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antibiotics

isolation, separation and purification

edited by
M.J. Weinstein and G.H. Wagman

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PREFACE

The most recent developments in antibiotic chemotherapy have been accomplished through the creation of new effective agents resulting from chemical manipulations. Active substances, as well as inactive molecular fragments, have been used as substrates to accomplish dramatic changes in biological activity.

New chemical reactions, developed in recent years, have enabled the research laboratory to provide a steady flow of interesting semi-synthetic antibiotics. In the aminocyclitol family, several very promising antibiotics have reached the clinic either with improved activity against resistant bacteria or with reduced toxicity. Certain derivatives have shown improvement in both of these parameters. Among the beta-lactam antibiotics, the flow of new agents is better characterized as a flood. Modification of the 6-aminopenicillanic acid, 7-aminocephalosporanic acid and 7-methoxy, 7-aminocephalosporanic acid nucleus has resulted in a plethora of agents that could alter the ego of any chemist! These new derivatives are too numerous to name, and such an effort would likely omit one destined to be the major bullet. More recently the thienamycin, nocardicin and clavulanic acid class of beta-lactams have become the chemist's playground.

We know this is not the beginning--nor is the end in sight. In earlier days, chemists tried their hands at doctoring the now-ancient warriors such as streptomycin, neomycin, the tetracyclines, chloramphenicol and erythromycin, always changing, but rarely improving the patient, literally and figuratively. The latest breed of chemist armed with new technology, new reactions and novel substrates has made remarkable strides in new, useful drug development...and the better is yet to come!

It is with these thoughts in mind that we have asked the most competent scientists in the field of antibiotic isolation to contribute to this latest *vade mecum*. We have chosen key chemical families to be represented, and in each chapter the authors write from hard personal experience.

This volume anticipates a continuation of this renaissance in the field of antibiotic discovery. Old substrates will be worked again for nuggets of new activity and the only source for many of these starting materials is the fermentation vessel. For those who would venture forth in this fermentation broth, this text will present the latest techniques for transferring the hopeful substrates from the fermentor to the retort.

We are convinced that a worthwhile contribution to the antibiotic armamentarium lies somewhere in the molecular manipulation of the various chemical types reviewed in this publication and will feel a certain satisfaction in having eased the way for that happening to occur.

Bloomfield, N.J., USA
May 1978

Marvin J. Weinstein
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CONTENTS

Actinomycins

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1. Techniques for Separation of Actinomycin Mixtures

1.1 Introduction

The actinomycins are a series of chromopeptide lactone antibiotics with a common structural format differing only in certain amino acid residues (Fig. 1; Table 1, following page)[1].

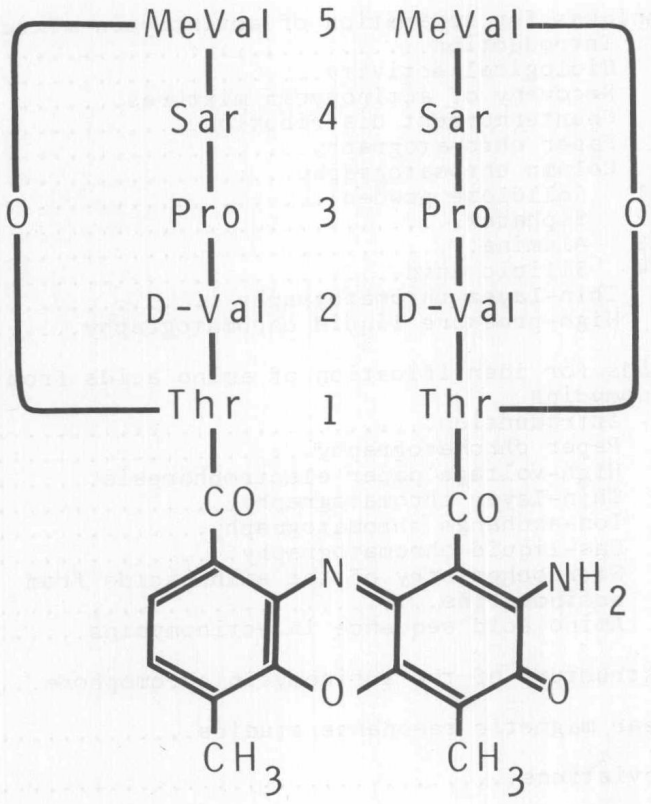

Figure 1. Structure of actinomycin D

The chromophore, actinocin, is 2-amino-4,6-dimethylphenoxazinone (3)-1,9-dicarboxylic acid. Examination of the structures in the table reveals the extent of the variations in amino acid composition of the naturally occurring actinomycins. Thus, in site 1 of the peptide chain, L-threonine may be replaced by α-amino-β,γ-dihydroxybutyric acid (hydroxythreonine); in site 2 there may be present D-valine and/or D-alloisoleucine. L-Proline, in site 3, may be substituted for by 4-hydroxy-L-proline, 4-keto-L-proline, sarcosine, 4-keto-5-methylproline, 5-methylproline or 3-hydroxy-5-methylproline. Site 4 is always occupied

TABLE 1

Amino Acid Sequences of Actinomycins

Actinomycins produced by *Streptomyces* species	Site of Actinomycin Peptides				
	1	2	3	4	5
I, A_I, B_I, $X_{O\beta}$	Thr	Val	Pro	Sar	MeVal
	Thr	Val	HyPro	Sar	MeVal
$X_{O\delta}$	Thr	Val	Pro	Sar	MeVal
	Thr	Val	aHyPro	Sar	MeVal
II, A_{II}, B_{II}, F_8	Thr	Val	Sar	Sar	MeVal
	Thr	Val	Sar	Sar	MeVal
III, A_{III}, B_{III}, F_9, $X_{O\gamma}$	Thr	Val	Pro	Sar	MeVal
	Thr	Val	Sar	Sar	MeVal
IV, A_{IV}, B_{IV}, D, C_1, I_1, X_1	Thr	Val	Pro	Sar	MeVal
	Thr	Val	Pro	Sar	MeVal
X_{1a}	Thr	Val	Sar	Sar	MeVal
	Thr	Val	KetoPro	Sar	MeVal
V, A_V, B_V, X_2	Thr	Val	Pro	Sar	MeVal
	Thr	Val	KetoPro	Sar	MeVal
VI, C_2, I_2	Thr	Val	Pro	Sar	MeVal
	Thr	aIleu	Pro	Sar	MeVal
C_{2a}	Thr	aIleu	Pro	Sar	MeVal
	Thr	Val	Pro	Sar	MeVal

TABLE 1 (Continued)

Actinomycins produced by *Streptomyces* species	Site of Actinomycin Peptides 1	2	3	4	5
VII, C_3	Thr	aIleu	Pro	Sar	MeVal
	Thr	aIleu	Pro	Sar	MeVal
Z_1	Thr	Val	4-Keto-5-MePro	Sar	MeVal
	HyThr	Val	3-Hy-5-MePro	Sar	MeAla
Z_5	Thr	Val	4-Keto-5-MePro	Sar	MeVal
	HyThr	Val	5-MePro	Sar	MeAla
Actinomycins produced by *Micromonospora floridensis*					
Schering 1a	Thr	Val	4-Keto-5-MePro	Sar	MeVal
	Thr	Val	5-MePro	Sar	MeVal
Schering 1b	Thr	Val	4-Keto-5-MePro	Sar	MeVal
	Thr	Val	5-MePro	Sar	MeAla
Schering 1c	Thr	Val	4-Keto-5-MePro	Sar	MeVal
	Thr	Val	3-Hy-5-MePro	Sar	MeVal
Schering 6a	Thr	Val	4-Keto-5-MePro	Sar	MeVal
	Thr	Val	3-Hy-5-MePro	Sar	MeAla
Schering 10	Thr	Val	4-Keto-5-MePro	Sar	MeVal
	HyThr	Val	5-MePro	Sar	MeAla
Directed biosynthesis with pipecolic acid					
Pip 2	Thr	Val	Pip	Sar	MeVal
	Thr	Val	Pip	Sar	MeVal

TABLE 1 (Continued)

Directed biosynthesis with pipecolic acid	Site of Actinomycin Peptides 1	2	3	4	5
Pip 1β	Thr	Val	Pip	Sar	MeVal
	Thr	Val	Pro	Sar	MeVal
Pip 1α	Thr	Val	4-KetoPip	Sar	MeVal
	Thr	Val	Pip	Sar	MeVal
Pip 1δ	Thr	Val	4-KetoPip	Sar	MeVal
	Thr	Val	Pro	Sar	MeVal
Pip 1γ	Thr	Val	4-HyPip	Sar	MeVal
	Thr	Val	Pip	Sar	MeVal
Pip 1ε	Thr	Val	4-HyPip	Sar	MeVal
	Thr	Val	Pro	Sar	MeVal
Directed biosynthesis with sarcosine					
F_1	Thr	aIleu	Sar	Sar	MeVal
	Thr	Val	Sar	Sar	MeVal
F_2	Thr	aIleu	Sar	Sar	MeVal
	Thr	Val	Pro	Sar	MeVal
F_3	Thr	aIleu	Sar	Sar	MeVal
	Thr	aIleu	Sar	Sar	MeVal
F_4	Thr	aIleu	Sar	Sar	MeVal
	Thr	aIleu	Pro	Sar	MeVal

TABLE 1 (Continued)

Directed biosynthesis with sarcosine	Site of Actinomycin Peptides 1	2	3	4	5
F_8			See actinomycin II		
F_9			See actinomycin III		
Directed biosynthesis with isoleucine stereoisomers*					
E_1 (*S. chrysomallus*)	Thr	{aIleu} / {Val}	Pro	Sar	MeVal
	Thr	{Ileu}	Pro	Sar	MeaIleu
E_2 (*S. chrysomallus*)	Thr	aIleu	Pro	Sar	MeaIleu
	Thr	Ileu	Pro	Sar	MeaIleu
E_2 (*S. parvulus*)	Thr	Ileu	Pro	Sar	MeaIleu
	Thr	Ileu	Pro	Sar	MeaIleu
Directed biosynthesis with *cis* or *trans*-4-methylproline					
K_1c	Thr	Val	Pro	Sar	MeVal
	Thr	Val	*cis*-4-MePro	Sar	MeVal
K_2c	Thr	Val	*cis*-4-MePro	Sar	MeVal
	Thr	Val	*cis*-4-MePro	Sar	MeVal
K_1t	Thr	Val	Pro	Sar	MeVal
	Thr	Val	*trans*-4-MePro	Sar	MeVal
K_2t	Thr	Val	*trans*-4-MePro	Sar	MeVal
	Thr	Val	*trans*-4-MePro	Sar	MeVal

TABLE 1 (Continued)

Directed biosynthesis with azetidine-2-carboxylic acid	Site of Actinomycin Peptides 1	2	3	4	5
Azetomycin I, Azβ	Thr	Val	Pro	Sar	MeVal
	Thr	Val	Azet	Sar	MeVal
Azetomycin II, Azα	Thr	Val	Azet	Sar	MeVal
	Thr	Val	Azet	Sar	MeVal
Directed biosynthesis with *cis*- or *trans*-4-chloroproline					
cis-chloro A	Thr	Val	*cis*-4-ClPro	Sar	MeVal
	Thr	Val	*cis*-4-ClPro	Sar	MeVal
cis-chloro B	Thr	Val	Pro	Sar	MeVal
	Thr	Val	*cis*-4-ClPro	Sar	MeVal
trans-chloro A	Thr	Val	*trans*-4-ClPro	Sar	MeVal
	Thr	Val	*trans*-4-ClPro	Sar	MeVal
trans-chloro B	Thr	Val	Pro	Sar	MeVal
	Thr	Val	*trans*-4-ClPro	Sar	MeVal

*Recent investigations with isoleucine stereoisomers[66] do not confirm an earlier report[34].

by sarcosine; finally, the amino acid at site 5, N-methyl-L-valine, can be replaced by N-methylalanine in some actinomycins of the Z series. Amino acid substitutions in the peptide chains are responsible for the quantitative differences observed in the biological activities of natural actinomycins and those formed via directed biosynthesis.

With the exception of actinomycin D (Am D), the naturally occurring actinomycin preparations were shown by the techniques described here to be mixtures of at least three components. Initially, such complexes were designated as A, B, C, D, X, etc. according to the producing organism. However, A, B and X contain the same components (I-V) and differ only in their relative abundance, which can also vary with culture conditions. The sole component of Am D (= IV) is present in all the above-mentioned complexes. The Z complex, in contrast, has no components in common with the above mixtures. In addition to the naturally produced actinomycin complexes, mixtures containing novel components have been produced by directed biosynthesis, in which an added amino acid or amino acid analogue competes for incorporation into specific sites in the peptide moieties[2].

Separation of mixtures of such closely-related compounds represents a formidable task. Partition chromatography has in general proved more useful than adsorption chromatography, particularly in the separation of components differing only in alkyl side chains (for example, D-*allo*isoleucine in place of D-valine). Historically, the first separations (1951) were accomplished by countercurrent distribution, with partition chromatography on paper or on columns of cellulose or Sephadex developed soon thereafter. More recent developments include the use of thin-layer and high-pressure liquid chromatography.

Abbreviations used in this chapter will be found in Section 5.

1.2 Biological activity

Investigations concerning the mode of action of Am D have revealed that the antibiotic is a potent inhibitor of deoxyribonucleic acid-dependent RNA synthesis. Consequently, the antibiotic has become an extremely useful probe for investigations relating to the synthesis of messenger RNA and protein as well as virus replication. Clinically, Am D is of considerable importance in the treatment of Wilm's tumor, gestational choriocarcinoma and mixed metastatic carcinoma of the testes.

1.3 Recovery of actinomycin mixtures

The recovery of actinomycins from culture filtrates after fermentations has usually been effected by extraction with organic solvents such as ethyl acetate or butyl acetate. The mycelium which generally contains relatively small amounts of the antibiotics may be extracted with n-butanol overnight. After evaporation under reduced pressure, the crude actinomycin mixtures so obtained are then subjected to the procedures described below.