosomes for protein synthesis, RNA synthesis, DNA synthesis, regulation of purine and pyrimidine nucleotide synthesis, mechanisms of enzymatic reactions, subcellular organization, intermediary metabolism, and cell wall biosynthesis. The close structural relationship of the nucleoside antibiotics to purine and pyrimidine nucleosides and nucleotides have made them useful as structural analogs and inhibitors. Finally, the studies that have been made on the biosynthesis of eleven of the nucleoside antibiotics add another role for preformed purine or pyrimidine nucleosides and nucleotides in cellular reactions.

This book attempts to provide the first clear, comprehensive, and up-to-date review of the nucleoside antibiotics. It is my hope that the contents will serve as a useful reference for teaching and research. The extraordinary growth of the research related to the nucleoside antibiotics is vividly illustrated by a comparison of the material available for this review with that of recent reviews (Cohen, *Progress in Nucleic Acid Research and Molecular Biology*, Academic Press, New York, 1966; Fox, Watanabe and Bloch, *Nucleoside Antibiotics*, Academic Press, New York, 1966; Korzyski, Kowszyk-Gindifer, and Kurylowicz, *Antibiotics*, Vols. I and II, Pergamon Press, New York 1967; Gottlieb and Shaw, *The Antibiotics*, Vols. I and II, Springer-Verlag, New York, 1967; Umezawa, *Recent Advances in Chemistry and Biochemistry of Antibiotics*, Nissin Tosho Insatsu Co., Ltd., Tokyo, 1964; Umezawa, *Index of Antibiotics From Actinomycetes*, University Park Press, 1970 ;Rinehart, *The Neomycins and Related Antibiotics*, 1964; John Wiley & Sons, Inc.; Roy-Burman, Recent Results in Cancer Research, **25**, Springer Verlag, 1970).

The structural features of the nucleoside antibiotics provided a convenient basis for grouping these compounds into eleven chapters in this book. In

general, the first five chapters deal with those nucleosides in which structural modifications occur in which ribose is replaced by a 3′-deoxyribose, ketohexoses, D-arabinose, or 4- or 5-aminohexuronic sugars. With aristeromycin (Sect. 6.1), the oxygen in the ribofuranosyl moiety has been changed to a methylene group. The remaining chapters are concerned with those nucleosides in which structural modifications occur in the aglycone moiety, such as the substitution of a *s*-triazine, a pyrrolopyrimidine, a pyrazolopyrimidine, a maleimide, or an isoguanine ring for the normal purine or pyrimidine base. For consistency, each of the 35 nucleosides reviewed has the following general outline: Introduction; Discovery, Isolation, and Production; Physical and Chemical Properties; Structural Elucidation; Chemical Synthesis; Synthesis of Analogs; Inhibition of Growth; Biosynthesis; Biochemical Properties; Summary; References.

Seven nucleosides discussed in this book have not exhibited any antibiotic properties. They are 3′-acetamido-3′-deoxyadenosine, spongosine, arabinosyluridine, nebularine, crotonoside, pseudouridine, and oxoformycin. They are included since they represent naturally occurring nucleosides which are closely related to the nucleoside antibiotics that have been isolated and studied. Septicidin, a N^6-glycosyl adenine antibiotic, is reviewed since it consists of the adenine chromophore and a unique seven-carbon sugar.

Finally, with the exception of very few references, I have carefully read all the original publications cited in this book. Indeed, the writing of this book was made possible by the reprints, preprints, and personal communications from those scientists who have contributed to the studies on the nucleoside antibiotics. Therefore, I am extremely grateful to those principal investigators throughout the world who have sent me their material and who have generously given time to read, criticize, and correct the chapters in this book. However, I assume responsibility for any shortcomings in the material cited.

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R. J. S.

Contents

CHAPTER 1

3′-Deoxypurine Nucleosides

ABBREVIATIONS

5′-Adenylylmethylene-diphosphonate: AMP-PCP; 5′guanylylmethylene-diphosphonate: GMP-PCP; formylmethionine: F-met; methionine: met; acetylmethionine: acetyl-met; transfer RNA: tRNA; trinucleoside diphosphates: AUG, GUG, UUG, CUG; formylatable species of tRNA are represented by $tRNA_F$; nonformylatable species of tRNA by $tRNA_M$.

Structural changes at C-3′ of purine nucleosides result in nucleosides with marked changes in their biological properties. To date five 3′-deoxyadenine nucleoside antibiotics have been isolated from the *Streptomyces* and fungi that are extremely toxic to bacteria, animal cells in culture, tumors, and viruses. They are puromycin, cordycepin (3′-deoxyadenosine), 3′-amino-3′-deoxyadenosine, homocitrullylaminoadenosine, and lysylamino adenosine. The 3′-deoxynucleoside (3′-acetamido-3′-deoxyadenosine) is not toxic to bacteria or tumor cells. These nucleosides have been excellent biochemical tools for studying numerous reactions with partially purified enzymes or cellular processes. This chapter reviews the chemical and biochemical properties of these six naturally occurring adenine nucleosides.

1.1. PUROMYCIN

INTRODUCTION

Puromycin, 6-dimethylamino-9-[3-(*p*-methoxy-L-β-phenylalanylamino)-3-deoxy-β-D-ribofuranosyl]purine (Fig. 1.1*A*), is a nucleoside antibiotic elaborated by *Streptomyces albonigęr*. The structure and total chemical synthesis have been reported. Puromycin is structurally similar to the 3′-terminus of aminoacyl-tRNA (Fig. 1.1*B*). Puromycin has been a valuable biochemical tool in molecular biology for the elucidation of the mechanisms involved in reading the genetic message by which the nascent polypeptide chain on the peptidyl site is transferred to the aminoacyl-tRNA on the

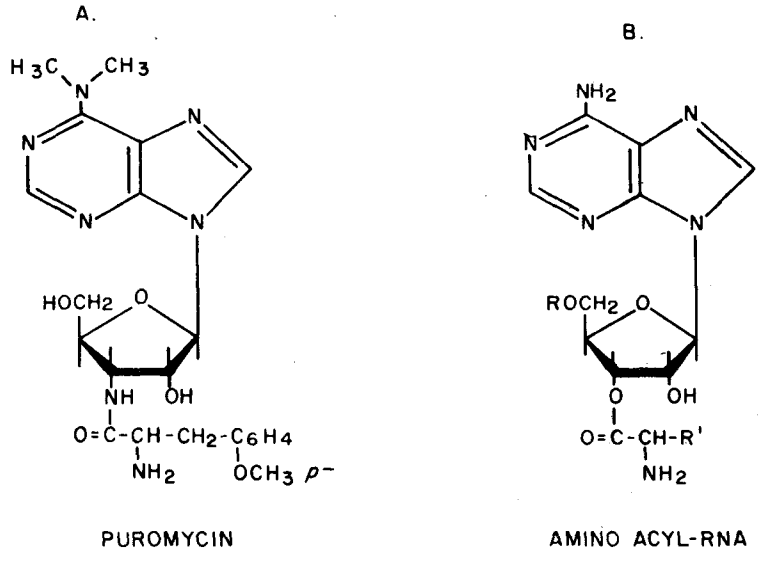

FIGURE 1.1 (*A*) *Structure of puromycin.* (*B*) *3′-Terminus of aminoacyl-tRNA. R = tRNA; R′ = H; alkyl group of amino acids.*

aminoacyl site on the ribosomes. The use of puromycin in the elucidation of initiation and elongation as related to protein synthesis will be discussed. Puromycin inhibits a wide spectrum of organisms. The final enzymatic reaction in the biosynthesis of puromycin has been reported.

DISCOVERY, PRODUCTION, AND ISOLATION

Puromycin (achromycin or stylomycin) (Fig. 1*A*) was isolated from the culture filtrates of *S. alboniger* (ATCC, 12,462) by Porter et al. (1952). The medium reported by Porter et al. (1956) to produce puromycin was modified by Szumski and Goodman (1957). The composition of the medium is as follows: 6% corn steep liquor (50% solids), 4% corn starch, 0.7% calcium carbonate, and 1% lard oil (to prevent foaming) (Szumski and Goodman, 1957). The pH was 6.0–8.5. The addition of the purinyl radical (uric acid, adenine, hypoxanthine, or xanthine) increased the yield of puromycin from 627 to 479 μg/ml. Seed flasks (100 ml medium/500 ml flask) were inoculated with spores from an agar slant (suspended in water). The flasks were maintained on an incubator shaker at 27°C for 2 days. A 2.5% inoculum was used to inoculate larger volumes of the same medium (pH 7.0) (Porter et al., 1956). Puromycin was isolated from the fermentation medium 70 hr after inoculation by adjustment of the pH to 4.0–4.5, filtration, readjustment of the pH to 9.0–9.5, and extraction with 1-butanol. The butanol phase was extracted with distilled water (pH 1.5–2.0). The acid–water was concen-

trated *in vacuo*, and puromycin crystallized in the cold. It was recrystallized by dissolving in water at 40°C, pH 2–4 in a 10% solution. Hydrochloric acid was added to make a 1 *N* solution. Puromycin hydrochloride crystallized on standing. The free base has been isolated by addition of NaOH to pH 7.0 to the hydrochloride salt (Porter et al., 1956; Szumski and Goodman, 1957).

PHYSICAL AND CHEMICAL PROPERTIES

The molecular formula for puromycin is $C_{22}H_{29}N_7O_5$; mp 175.5–177°C; $[\alpha]_D^2$ −11° (in ethanol); λ_{max} 267.5 mμ (ϵ = 19,500) in 0.1 *N* HCl; λ_{max} 275 mμ (ϵ = 20,300) in 0.1 *N* NaOH. It is hydrolyzed to 6-dimethylaminopurine, *O*-methyl-L-tyrosine and 3-amino-3-deoxyribose in acid (Waller et al., 1953). The mass spectra of puromycin have been reported by Eggers et al. (1966). The fragmentation pattern of puromycin is in agreement with the structure assigned and can be compared with the mass spectra of the purine nucleoside analogs cordycepin, 3′-amino-3′-deoxyadenosine, and 3′-acetamido-3′-deoxyadenosine (see pp. 55, 77, and 88).

STRUCTURAL ELUCIDATION AND CHEMICAL SYNTHESIS

The partial structure of puromycin was reported by Waller et al. (1953.) When puromycin (**1**) was treated with ethanolic HCl, three compounds were formed (Fig. 1.2): 6-dimethylaminopurine (**2**), *O*-methyl-L-tyrosine (**3**), and an aminopentose (**4**) that consumed periodic acid (Waller et al., 1953). The amino pentose was shown to be identical with synthetic 3-amino-3-deoxyribose synthesized by Baker and Schaub (1954) and Baker et al. (1955a). When puromycin was treated with phenyl isothiocyanate and sodium methoxide, aminonucleoside (**5**) was isolated (Baker et al., 1955b). This compound (**5**) consumed 1 mole of periodic acid, which established the furanoid structure for the 3-amino-3-deoxyribose moiety. Since puromycin failed to consume periodic acid (Waller et al., 1953), the carboxyl group of *O*-methyl-L-tyrosine was covalently linked to the amino group on carbon-3′ of the pentose. The total chemical synthesis of 3-amino-3-deoxy-D-ribose made possible the synthesis of the aminonucleoside (Baker et al., 1955c). When the titanium chloride complex of the amino sugar was treated with the chloromercuri derivative of 2-methylmercapto-6-dimethylaminopurine, followed by desulfurization with Raney nickel and de-*O*-benzoylation, the *N*-acetyl derivative of the aminonucleoside was obtained. This derivative was then converted to the aminonucleoside (**5**), which was converted to puromycin (Baker et al., 1955b). The glycosidic bond of puromycin has the β configuration (Baker et al., 1954; Baker and Joseph, 1955).

The syntheses of various analogs of puromycin with varying amino acids substituted for *O*-methyl-L-tyrosine have been described (Montgomery and Thomas, 1962; Nathans and Neidle, 1963; Symons et al., 1969). At 3×10^{-4}

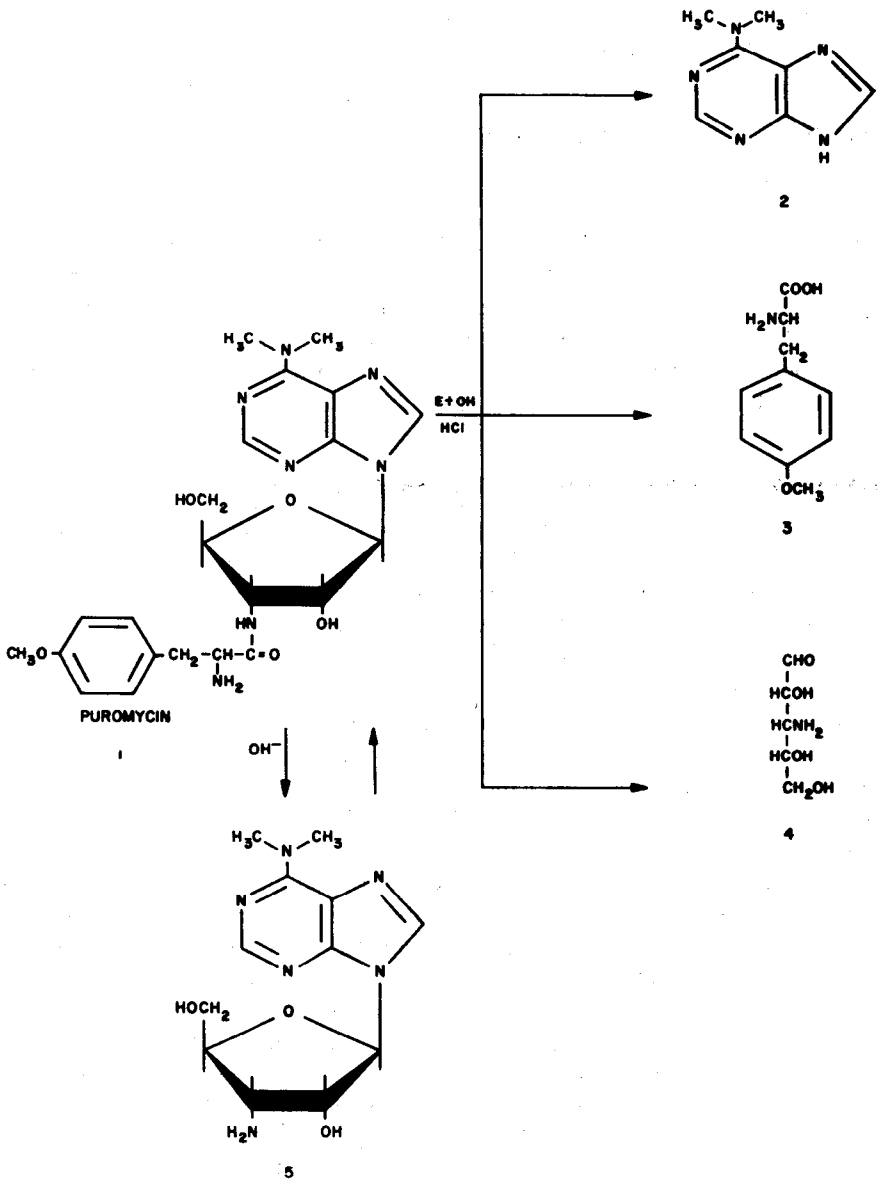

FIGURE 1.2 *Hydrolysis of puromycin.* (*From Waller et al., 1953; Baker et al., 1955b.*)

M, L-phenylalanine, *S*-benzyl-L-cysteine, and L-tyrosine were respectively, 99, 78, and 62% as inhibitory as puromycin (Symons et al., 1969). It is of interest that the X-ray structure of puromycin dihydrochloride reveals that the N^6,N^6-dimethyladenine and the *p*-methoxyl group in tyrosine form alternate stacks such that the *p*-methoxyl group underlies the adenine ring. This type stacking suggests a hydrophobic interaction between the *p*-methoxyl group and the adenine ring and might explain the function of puromycin as an inhibitor of protein synthesis when this analog interacts with the CCA end of tRNA. These findings may explain the lower activity of demethoxypuromycin (Sundaralingum, private communication). Carbon-14, tritium-labeled, and ^{32}P-labeled puromycin have also been synthesized (Allen and Zamecnik, 1962; Shelton and Clark, 1967; Smith et al., 1965). Fisher, Lee, and Goodman have prepared a number of puromycin analogs (1970).

INHIBITION OF GROWTH

Puromycin is a strong inhibitor of gram-positive organisms, but weakly active against the gram-negatives and acid-fast bacilli. The gram-positive organisms are generally more sensitive. It is also effective *in vivo* against *Trypanosoma equiperdum*, *Entamoeba histolytica*, oxyurids, tapeworms, a glioblastoma cultivated in chick embryos, a mammary adenocarcinoma of C_3H mice, viruses, and HeLa cells (Hewitt et al., 1954, 1955). The unusual broad spectrum of inhibition of puromycin may probably be attributed to the fact that puromycin has been shown to be an inhibitor of peptide synthesis.

White and White (1964) reported that puromycin is bacteriostatic and not bacteriocidal. However, the concentration of puromycin added to bacterial cultures is important.

Since puromycin is an effective inhibitor of peptide synthesis, it is also very toxic in higher animals. The 50% lethal dose for mice by the intravenous route is 335 mg/kg; while that supplied intraperitoneally is 580 mg/kg (Sherman et al., 1954/1955). Intravenous injections of 25 mg/kg in cats cause a drop in blood pressure. In rats, intraperitoneal doses of 25 mg/kg caused the animals to lose weight and evoked weakness. At 100 mg/kg, there was renal and bone marrow impairment.

BIOSYNTHESIS

Pogell and his co-workers reported on the biosynthesis of 2-aminoribose-5-phosphate and 2-aminolyxose-5-phosphate with cell-free extracts from *S. alboniger* (Rebello et al., 1969). They reported that D-ribose-5-phosphate is enzymatically converted to 2-amino-2-deoxy-D-ribose-5-phosphate and 2-amino-2-deoxy-D-lyxose-5-phosphate. The specificity for the carbon donor for aminopentose phosphate synthesis with the dialyzed crude super-

natant of *S. alboniger* was shown to be ribose-5-phosphate. When ribose was the substrate, ATP was an essential energy source. Glucose-6-phosphate, fructose-6-phosphate, and ribose were not precursors for amino pentose biosynthesis. Linearity of product formation with time was observed with the partially purified enzyme. A very active phosphoriboisomerase is present in the cell-free extracts. Evidence for the presence of two phosphorylated pentoses was established by paper chromatography following enzyme incubation. Rebello et al. (1969) concluded that the aminopentose phosphates formed from ribose-5-phosphate were 2-amino-2-deoxyribose-5-phosphate and 2-amino-2-deoxylyxose 5-phosphate. The significance of 2-amino-pentose-5-phosphates in extracts of *S. alboniger* as related to the biosynthesis of puromycin is not known. The 2-aminoribose may be an important precursor for the biosynthesis of the 3-aminoribose moiety of puromycin. Additional *in vitro* studies are necessary to establish this biosynthetic relationship.

Rao et al. (1969) have also isolated and partially purified the enzyme from sonicated extracts of *S. alboniger* that catalyzed the enzymatic methylation of *O*-demethylpuromycin. No cofactors were needed. The physical and chemical properties of this methylated puromycin established unequivocally that *O*-methylation of *O*-demethylpuromycin had occurred. Pattabiraman and Pogell (1969) have isolated and identified *O*-demethylpuromycin as a contaminant of crystalline puromycin. These data strongly suggest that the final step in puromycin biosynthesis is the methylation of the tyrosine moiety of *O*-demethylpuromycin.

BIOCHEMICAL PROPERTIES

Since puromycin has been used in a number of cellular reactions, this section on the biochemical properties of this nucleoside antibiotic will be divided into sub-headings applicable to each study. Lipmann (1969), Ono et al. (1969), Vazquez and Monro (1968), Monro (1969), Pestka (1970c), Coutsogeorgopoulos (1970), Nathans (1967), and Lengyel and Söll (1969) have recently published and reviewed polypeptide chain elongations in protein synthesis and the role that puromycin and other antibiotics have played in understanding the mechanisms involved in reading the genetic message. For additional information on the mechanism of protein synthesis, the reader should consult volume 34 of the Cold Spring Harbor Symposia, 1970, The Mechanism of Protein Synthesis.

I. Site of Action of Puromycin

1. *Puromycin: A Biochemical Tool for Studying Protein Synthesis*

Puromycin has been used extensively in the study of the mechanism of protein biosynthesis in both mammalian and bacterial cell-free ribosomal and non-ribosomal systems. The results from these studies indicate that puromycin releases only those peptides from ribosomes that are bound to the

so-called donor site, or peptide binding site (Heintz et al., 1966). Peptidyl-tRNA on the acceptor site (aminoacyl-tRNA binding site), will not react with puromycin. The terms "donor" and "acceptor" reflect the idea that the tRNA on the donor site "donates" its peptidyl moiety to the acceptor, aminoacyl-tRNA, that is bound on the acceptor site. Hence, growth of the polypeptide is from amino to carboxyl end.

Yarmolinsky and de la Haba (1959) were the first to recognize the close structural similarity between puromycin and the aminoacyl end of amino-acyl-tRNA. They showed that the linkage of the carboxyl group of amino acids to the 2′ or 3′ hydroxyl group of adenosine at the 3′-terminus of tRNA is structurally similar to the *p*-methoxytyrosine in which the carboxyl group is covalently bound to the 3′-amino group of puromycin. They reported that puromycin inhibited peptide synthesis in rat liver preparations by blocking the transfer of ^{14}C-leucine from ^{14}C-leucyl-tRNA to the nascent peptide bound to the ribosome. Nathans and Lipmann (1961) subsequently confirmed these results using a cell-free preparation from *E. coli*. Gardner et al. (1962) and Nathans and Neidle (1963) showed that the inhibition of protein synthesis by puromycin was not amino acid specific. Both polylysine and polyphenylalanine synthesis were inhibited. A subsequent paper by Morris et al. (1963) revealed several characteristics of the mode of action of puromycin in hemoglobin biosynthesis. They showed that the amount of TCA-insoluble polypeptide formed was reduced in the presence of puromycin. The release of ^{14}C-labeled peptide from the ribosomes by puromycin occurred in the presence of the supernatant enzyme fraction with no measurable breakdown of ribosomes. When puromycin was added to intact cells, there was a release of polypeptide that contained valine in the *N*-terminal residues. These results showed that puromycin released incomplete globin chains. One molecule of puromycin was covalently bound for each *N*-terminal valine.

Allen and Zamecnik (1962) were the first to suggest that the amino group of the *p*-methoxyphenylalanyl moiety of puromycin attacked the *C*-terminal acyl group of the growing peptide chain with the displacement of tRNA. Additional proof for the release of shorter, acid-soluble and alcohol-soluble peptides by puromycin was supplied by Nirenberg et al. (1962) and Nathans et al. (1963). Gilbert (1963) similarly showed that puromycin released polypeptides from peptidyl-tRNA bound to the 50-S subunit by reacting with the ester bond of the peptidyl-tRNA. The composition of the peptidyl–puromycin reaction was determined by studying the puromycin reaction in the polyadenylate-directed synthesis of polylysine with ^{32}P-β-cyanoethylphosphate 5′-puromycin (Smith et al., 1965). Each of the peptides released to the supernatant contained one molecule of puromycin. Hydrolysis of dilysyl-puromycin with trypsin resulted in the isolation of lysyl-lysine. Lysyl-puromycin was not a product released to the supernatant. These studies clearly showed that the carboxyl end of the endogenous