Russian Chemical Reviews **65** (4) 355 – 378 (1996)
© 1996 Russian Academy of Sciences and Turpion Ltd

UDC 615.779.925 + 577.113.4

Interaction of bleomycin and its oligonucleotide derivatives with nucleic acids

D S Sergeev, V F Zarytova

Contents

I. Introduction 355
II. Binding of bleomycin to nucleic acids 356
III. Cleavage of nucleic acids by bleomycin 363
IV. Bleomycin derivatives of oligonucleotides. Synthesis and site-specific cleavage of DNA 369

Abstract. Various aspects of interaction of the antitumour glycopeptide antibiotic bleomycin with nucleic acids are considered. Data on equilibrium binding parameters obtained by various physicochemical methods have been collected and compared. The contribution of N- and C-terminal domains of the glycopeptide molecule to the binding with DNA and sequence specificity of DNA cleavage are discussed. Data on a recently created new class of compounds — bleomycin – oligonucleotide conjugates — are presented. These compounds, like antibiotics, possess DNA-cleaving activity (also in a catalytic manner) together with high selectivity towards a selected nucleotide sequence. The bibliography includes 267 references.

I. Introduction

1. A brief review of the bleomycin antibiotics family

The discovery and pioneering studies on the family of bleomycin antibiotics date back to 1963 when a group of Japanese investigators headed by Umezawa detected a substance possessing a high antibiotic activity in the culture fluid of the mutant strain of the soil bacterium *Streptomyces verticillus*. Just three years later, in 1966, a wide range of structurally related compounds, given common name of bleomycins, were isolated.[1,2] Owing to their beneficial therapeutic effects, these compounds have found wide use in the clinical practice as valuable pharmaceutical drugs endowed with both antibacterial and, even more important, antitumour activities.[3–8] The major area for clinical application of bleomycins is in the chemotherapy of malignant tumours, particularly of lymphomas and squamous carcinomas.[9–12] Along with their high antitumour activity, bleomycins are characterized by relatively low toxicity and lack of immunodepressive action which makes them helpful tools in combined chemotherapy.[13,14] Applications of bleomycins in clinical oncology have been described in numerous reviews and special publications.[13–20]

Simultaneously with practical applications of bleomycin antibiotics, in-depth studies into their actions on the cell were under way. It turned out that the main target for bleomycin antibiotic action in the cell is DNA.[21,22] Bleomycin has a number of actions coupled to DNA degradation, namely, the loss by animal and bacterial cells of their ability to form colonies,[5] inhibition of DNA synthesis,[6,23] chromosomal aberrations,[24] intracellular DNA degradation to acid-soluble products,[25] and DNA dissociation from the membrane complex.[26] These findings prompted extensive research concerned with bleomycin interactions with DNA with special emphasis on its binding to DNA, action mechanisms, cofactors required, etc. Recent studies have demonstrated that bleomycin can also cleave RNA. This important discovery gave a strong impetus to further studies into the action mechanisms of bleomycin and the basis for its biological activities.

To date, a great body of information on this antibiotic has been accumulated in the literature. Some of the data concerning the nature of bleomycin interactions with DNA are presented in this review.

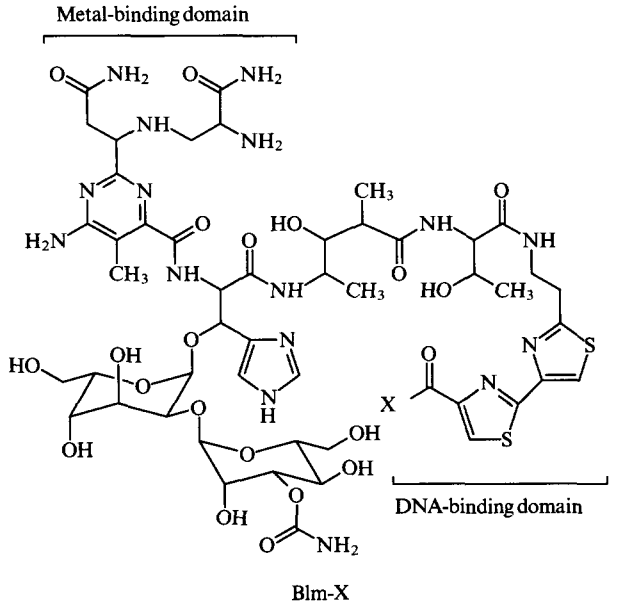
2. Functional domains of bleomycin

Since the initial isolation of individual bleomycins, intensive studies have taken place with the aim of establishing the structure, the elucidation of which required a vast variety of chemical and physicochemical methods including NMR spectroscopy and X-ray analysis. For general features, this work was completed by 1972.[27] In 1978, the bleomycin structure was finally elucidated;[28] total chemical synthesis of bleomycin A_2 was carried out in 1982.[29]

Bleomycins are glycopeptides with a molecular mass of about 1500 Da. The fragment of the molecule (termed as bleomycinic acid) common to all bleomycins consists of pyrimidoblaminic acid, β-hydroxyhistidine, 4-amino-3-hydroxy-2-methylpentanoic acid, threonine, 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylic acid linked by peptide bonds, and a carbohydrate moiety containing 3-(*O*-carbamoyl)-D-mannose and L-gulose attached to the hydroxyl group of β-hydroxyhistidine. The structures of bleomycin (Blm) and C-terminal amines of the most commonly occurring bleomycins are given below. Individual bleomycins differ from one another by the terminal amine bound to the carboxyl group of the bithiazole residue. Bleomycinic acid (Blm–OH) represents a compound with OH instead of NHX. Depending on the composition of their terminal amines, bleomycins are divided into two subgroups: A and B. Group A bleomycins carry in their terminal amine both primary and secondary amino groups with the exception of bleomycins A_1 and A_2; group B bleomycins have guanidinium groups. Studies on bleomycin carried out abroad were largely performed on bleomycin A_2 and blenoxane, a mixture of bleomycins A_2 (60% – 65%), bleomycin B_2 (30%) and minor components (B_4, A_5, etc.). In our country, bleomycin A_5 is routinely used in clinical practice and in laboratory studies.

D S Sergeev, V F Zarytova Division of Chemistry of Nucleic Acids, Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences, prosp. Akad. Lavrent'eva 8, 630090 Novosibirsk, Russian Federation. Fax: (7-383) 235 16 65, tel: (7-383) 239 62 24, E-mail: zarytova@modul.bioch.nsk.su

Received 28 August 1995
Uspekhi Khimii **65** (4) 377 – 402 (1996); translated by R L Birnova



Blm-X

X	Name	Designation
$NH(CH_2)_3S^+(CH_3)_2$	Bleomycin A_2	(Blm-A_2)
$NH(CH_2)_3NH(CH_2)_4NH_2$	Bleomycin A_5	(Blm-A_5)
$NH(CH_2)_3SCH_3$	Demethylbleomycin A_2	(DMBlm-A_2)
NH_2	Bleomycin B'_2	(Blm-B'_2)
$NH(CH_2)_4NHC(NH)NH_2$	Bleomycin B_2	(Blm-B_2)
$NH(CH_2)_3NH(CH_2)_3N(CH_2)_4$	Bleomycin BAPP	(Blm-BAPP)

In parallel with the determination of the bleomycin structure, general concepts of molecular mechanisms of their biological activity were developed. It was found, in particular, that bleomycin can interact with DNA and form strong noncovalent complexes with it.[30-34] Bleomycin can also form complexes with metal ions and cause DNA degradation in the presence of Fe(II) ions and molecular oxygen.[35-37] Based on these findings, the bleomycin molecule was conventionally divided into domains in accordance with their specific function.[38-40] Pyrimidoblaminic acid and β-hydroxyhistidine are involved in the metal-binding domain. The primary and secondary amino groups present in this domain provide for the formation of a tight complex with many transient metals.[41-44] The bithiazole residue and the C-terminal amine responsible for the binding to DNA are termed 'the DNA-binding domain'. 4-Amino-3-hydroxy-2-methylpentanoic acid and threonine determine the reciprocal arrangement of these two domains needed for the tight binding and effective cleavage of DNA. Finally, the disaccharide favours the binding of molecular oxygen which, in turn, is necessary for oxidative cleavage of DNA.[45,46]

II. Binding of bleomycin to nucleic acids

1. Parameters of equilibrium binding

An assumption that bleomycin binds to DNA was made as long ago as 1969[30,33] and the first evidence for the binding of tritium-labelled bleomycin A_2 to *E. coli* DNA was obtained a year thereafter.[34] However, systematic studies of this process employing physicochemical methods were begun only in the late 1970s. The discovery of quenching of bleomycin fluorescence in the binding of the antibiotic to DNA was a boon for experimenters.[38] Being light-excited at the wavelength of 300 nm, bleomycins produce two emission bands with maxima at 353 and 405 nm. Light with a wavelength of 353 nm is emitted by the bithiazole residue and exceeds in intensity the emission of the 4-aminopyrimidine ring at 405 nm. Addition of calf thymus DNA to bleomycin solutions results in significant (up to 50%) quenching of the fluorescence at 353 nm. This finding made it possible to quantify both bound and free bleomycin and to calculate, at variable concentrations of DNA, the apparent equilibrium binding constant, K_{app}, and the number of DNA nucleotides per bound molecule of the antibiotic, n. The K_{app} and n values were also determined using equilibrium dialysis,[47-49] gel filtration[49] and circular dichroism spectroscopy (CD).[50-52] The values of K_{app} and n determined by different methods are summarized in Table 1.

As can be seen from Table 1, the binding constants determined by different authors for metal-free bleomycin A_2 vary from 10^5 to 10^6 dm^3 mol^{-1}. The number of DNA base pairs per bound antibiotic molecule also varies significantly in different studies, being equal, on average, to 3 – 10 pairs. The lower values cited in the most recent papers[52,55] appear to be far more reliable. An increase in ionic strength does not affect the K_{app} values but increases the n value, thereby decreasing the number of accessible binding sites.[38,54] Interestingly, bleomycin binding to homogenous DNA of poly(dG)·poly(dC) and poly(dA)·poly-(dT) appears to be stronger than that to heterogeneous DNA, and parameter n[49] is greater in the former case. The binding of bleomycin to the bivalent copper ion slightly decreases the binding constant but increases the number of accessible binding sites.[49,57] At the same time, the binding constant for the blenoxane – Fe(III) complex is twice as high as that for blenoxane itself, while the number of accessible binding sites is the same in both cases.[56] Bleomycin complexes with Co(III) are characterized by K_{app} of 10^5 to 10^7 dm^3 mol^{-1} depending on the nature of the chelated ligand.[50,59] An increase in ionic strength strongly decreases the constant of binding of bleomycin – copper and cobalt complexes to DNA without any change in the number of base pairs per bound molecule,[48,59] thus suggesting that electrostatic interactions have a role in this process.

Evidence concerning the parameters of bleomycin binding to RNA is very sparse in the current literature except for a study,[61] in which K_{app} and n values for blenoxane binding to yeast phenylalanine tRNA were determined by the fluorescence quenching method. The parameters of binding to this tRNA are close to those for DNA ($K_{app} = 0.86 \times 10^5$ dm^3 mol^{-1} and $n = 25$ in 5 mmol dm^{-3} phosphate buffer pH 7.0).

Most of the data listed in Table 1 were obtained using the method of quenching the fluorescence of bithiazole rings. This widely used procedure, which has a number of indisputable advantages (simplicity, rapidity, etc.), also has some limitations. Firstly, the quantum yield of this fluorescence is rather low (ca. 0.01).[38] Chelation of the metal ion leads to a further decrease in bleomycin fluorescence,[54,57] which imposes certain restrictions on the accuracy of the experimental results. Secondly, according to recent data,[55] upon excitation by light with a wavelength of 300 nm, the intensity of bleomycin fluorescence decreases significantly with time due to the decay or phototransformation of the bithiazole residue that has been neglected in earlier studies. Finally, bleomycin fluorescence is effectively quenched not only by double-stranded DNA (binding data are given in Table 1) but also by single-stranded DNA and RNA.[54,57] However, it is known that neither single-stranded DNA nor RNA protect double-stranded DNA from bleomycin action.[62-65] Apparently, the mode of bleomycin fluorescence quenching by these substrates depends on the binding type and affinity. The fact that, after incubation under identical conditions and subsequent dialysis,[34] the amount of tritium-labelled bleomycin on double-stranded *E. coli* DNA exceeds 30-fold that on ribosomal RNA, provides additional evidence that fluorescence quenching does not indicate strong binding. Moreover, the fluorescence quenching itself depends, in turn, on the composition of DNA: poly(dG)- and poly(dG)·poly(dC) sequences quench the fluorescence to a greater extent.[54,57] Presumably, the K_{app} and n values listed in Table 1 are mean values for different binding sites displaying different affinity.

Table 1. Parameters for equilibrium binding of bleomycins, metal-containing bleomycins, and bleomycin A_2 C-terminal fragments to DNA.

Compound	$10^5 K_b/dm^3 mol^{-1}$ [a]	n [a]	Buffer conditions	DNA	Method [b]	Ref.
Heterogeneous DNA at low ionic strength						
Blm-A_2	1.2 ± 0.2	11 ± 1	1.2 mmol dm^{-3} NaCl, 2.5 mmol dm^{-3} Tris – HCl (pH 8.4)	Calf thymus	F	38
	0.34 ± 0.04	3.4 ± 0.5	1.2 mmol dm^{-3} NaCl, 2.5 mmol dm^{-3} Tris – HCl (pH 8.4)	Salmon sperm	F	53
	7.8 ± 0.5	25 ± 3	1.2 mmol dm^{-3} NaCl, 2.5 mmol dm^{-3} Tris – HCl (pH 8.4)	Calf thymus	F	54
	1.0	7.6	1.2 mmol dm^{-3} NaCl, 2.5 mmol dm^{-3} Tris – HCl (pH 8.4)	"	F	55
Blenoxane [á]	9.3	6	0.8 mmol dm^{-3} sodium citrate (pH 5.5)	*Col E1*	E	47
	4.1	10	1.0 mmol dm^{-3} Tris – HCl (pH 8.0)	Calf thymus	F	56
Heterogeneous DNA at ionic strength of 25 – 50 mmol dm^{-3}						
Blm-A_2	0.92 ± 0.01	52 ± 4	1.2 mmol dm^{-3} NaCl, 25 mmol dm^{-3} Tris – HCl (pH 8.4)	Calf thymus	F	38
	3.2	417	15 mmol dm^{-3} NaCl, 15 mmol dm^{-3} Tris – HCl (pH 8.4), 1.5 mmol dm^{-3} EDTA	"	F	57
	8.1	36	50 mmol dm^{-3} NaCl, 1.5 mmol dm^{-3} Tris – HCl (pH 8.4)	"	F	54
	5.7 ± 0.5	7.4 ± 0.6	25 mmol dm^{-3} Na_2HPO_4 (pH 7.0)	"	G	49
	6.8 ± 0.4 2.5 ± 0.1	7.8 ± 0.4 6.6 ± 0.6	25 mmol dm^{-3} Na_2HPO_4 (pH 7.0)	"	E	49
Homogeneous DNA						
Blm-A_2	26 ± 1 3.9 ± 0.2	22.8 ± 0.8 9.0 ± 0.6	25 mmol dm^{-3} Na_2HPO_4 (pH 7.0)	poly(dG) · poly(dC)	E	49
	19 ± 1 2.7 ± 0.1	29.4 ± 0.6 13.2 ± 0.4	25 mmol dm^{-3} Na_2HPO_4 (pH 7.0)	poly(dA) · poly(dT)	E	49
	69.5 0.51	19.2 2.7	25 mmol dm^{-3} NaCl 10 mmol dm^{-3} Na_2HPO_4 (pH 6.8)	poly(dA) · poly(dT)	F	58
	87.0 0.50	20.4 2.6	100 mmol dm^{-3} NaCl, 10 mmol dm^{-3} Na_2HPO_4 (pH 6.8)	poly(dA) · poly(dT)	F	58
Heterogeneous DNA						
Cu(II)Blm-A_2	2.3	77	15 mmol dm^{-3} NaCl, 15 mmol dm^{-3} Tris – HCl (pH 8.4), 1.5 mmol dm^{-3} EDTA	Calf thymus	F	57
	3.9 ± 0.3	5.6 ± 0.4	25 mmol dm^{-3} Na_2HPO_4 (pH 7.0)	"	G	49
	4.4 ± 0.4 1.7 ± 0.1	5.6 ± 0.4 4.2 ± 0.2	25 mmol dm^{-3} Na_2HPO_4 (pH 7.0)	"	E	49
	18 ± 1 2.6 ± 0.1	20.2 ± 0.4 8.2 ± 0.6	25 mmol dm^{-3} Na_2HPO_4 (pH 7.0)	poly(dG) · poly (dC)	E	49
	10 ± 1 1.7 ± 0.1	22.0 ± 0.6 12.0 ± 0.6	25 mmol dm^{-3} Na_2HPO_4 (pH 7.0)	poly(dA) · poly(dT)	E	49

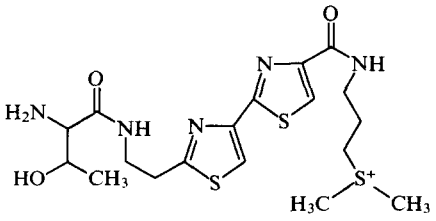
Table 1 (continued).

Compound	$10^5 K_b/dm^3 mol^{-1}$ [a]	n [a]	Buffer conditions	DNA	Method [b]	Ref.
Cu(II)Blenoxane	0.2	8	25 mmol dm^{-3} Tris – HCl (pH 7.0)	Calf thymus	F	48
	0.01	8	50 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris – HCl (pH 8.0)	"	F	48
	0.012	8	50 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris – HCl (pH 8.0)	"	E	48
Fe(III)Blenoxane	9.6	10	1 mmol dm^{-3} Tris – HCl (pH 8.0)	"	F	56
Fe(III)Blm-A_2	3.5 (pH 7.4)	5.2	50 mmol dm^{-3} Hepes	"	CD	51
Co(III)Blm-A_2	4.7 ± 0.4	10 ± 2	1 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris – HCl (pH 8.0), 1 mmol dm^{-3} EDTA	"	F	59
	1.5 ± 0.2	10 ± 2	20 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris – HCl (pH 8.0), 1 mmol dm^{-3} EDTA	"	F	59
HO_2^- – Co(III)Blm-A_2	130 ± 30	8 ± 2	20 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris – HCl (pH 8.0), 1 mmol dm^{-3} EDTA	"	F	59
H_2O – Co(III)Blenoxane	500 ± 100	6 ± 2	1 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris – HCl (pH 8.0), 1 mmol dm^{-3} EDTA	"	F	59
	19 ± 3	26 ± 2	140 mmol dm^{-3} NaCl, 20 mmol dm^{-3} Tris – HCl (pH 8.0), 1 mmol dm^{-3} EDTA	"	F	59
Cu(II)Blm-A_5	3	14	15 mmol dm^{-3} NaCl, 15 mmol dm^{-3} Tris – HCl (pH 8.4), 1.5 mmol dm^{-3} EDTA	"	F	57
Ni(II)Blm-B_2	4	–	100 mmol dm^{-3} NaCl, 10 mmol dm^{-3} Na_2HPO_4 (pH 7.5)	12-Meric duplex	CD	52
Tripeptide S [d]	1.95 ± 0.04	8 ± 1	1.2 mmol dm^{-3} NaCl, 2.5 mmol dm^{-3} Tris – HCl (pH 8.4)	Calf thymus	F	38
	0.59 ± 0.01	35 ± 4	1.2 mmol dm^{-3} NaCl, 25 mmol dm^{-3} Tris – HCl (pH 8.4)	"	F	38
	4.5	5	1.25 mmol dm^{-3} NaCl, 2.5 mmol dm^{-3} Tris – HCl (pH 8.4)	"	F	55
	10	5	0.8 mmol dm^{-3} sodium citrate (pH 5.5)	*Col E1*	F	47
	1.5	5	2 mmol dm^{-3} Tris – HCl (pH 8.0), 1 mmol dm^{-3} EDTA	"	F	47
Acetyldipeptide [e]	7.5	16	0.1 mmol dm^{-3} EDTA, 10 mmol dm^{-3} sodium cacodylate (pH 7.0)	Calf thymus	F	60
	5	16	0.1 mmol dm^{-3} EDTA, 10 mmol dm^{-3} sodium cacodylate (pH 7.0)	poly(dA) · poly(dT)	F	60

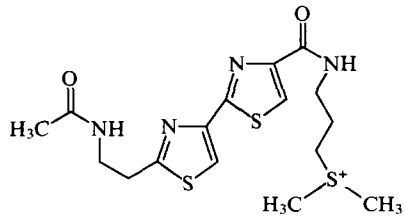
Table 1 (continued).

Compound	$10^5 K_b$/dm^3 mol^{-1} [a]	n [a]	Buffer conditions	DNA	Method [b]	Ref.
Acetyldipeptide [e]	27.0 0.66	20.8 4.8	25 mmol dm^{-3} NaCl, 10 mmol dm^{-3} Na_2HPO_4 (pH 6.8)	poly(dA)·poly(dT)	F	58
	74.9 0.28	41.7 5.0	100 mmol dm^{-3} NaCl, 10 mmol dm^{-3} Na_2HPO_4 (pH 6.8)	poly(dA)·poly(dT)	F	58

[a] Where two values are indicated, the former corresponds to strong binding, and the latter to weak binding.
[b] F is fluorescence quenching; E is equilibrium dialysis; G is gel filtration; CD is circular dichroism spectroscopy.
[c] Blenoxane is a mixture of bleomycin A_2 (60% – 70%), bleomycin B_2 (30%) and minor components (bleomycin A_5, bleomycin B_4, demethylbleomycin A_2, etc.) used as a chemotherapeutic drug.
[d] Tripeptide S:



[e] Acetyldipeptide:



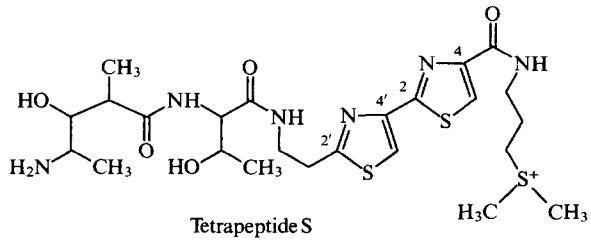
Another method of using the fluorescence quenching technique for a determination of the parameters of bleomycin binding to DNA has been described by Sakai et al.[58] In this study, the fluorescence was excited at a wavelength of 260 nm (not 300 nm) as usual. This made it possible to increase the difference between the fluorescence of DNA-bound and free bleomycin and thus to increase the sensitivity of the method, as a result of which two binding constants could be obtained for bleomycin binding to poly(dA)·poly(dT). One of them, which reflects the binding of one molecule of the antibiotic per 10 base pairs, corresponds to stronger binding and is equal to $(7-9) \times 10^6$ dm^3 mol^{-1}, whereas the other one, which reflects the binding of one bleomycin molecule per 1.5 base pairs, is 5×10^4 dm^3 mol^{-1}. The existence of different binding sites was also demonstrated by applying the method of equilibrium dialysis to tritium-labelled bleomycin A_2 and its copper complex.[49] The difference between K^1_{app} and K^2_{app} determined for calf thymus DNA appeared to be insignificant (6.8×10^5 and 2.5×10^5 dm^3 mol^{-1}). A greater difference between the binding constants was observed when bleomycin A_2 was bound to the homopolynucleotides poly(dG)·poly(dC) and poly(dA)·poly(dT) (Table 1).

Hence, the parameters of equilibrium binding obtained by various methods suggest a significant affinity of bleomycin and its metal complexes for double-stranded DNA. Strong binding of the antibiotic to the double helix precedes a cascade of events resulting in oxidative degradation of the DNA chain.[41,66]

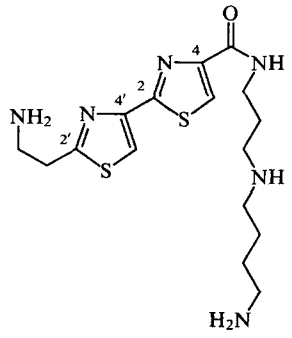
2. The role of domains in binding

a. The role of the bithiazole residue and C-terminal amine in bleomycin binding to DNA

Historically, NMR spectroscopy was the first and the most informative method to shed some light on the involvement of certain parts of the bleomycin molecule in the binding to DNA. Bleomycin interaction with calf thymus DNA was accompanied, first of all, by broadening and changes in the intensity of the signals in the ^{1}H NMR spectrum of the bithiazole residue and the dimethylsulfonium group, thus suggesting the strongest binding of these fragments of the bleomycin A_2 molecule to DNA.[38] The phenomenon of quenching bithiazole ring fluorescence detected by Horwitz et al. also argues for the interaction of the bithiazole residue with DNA. This pioneering study was followed by a vast series of NMR investigations into interactions of bleomycin A_2 and its derivatives with poly(dA)·poly(dT).[40,58,60,67-71] It was found that significant spectral changes were observed only in the binding of bleomycin to double-stranded DNA, being maximal for the signals for the aromatic protons of the bithiazole residue, which upon interaction with poly(dA)·poly(dT) broadened and shifted upfield. An upfield shift was also noted for adjacent protons: CH_3-S^+ and CH_2-S^+ of the terminal amine, CH_2-N of the 2′-substituent of the bithiazole residue, and $CH(CH_3)CO$ of methylpentanoic acid.[67]



Tetrapeptide S



C-Terminal fragment Blm-A_5

Similar results were obtained in the study of binding of bleomycin and its metal complexes to short duplex fragments of DNA.[52,72-75]

The use of the nuclear Overhauser effect (NOE technique) made it possible to detect complex formation between bleomycin containing a Zn(II) or Fe(II) ion in the metal-binding domain, and DNA.[68] In this case, bleomycin acts as a spacer between DNA and the metal ion. The general changes in the chemical shifts for the protons of bleomycin during the formation of such a complex virtually represent overall changes produced by chelation of the

metal ion and its binding to DNA.[40] The signals for the protons of the N-terminal fragment of the molecule comprising β-hydroxyhistidine and disaccharide change only upon their interaction with the metal; those in the C-terminal dipeptide, only during the binding to DNA, whereas the signals for the protons of 4-amino-3-hydroxy-2-methylpentanoic acid and threonine are equally sensitive to the both interactions.

The same research group studied the binding of C-terminal fragment of bleomycin A_2, C-terminal fragments of Blm-A_2 (acetyldipeptide, tripeptide S, tetrapeptide S) and Blm-A_5, to DNA. The nature of changes in the signals for bithiazole ring and terminal amine protons was identical for both the entire bleomycin molecule and its C-terminal fragments.[58,60,67,69] Thus, the NMR spectroscopy data suggest that the C-terminal fragment of the bleomycin molecule containing 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylic acid and the terminal amine binds to DNA to a greater degree and apparently determines the strength of binding.

This conclusion was supported by the data obtained by equilibrium methods. The parameters of binding of tripeptide S and acetyldipeptide to DNA practically coincide with those for bleomycin A_2 (Table 1).[38,47,55,58,60] An attempt was made[55] to establish a correlation between the values of binding constants for the C-terminal fragments of bleomycin A_2 and their increasing lengths, beginning with the dipeptide fragment. The latter plays a crucial role in the binding; L-threonine increases the binding constant by a factor of 2, while 4-amino-3-hydroxy-3-methylpentanoic acid and β-hydroxyhistidine have no effect on the magnitude of the binding constant.

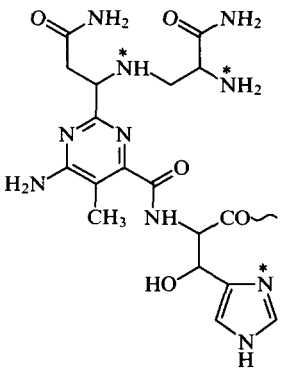
This finding is consistent with the ability of bleomycin C-terminal fragments to inhibit DNA cleavage by the complete antibiotic.[76,77] Unlike other inhibitors, C-terminal fragments diminish the cleavage proportionally to each cleavage site, i.e., the inhibition is competitive.[76] It is necessary to mention in this connection that bleomycin analogues devoid of the bithiazole residue or C-terminal amine cleave DNA far less effectively than the bleomycin itself.[78-81] At the same time, they can chelate the iron ion and effectively oxidise some organic substrates, e.g., *cis*-olefins.[78]

b. The role of electrostatic interactions in bleomycin binding to DNA

As can be seen from Table 1, the parameters of bleomycin binding to DNA depend on ionic strength, thus implicating electrostatic interactions between bleomycin and DNA. An increase in ionic strength also leads to a loss of the upper threshold of fluorescence quenching.[54] Similarly, an increase in ionic strength from 10 to 150 mmol dm^{-3} strongly attenuates the changes in the chemical shift of the aromatic protons H(5) and H(5′) of the bithiazole residue and methyl CH_3-S^+ protons of bleomycin A_2 interacting with poly(dA)·poly(dT). Under these conditions, the changes in the chemical shifts of protons CH_2-N of the substituent at position 2′, of bithiazole and CH_2-S^+ of the C-terminal amine, are completely suppressed. No changes in the ^1H NMR spectrum of bleomycin added to DNA are observed at an ionic strength of 1.0 mol dm^{-3}.[71]

It should be noted that the bleomycin molecule (devoid of the terminal amine) has several ionogenic groups at its N-terminus: a secondary NH group of β-aminoalanine within the structure of the pyrimidoblaminic acid ($pK_a = 2.7-2.9$), an N(1) atom of the imidazole ring of β-hydroxyhistidine ($pK_a = 4.7-4.9$) and a primary NH_2 group of β-aminoalanine ($pK_a = 7.3-7.7$).[27,82,83]

The structure of the N-terminal fragment of Blm is given below (the ionogenic groups within the pH range of 2–8 are marked with asterisks).



This indicates that the positive charge of the bleomycin molecule at pH 7.5 is mainly localised at the C-terminal amine which provides for electrostatic interactions with the negatively charged DNA backbone.

Evidence for this assumption can be derived from the following data.

1. Bleomycinic acid bearing a negative charge at its C-end only weakly cleaves DNA.[57,63,84]

2. The extent of fluorescence quenching upon binding to DNA increases in the following order: bleomycinic acid (negatively charged C-end), bleomycin B_1 (neutral C-end), bleomycin A_2 (positively charged C-end).[54,57]

3. Demethylbleomycin A_2 and deglycobleomycin A_1 with neutral C-terminal fragments cleave DNA much more weakly than does bleomycin A_2.[69,70,81,84] No changes in the NMR spectrum of demethylbleomycin A_2 were found upon its interaction with DNA.[69,70]

4. The copper complex of demethylbleomycin A_2 (1×10^{-4} mol dm^{-3}) does not cause any unfolding of the supercoiled plasmid DNA, whereas the copper complex of bleomycin A_2 provides its effective unfolding.[85]

At the same time, there is evidence that the positive charge at the N-end of the bleomycin molecule influences the binding of bleomycin to DNA. Acetylation of the primary amino group of β-aminoalanine gives acetylbleomycin A_2, whose N-end is neutral at pH 6.8. Virtually no changes in the NMR spectrum of this derivative occur in its interaction with poly(dA)·poly(dT).[69] The appearance of a positive charge at the N-end of the bleomycin A_2 molecule at pH<7 (6.8 and 4.5) results in a higher upfield shift of bithiazole proton signals; however, this does not affect the shift of proton signals in the N-terminal fragment of the bleomycin molecule upon its interaction with DNA.[71] Chelation of the metal ion, e.g., Cu(II) or Fe(III), which also provokes the appearance of a positive charge in the N-terminal fragment of the antibiotic molecule,[83] only weakly reflects on the value of the binding constant.[49,56,57] However, the appearance of an extra charge at the C-end of the bleomycin A_5 molecule increases the degree of fluorescence quenching and affinity of the antibiotic for DNA.[54,57]

The results presented above testify to the fact that electrostatic interactions are a necessary prerequisite for bleomycin binding to DNA. A crucial role in providing strong binding to DNA is played by the positive charge at the C-terminal amine.

c. Interaction of the N-terminal fragment of the bleomycin molecule with DNA

In late 1970s, a definite concept was formed concerning the relationship between the structure of the bleomycin molecule and its biological activity, namely, that the C-terminal fragment of the antibiotic binds to DNA, while its N-end chelates the metal (iron) ion which interacts with molecular oxygen, eventually resulting in DNA decay.[38-40] According to this concept, the interaction between the metal-binding domain and DNA was thought to be very weak. However, the ^1H NMR spectra of the bleomycin N-terminal fragment and the Zn(II)-bleomycin complex reveal small, though persistent changes upon their interaction with poly(dA)·poly(dT).[40] These changes are even more pronounced in the case of the CO – Fe(II)Blm-A_2 complex binding to DNA. In parallel with the broadening and upfield shift of signals for the protons of the bithiazole residue and the terminal amine, the signals for the aromatic protons of β-hydroxyhistidine also tend to

broaden considerably with a small shift towards the higher field. The fact that the signals for the pyrimidine methyl protons also shift upfield (by 0.08 ppm)[86] is also suggestive of interactions between the N-terminal fragment of the bleomycin molecule and the DNA backbone.

^{1}H NMR data on the binding of bleomycin to short oligonucleotide duplexes provide support for the interaction of the metal-binding domain with the double helix.[52,73-75] The most interesting results were obtained in the study in which the binding of bleomycin A_5 to the 8-meric duplex $d(CGCTAGGCG)_2$ was analysed[75] using the NOE technique. The authors succeeded in establishing intermolecular interactions between the protons of the α-CH_2-group of β-hydroxyhistidine and H(4′) of the deoxyribose C(7) residue. The formation of a hydrogen bond between the NH_2 group of β-aminoalanine and the oxygen atom of the ribose – phosphate backbone in the oligonucleotide chain was also postulated in this study.

EPR data provide additional support in favour of the metal-binding domain interaction with DNA. Thus, the interaction of the NO – Fe(II)Blm complex with calf thymus DNA was accompanied by marked spectral shifts indicating changes in the Fe(II) microenvironment.[45,87,88] Evidence for direct interaction of the metal-binding domain with DNA can be derived from changes in the EPR spectrum of 'activated bleomycin' [HOO – Fe(III)Blm] upon its binding to the double helix.[89] The binding to DNA converts the Fe(III)Blm complex from the high spin ($g = 4.3$) to the low spin state ($g = 2.45$, 2.18, 1.89). In this case, native double-stranded DNA stabilises the low spin state with a greater efficiency than the denatured one.[89]

Characteristic changes in EPR spectra are produced upon addition of DNA to the O_2 – Co(II)Blm-A_2 complex which mimics the active O_2 – Fe(II)Blm-A_2 complex.[90,91] This may be due to the alteration of the oxygen molecule orientation relative to the plane containing the cobalt ion. Recent studies[92] on this complex revealed significant restrictions in the orientation of the O – O bond, which is almost perpendicular to the main axis of the DNA helix.

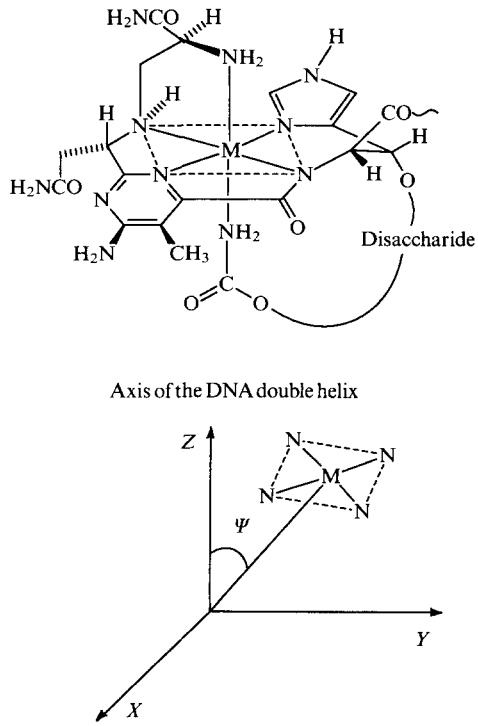


Figure 1. The structure of the site of the bleomycin molecule that chelates the copper ion (*a*)[93,94] and the hypothetical position of the plane containing a metal ion relative to the axis of the DNA helix (*b*).[96,97]

Conclusive evidence in favour of tight contacts of the metal-binding domain and DNA is the displacement of ligand L from the coordination sphere of the L – Fe(III)Blm complex (where L is the ligand of the N_3^-, $S_2O_3^{2-}$ or SCN^- type).[51]

The influence of the positive charge of the bleomycin N-terminal fragment on its binding to DNA can also be regarded as evidence of interaction of the metal-binding domain with DNA. In this context, mention should be made of the study[85] in which chelation of the Cu(II) ion by bleomycin A_2 produced qualitative changes in the binding of the antibiotic to supercoiled plasmid DNA. Whereas metal-free bleomycin A_2 is practically incapable of unfolding such DNA under conditions excluding DNA cleavage, its copper-containing counterpart effectively eliminates the coils in the DNA superhelix. It should be noted also that Cu(II) and Fe(III) complexes of bleomycin bind to DNA at different rates which also points to the involvement of the bleomycin N-terminal fragment in the binding to the polymer.[48]

The application of the EPR method to oriented DNA fibres provides valuable information on the position of the metal-binding domain relative to the axis of the DNA double helix. As is known, the nitrogen atoms in the N-terminal moiety of the bleomycin molecule which chelate copper or iron ions, *viz.*, the nitrogen atom of the secondary amino group of β-aminoalanine, the N(1) atom of pyrimidine, the N(1) atom of imidazole, and the nitrogen atom of β-hydroxyhistidine forming a peptide bond,[39,93,94] lie in one plane with the metal ion (Fig. 1). For the bleomycin molecule bound to DNA, the angle Ψ between the normal (perpendicular) to this plane and the axis of the DNA helix has been found to be 15° for the Cu(II)Blm complex, with rotational freedom of the normal around one of the axes lying in the plane of base pairs of ±80°.[95] However, recent studies using the same model gave different results, namely, the angle between the normal and the axis of the DNA helix is 25° with deviations in the range of ±30°.[96] This is consistent with the earlier data for the Fe(III)Blm complex bound to oriented DNA fibres (the Ψ value for this model is 15 – 30° with variations of ±26°).[97]

Thus, the great body of experimental evidence testifies to tight contacts of the bleomycin N-terminal fragment with DNA. These contacts are manifested also in the influence of the metal-binding domain on the specificity of DNA cleavage (see below).

3. Models of bleomycin binding to DNA

The data obtained by physicochemical methods suggest that the interaction of bleomycin with DNA involves both the metal-binding domain and the C-terminal fragment of the antibiotic molecule. It is the latter, which contains the bithiazole residue and the terminal amine and is especially tightly bound to DNA, that is responsible for the binding to the polymer. We have already noted that electrostatic interactions between the positively charged terminal amine and the negatively charged sugar – phosphate backbone contribute to the binding of bleomycin to DNA. However, there exist other interactions that can also play a role in the binding of bithiazole rings to DNA. The three hypotheses postulating the nature of this binding are as follows: the intercalation model, the partial intercalation model and binding in the minor groove of DNA.

Both heterocyclic rings of the bleomycin bithiazole fragment lie in the same plane[98] and can theoretically intercalate between the base pairs of DNA. First experimental evidence for intercalation binding of bleomycin was obtained in 1979.[47] It was found that bleomycin A_2 and its C-terminal fragment, tripeptide S, cause the unfolding of covalently closed circular double-stranded DNA and increase the length of short rod-like DNA molecules as determined by their orientation time in an electric field. Moreover, they favour parallel arrangement of the bithiazole rings and base pairs as evidenced by linear dichroism data.[47] Changes of this kind usually occur in intercalation binding.[99] Metal complexes of bleomycin reveal a similar capacity upon their binding to DNA.

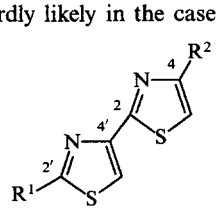
It has already been mentioned that the copper-containing complex of bleomycin A_2 can produce the relaxation of supercoiled DNA.[85] According to Povirk et al.,[48] the length of short-chain DNA (150 base pairs) increases by 0.46 nm upon binding to the Cu(II)Blm molecule. For the Fe(III)Blm complex, this value is equal to 0.32 nm, and for Blm, 0.31 nm;[47] such elongation is characteristic of typical intercalators.[99,100] At the same time, the copper-containing complex of the bleomycin analogue phleomycin, whose thiazole ring is partially reduced,[101] does not produce any elongation of DNA upon binding.[48] It follows, therefore, that DNA elongation can only be achieved in the case of a planar chromophore.

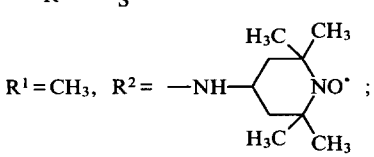
In 1981, Lin and Grollman [102] proposed, on the basis of NMR data, a model for complete intercalation binding of the bleomycin C-terminal fragment to dinucleotides. In this study, the greatest changes in the chemical shift (0.4 ppm) were observed for aromatic protons of the bithiazole residue upon binding to pdG-dC. According to this model, the bithiazole residue is 'stacked' to base pairs in such a way that its aromatic protons appear to be oriented towards the minor groove of DNA. Theoretical calculations confirmed the indisputable energetic superiority of the proposed intercalation model for binding of the terminal positively charged amine in the minor groove of DNA.[103]

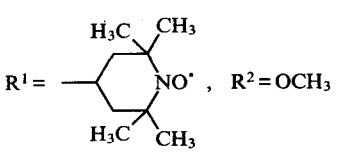
In a recent work by Wu et al.,[74] two-dimensional NMR was used to study the interaction of the cobalt-containing bleomycin A_2 complex with the 10-meric DNA-duplex d(CCAGGCCTGG)$_2$. It was found that the bithiazole residue intercalate between GC pairs corresponding to positions C(6) and C(7). In this case, the upfield shifts of the signals for bithiazole protons were 0.9 and 0.61 ppm for H(5) and H(5′), respectively.

However, the model of complete intercalation binding does not agree with numerous experimental data. Thus, the angle of unfolding of supercoiled DNA upon its binding to the bleomycin molecule is 12°,[47] whereas for the majority of intercalators it constitutes 17 – 26°.[100] Bleomycin interaction with sonicated DNA did not increase significantly the solution viscosity and, correspondingly, the length of the DNA chain,[38] which is in conflict with the results reported by Povirk et al.[47] Moreover, viscosimetric data for the binding of the bleomycin C-terminal fragment to calf thymus DNA suggest that the length of the DNA molecules decreases slightly.[104] Laser scattering data point to the length reduction of short rod-like DNAs upon their interaction with the antibiotic.[105]

Incorporation of spin labels into bleomycin molecules is a convenient tool for determining the parameters of their binding to DNA.[106,107] The EPR spectrum of such a label (2,2,6,6-tetramethyl-1-piperidine-*N*-oxide) incorporated at position 4 or 2′ of bithiazole, practically did not change upon its interaction with DNA which is hardly likely in the case of complete intercalation binding.[104]





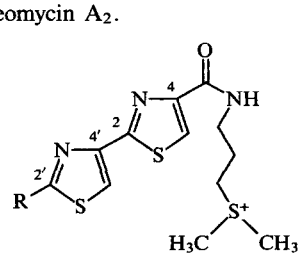


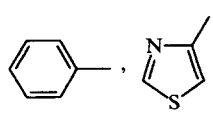
Changes in the ^{1}H NMR spectrum of bleomycin upon its binding to poly(dA)·poly(dT) are also incompatible with the complete intercalation model. Although the most pronounced changes are observed for the signals of bithiazole ring protons (their maximum upfield shift is 0.1 – 0.2 ppm),[38,40,67,69–71] for typical intercalators this varies from 0.4 to 1.0 ppm.[108,109] It should be noted also that the shift strongly depends on temperature, ranging from 0.02 to 0.05 ppm at 20 °C up to 0.1 – 0.2 ppm at temperatures just below the melting temperature for poly(dA)·poly(dT) (50 – 60 °C)[67,69,71] and thus cannot be explained within the framework of the complete intercalation model.

There are a number of publications dedicated to the analysis of interactions of bleomycin and its metal complexes with oligonucleotide duplexes using the NMR method.[52,72–75] Most of the data obtained are also contrary to the complete intercalation model. Hiroaki et al.[52] studied the binding of bleomycin to the duplex d(CCCCAGCTGGGG)$_2$ possessing a single site for the antibiotic-induced cleavage (underlined). The signals from non-exchangeable protons of the bithiazole residue and the bleomycin terminal amine displayed a characteristic shift towards higher field (0.15 to 0.2 ppm), however, without any changes in the chemical shifts of signals for imino protons and phosphorus nuclei of the oligonucleotide in the vicinity to the binding site. On the other hand, it is known that intercalating molecules produce significant changes in the chemical shifts of signals for these nuclei.[110,111] A somewhat greater upfield shift of the signals for bithiazole protons (0.2 – 0.4 ppm) was observed upon the interaction of the antibiotic with the duplex d(CGCGCG)$_2$.[72,73] In this case the temperature dependence of the chemical shifts characteristic for poly(dA)·poly(dT) was not observed, apparently, due to the stronger binding of bleomycin to GC sequences. The use of the NOE technique makes it possible to establish the intercalation type of binding.[112] The presence of cross-peaks along the whole length of the 8-meric duplex d(CGCTAGCG)$_2$ chain upon its interaction with bleomycin A_5 is suggestive of the non-intercalating type of antibiotic binding.[75]

In order to rationalise the experimental data in favour of intercalation and against it, an attempt was made to interpret them in terms of the hypothesis of partial intercalation of bithiazole rings proposed as early as in 1976.[113] According to this hypothesis, one of the bithiazole rings is inserted between the DNA bases, thereby producing a local bending in the helix. Its effect on the length and, correspondingly, hydrodynamic properties of DNA may be very different [114] as evidenced by viscosimetric [38,104] and laser scattering data.[105] This model provides an explanation for relatively small changes in the chemical shift of bithiazole proton signals as well as for temperature dependence of the chemical shift magnitude. A rise in temperature up to values just below the melting temperature for DNA chains causes local denaturation of the helix, thereby facilitating the incorporation of the bithiazole residue between the base pairs and enhancing the upfield shift of the signals for the protons.[71] A decrease in ionic strength, which causes destabili-sation of the double helix, would also favour partial intercalation of the bithiazole residue and, as a consequence, more intensive fluorescence quenching and changes in the chemical shift.[54,71]

Evidence for the improbability of complete intercalation of bithiazole rings into the DNA double helix can be derived from experiments with analogues of the C-terminal fragment of bleomycin A_2.



R = $CH_3CONHCH_2CH_2$, H, CH_3, H_2N, 

According to ^{1}H NMR data, the removal of the aliphatic substituent from position 2′ of the 2,4′-bithiazole derivative enhances the ability of bithiazole rings to intercalate and increases the binding constants for poly(dA)·poly(dT).[58,69] At the same time, the substitution of the aliphatic chain at position 2′ by an aromatic ring (thiazole or benzene) strongly changes the characteristics of bithiazole analogue binding to DNA, thus permitting their intercalation. Changes in the chemical shifts (by 0.5–0.8 ppm) produced by such analogues upon their binding to DNA are far more pronounced than those for bleomycin.[58,69,115,116] In contrast to bleomycin, its analogues increase the viscosity of DNA solutions [116] and cause effective unfolding and folding (positive supercoiling) of covalently bound circular DNA.[77] Moreover, they display a far greater affinity for DNA and are more potent competitive inhibitors of DNA cleavage.[77] Thus, despite its inability to undergo complete intercalation binding to DNA, the bithiazole residue can partly incorporate between the base pairs, the most accessible loci for such binding being the sites of local injuries to the double-helical structure (transition regions between denatured and native states of the DNA helix, bends and breaks on the helix, protrusions formed by an extra base, etc.).

Yet another model for bleomycin binding to DNA also provides a satisfactory explanation for the available experimental data. According to this model, the terminal amine and bithiazole rings of bleomycin bind in the minor groove of DNA. This model, which was first proposed in 1986,[117] is based on the structural similarity of the bleomycin C-terminal fragment and netropsin and the fluorescent dye Hoechst 33258, both of which bind in the minor groove of DNA. In favour of this model are experiments in which changes in site-specificity and efficiency of DNA cleavage by bleomycin were studied in the presence of ligands tightly bound to DNA. Thus, distamycin A, which binds to the sites enriched with AT sequences in the minor groove of DNA, can inhibit DNA cleavage at sequences GT and GA by increasing the cleavage selectivity at GC sequences.[118–120] By contrast, actinomycin D binding to GC sequences and blocking the minor groove of DNA by its peptide rings around the binding sites (by 5–6 base pairs)[121] produces an opposite effect: cleavage at GT and GA sequences increases, while that at sequences GC decreases. Covalent attachment of aflatoxin B to the N(7) atom of guanine or of mitomycin C to the O(6) atom of guanine in the major groove of DNA has practically no influence on the specificity or efficiency of DNA cleavage by bleomycin.[122] The presence of bulky glycosyloxymethyl residues in the major groove of DNA of the wild-type phage T4 also has insignificant effect on the specificity and efficiency of cleavage.[123] In addition, bleomycin displays a similar affinity for both glycosylated and nonglycosylated DNA of phage T4.[124] A crucial role in the antibiotic affinity for DNA is played by the nucleotide composition of DNA, particularly by the presence of guanine and the 5′-guanine–pyrimidine-3′ sequence. Thus, G- and GC-enriched DNA sequences quench bithiazole ring fluorescence far more effectively,[54,57] whereas poly- and oligonucleotides carrying sequences GC and GT appear to be the most effective inhibitors of the SV40 DNA cleavage by the antibiotic.[63,64] The value of the constant for bleomycin binding to poly(dG)·poly(dC) exceeds those for binding to poly(dI)·poly(dC) and poly(dA)·poly(dT) (3- and 5-fold, respectively).[124] It should be noted that the minor groove of the double helix of poly(dG)·poly(dC) contains a 2-amino group of guanine which is absent in inosine or adenine. The crucial role of this amino group in strong binding of bleomycin was confirmed by experiments with anthramycin-modified DNA. This antibiotic, being localised in the minor groove, can form a covalent bond with the 2-amino group of guanine without any appreciable effect on the B-form of DNA.[125] Treatment of DNA with anthramycin results in strong inhibition of DNA cleavage by bleomycin at sequences GC and GT.[124] On the basis of these data, Kuwahara and Suguira [124] proposed a model for bleomycin binding to the 6-meric duplex d(ATGCCA)·d(TGGCAT) possessing a site for effective cleavage (underlined). The terminal amine and the bithiazole residue are arranged along the DNA minor groove in the direction from the 5′- to the 3′-end relative to the chain to be cleaved. The binding occurs owing to electrostatic interactions and hydrogen bond formation between the 2-amino group of guanine and the nitrogen of the thiazole ring and/or oxygen of the carboxamide group. The bleomycin binding site comprises two to three base pairs which is in keeping with the data on DNA protection from cleavage by DNAse I.[126]

Two-dimensional NMR data also testify to the possibility of bleomycin binding in the minor groove of the double helix. Of particular interest in this respect are the results [75] which in many features provide support for the earlier hypothesis.[126] In this study, the NOE technique was used to establish the interactions between the protons of the zinc-containing bleomycin A_5 complex and the protons of the 8-meric duplex $d(CGCTAGCG)_2$ localised in the minor groove of the double helix. On the basis of the data obtained by molecular dynamics methods, a hypothetical spatial model of the complex was calculated. According to the calculations, the bleomycin A_5 molecule is localised in the minor groove, overlapping 4–5 base pairs. The complex is stabilised by hydrogen bonds between the amino groups of the C-terminal fragment of spermidine and the oxygen atoms of the phosphate group and the deoxyribose cycle, between the nitrogen atom of the thiazole ring and the 2-amino group of guanine as well as between the primary amino group of β-aminoalanine and the oxygen atom of the sugar–phosphate backbone. The authors of this model state, however, that this structural model is not unique: a considerable upfield shift of the signals for bithiazole protons by 0.18 ppm [H(5)] and 0.52 ppm [H(5′)] and for the imino protons of GC- and AT-pairs corresponding to positions G(2) and T(4) suggests partial intercalation of the bithiazole rings.[75]

Thus, there is no experimental evidence thus far allowing an unequivocal identification of the binding mode (complete or partial intercalation or binding in the minor groove) characteristic of bleomycin. It is quite probable that these binding modes are characteristic of different structures in the DNA double helix. Nevertheless, it is certain that the C-terminal fragment of the bleomycin molecule provides the binding of the antibiotic to DNA by 'anchoring' the rest of the molecule which interacts with the polymer upon oxidative cleavage of the sugar–phosphate backbone. Whatever the mode of C-terminal fragment binding, the mechanism of DNA oxidation (see below) implies the closest approximation of the bleomycin metal-binding domain to the minor groove of the DNA double helix.

III. Cleavage of nucleic acids by bleomycin

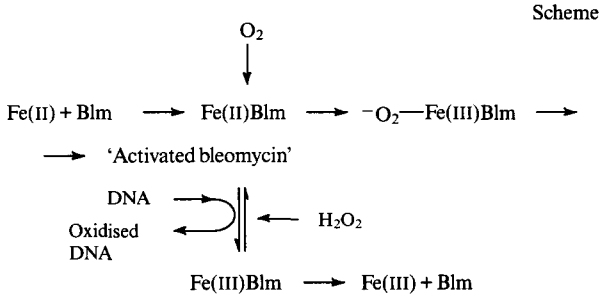
1. The mechanism of DNA cleavage

Degradation of DNA by treatment with bleomycin eventually resulting in the cleavage of the sugar–phosphate backbone was discovered as early as 1969;[30–33] however, the mechanism of action underlying this process has long remained obscure. The first theoretical speculations on the putative mechanism of DNA cleavage were made in 1973, and bleomycin was considered to be a hydrolytic enzyme.[113,127] Shortly thereafter, cofactors essential for the antibiotic activity were identified. In 1975, it was found that molecular oxygen favours the DNA degradation by bleomycin.[128] In the same period it was shown that Fe(II) and Fe(III) ions stimulate the DNA cleavage by bleomycin in the presence of 2-mercaptoethanol;[129,130] these two cofactors favouring DNA degradation were distinguished as being the most essential ones.[35–37] According to nuclear absorption data,[130] the previously observed degradation of DNA upon treatment with bleomycin was due to the constant presence of trace amounts of iron ions in the reaction mixture. The discovery that iron atoms and molecular oxygen are essential for DNA degradation was immediately followed by a hypothesis suggesting a free radical mechanism for this process.[37,56,130–132] Indeed, the Fe(II)Blm complex can generate ·OH radicals [133,134] that can interact with DNA. The enzyme superoxide dismutase converting the O_2^{2-} ion into O_2 and H_2O_2 inhibits DNA cleavage under the action of

bleomycin.[37,135,136] A correlation exists between the biological activity of bleomycin analogues and the amount of 'OH radicals generated by them.[132] However, it turned out soon afterwards that *tert*-butylphenylnitrone and dimethylsulfoxide, which act as 'traps' for 'OH radicals, do not inhibit the cleavage of DNA. Moreover, the efficiency of 'OH radical production by the Fe(II)Blm complex is 40 times lower than in the Fe(II) – H_2O_2 system.[137] Another group of investigators [136] succeeded in demonstrating that inhibition of DNA cleavage by superoxide dismutase is not related to the reduced amount of O_2^{2-} ions but is rather due to the association of the enzyme with DNA.

An alternative explanation for the bleomycin action mechanism is the formation of the so-called 'activated bleomycin' which causes direct oxidative degradation of DNA.[89,138,139] Electron spectroscopy and EPR data as well as the use of the Mössbauer effect made it possible to propose the following scheme for DNA cleavage by bleomycin with participation of the iron ion and molecular oxygen (Scheme 1).[89]

Scheme 1



The Fe(II)Blm complex formed in the first stage rapidly interacts with molecular oxygen. In this complex the bivalent iron atom undergoes oxidation, giving rise to the $^{-}O_2$ – Fe(III)Blm complex. The formation of 'activated bleomycin' requires an extra electron which is provided either by the reducing agent present in the solution or by an additional Fe(II)Blm molecule undergoing disproportionation.[139]

$$O_2^{-} - \text{Fe(III)Blm} + \text{Fe(II)Blm} \longrightarrow O_2^{2-} - \text{Fe(III)Blm} + \text{Fe(III)Blm}$$

'Activated bleomycin' can oxidise organic substrates and DNA to form a Fe(III)Blm complex which can be reactivated by peroxy compounds.[89,138–142]

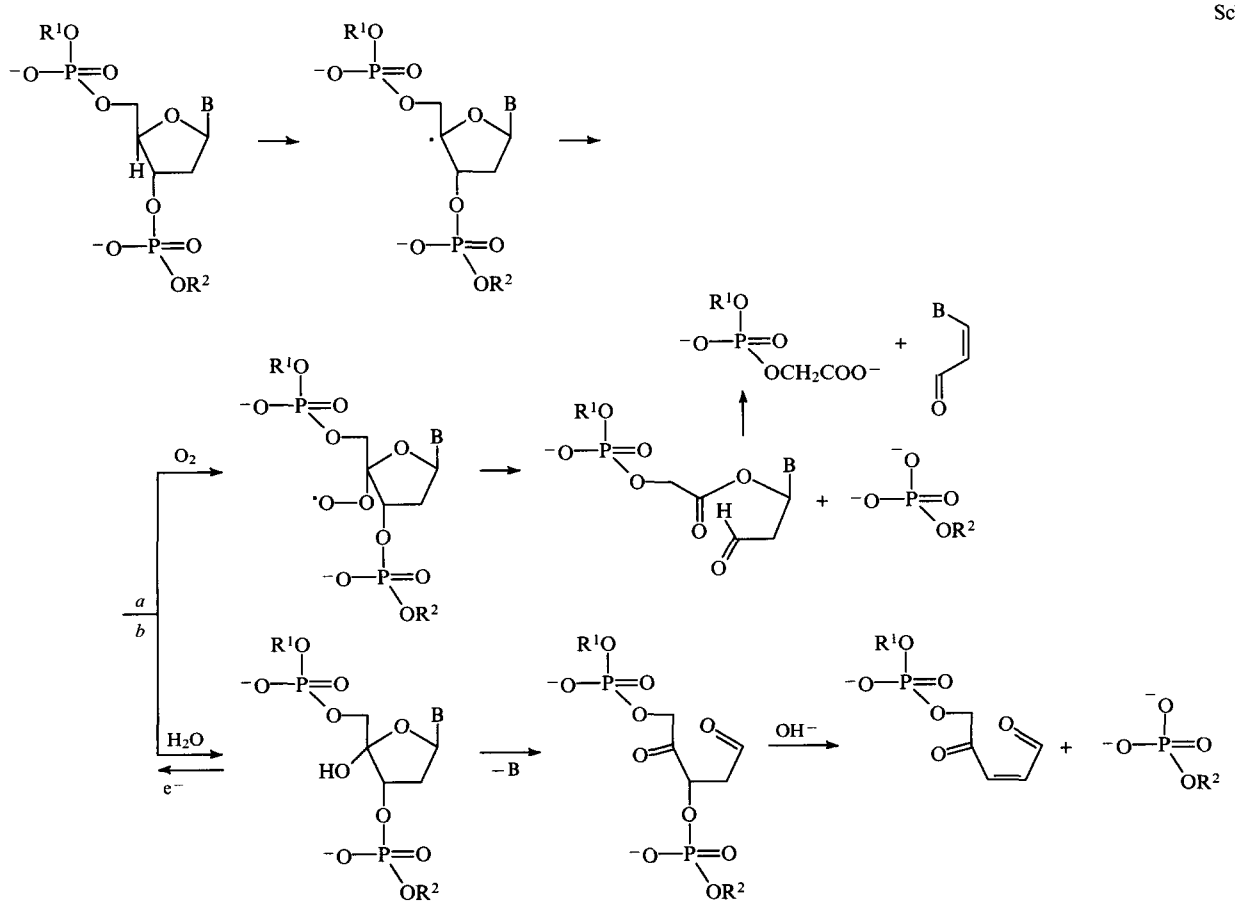
According to recent mass-spectrometry data, the structure of 'activated bleomycin' corresponds to the peroxy complex of the ferric ion, HOO – Fe(III)Blm.[143] This scheme, which depicts the bleomycin activity in the presence of iron ions and molecular oxygen, was confirmed experimentally and developed further.

Intensive studies of the mechanism of action of bleomycin led by late 1980s to the establishment of all the main stages of bleomycin activation and pathways of oxidative degradation of DNA. These were documented in detail in reviews and publications.[41,66,144,145] Therefore, in this review we give only a brief description of the main pathways of DNA oxidation by bleomycin.

Activated bleomycin induces oxidation at position 4′ of a carbon atom of the deoxyribose ring [146–148] which is accompanied by the splitting of the H(4′) atom and the formation of a 4′-radical (Scheme 2). The oxidation is the limiting step in the chain of subsequent conversions as unequivocally follows from the isotope effect produced by substitution of the hydrogen atom for deuterium or tritium (the rate of the end product formation is 2 – 7 and 7 – 12 times less, respectively).[148–150]

Further conversions of the 4′-radical proceed in two different ways (*a* and *b* in Scheme 2).[66] Under aerobic conditions (pathway *a*), an addition of molecular oxygen may occur, resulting in the formation of a 4′-peroxy radical.[146] The subsequent scission of the C(3′) – C(4′) bond results in the elimination of the base-substituted propenal and cleavage of the sugar – phosphate backbone of DNA, 5′-phosphate and 3′-phosphoglycollate being formed at the site of the chain scission.[146,151,152]

Scheme 2



Pathway *b* is associated with hydroxylation at position 4',[147] apparently as a result of oxidation of the 4'-radical to a carbocation and subsequent attachment of water.[153] Under physiological conditions, the hydroxylation results in the elimination of the free base without any scission of the DNA chain.[119,147,153-157] Under alkaline conditions β-elimination occurs, which results in the cleavage of the sugar–phosphate backbone and the formation of 5'-phosphate at the cleavage site. The oxidised deoxyribose residue is further converted into 2,4-dihydroxycyclopentenone which remains covalently bound to the 3'-phosphate group. If alkaline treatment is carried out in the presence of hydrazine, 3'-pyridazine is formed; treatment with alkylamines yields 3'-phosphate.[156]

Under normal conditions (e.g., at atmospheric pressure of O_2) DNA oxidation by bleomycin results in the formation of approximately equal amounts of free bases and their propenal derivatives,[158] i.e., both pathways of the 4'-radical conversion are possible. Under anaerobic conditions only free bases are formed.[148,158] Under O_2 pressure of 3 atm, the ratio of base-propenal derivatives to free bases is 7;[148] at 4 atm this is equal to 10.[159]

The high selectivity of bleomycin action with respect to oxidation of deoxyribose is worth noting. As it has been mentioned above, bleomycin can only slowly generate 'OH radicals.[137] Chromatomass-spectrometry data [160] demonstrate that treatment of DNA with the Fe(II)Blm complex in the presence of molecular oxygen and a reducing agent is practically unaccompanied by the formation of side products of DNA oxidation (as distinct from the products of the 4'-radical conversion). In particular, the major side product, 8-hydroxyguanine, constitutes only 2%–3% of the total amount of the bases and their propenal derivatives formed in the process.[160,161]

2. Specificity and efficiency of DNA cleavage by bleomycin

a. Effect of the primary sequence

Elaboration by Maxam and Gilbert in 1977 [162] of a convenient method for identifying the sequences of long-chain DNAs gave a strong impetus to a series of investigations of the sequence specificity of DNA cleavage by bleomycin and related compounds. It turned out that bleomycin displays marked specificity towards dinucleotide sequences carrying guanine at the 5'-end;[163,164] in this case deoxyribose of the second nucleotide undergoes oxidative degradation. The most preferred sequences for the cleavage are GT and GC; sequences GA, AT and GG are less susceptible to degradation. This finding correlates with the amount of liberated free bases: $T > C \gg A > G$.[37,164,165] Soon it was noted, however, that identical dinucleotide sequences are cleaved to a different degree. It was hypothesised that the specificity of DNA cleavage by bleomycin is determined by DNA sequences larger than dinucleotides.[166,167] Mirabelli et al.[168] found that pyrimidine present at the 5'-end of the GC sequence enhances the cleavage.[168] Statistical analysis of the sequence specificity of the bleomycin-induced cleavage confirmed that the base preceding the purine–pyrimidine sequence has a certain influence on the extent of cleavage.[169] Thymine enhances, whereas adenine diminishes, the cleavage in the sequences GT, GC, AT, and AC.

b. Effect of the double helix structure on bleomycin-induced cleavage

Despite the conclusions of Mirabelli et al.[168] (which further received experimental support) one cannot explain the difference in the DNA cleavage at different sites by the effect of the primary sequence alone. Lloyd et al.[170] demonstrated that bleomycin induces single- and double-strand scissions at a limited number of sites in bacteriophage PM2 DNA. Some of these sites, if present in DNA in a supercoiled form, were cleaved to a greater extent.[171,172] The specificity of cleavage of the linear pBR 322 DNA differed from that of the supercoiled DNA, the extent of cleavage being greater for the latter.[173] It is known that the supercoiled DNA of plasmid *Col E1* is cleaved 1.5 times more effectively than the relaxed form.[47] It was noted also that SV40 DNA contained regions which, by virtue of their secondary structure, were preferentially cleaved by bleomycin.[174] Interestingly, the intracellular DNA is cleaved by the antibiotic at the same preferential cleavage sites as the purified DNA.

The influence of structure on the cleavage efficiency manifests itself in the interaction of bleomycin with the modified DNA. Thus, covalent attachment of mitomycin C [122] or *cis*-diaminodichloroplatinum(II) [175] in the major groove of DNA enhances the bleomycin-induced cleavage. *cis*-Diaminochloroplatinum(II) also influences the specificity of cleavage,[175,176] apparently due to alterations in the structure of the DNA double helix resulting from intrastrand cross-links. Of special interest are recent findings testifying to the enhancement of the bleomycin effect:[120] distamycin A strongly (about 100-fold) enhances the bleomycin-induced cleavage of DNA at certain GC-rich sequences. Other compounds bound in the minor groove, such as Hoechst 33258, berenyl, 4',6-diamidino-2-phenylindol, only slightly increase the efficiency of DNA cleavage by the antibiotic. Presumably, the significant enhancement of DNA cleavage is associated with structural changes in the double helix upon binding of distamycin A.[120]

The presence of a protruding base has a strong effect on the specificity of the bleomycin-induced cleavage by directing its action to nucleotides located in close proximity to the given base.[177] It is quite probable that this effect is due to the ability of bithiazole rings to intercalate into sites with a distorted secondary structure.[71]

Convincing evidence for the sensitivity of the antibiotic to even minor changes in the target DNA structure was obtained in the studies by Murray et al.[178] when the cleavage of two virtually identical sequences in the human genome was compared. Substitution of 11 bases in a 340-meric double-stranded fragment produced significant changes in the mode of cleavage. Statistical analysis revealed that some of these changes may be attributed to the direct effect of the substituting base, whereas the changes in the cleavage at other sites located at a distance of 2–12 nucleotides from the substitution site result from structural changes in the substituted DNA.

The effect of the double helix structure on the bleomycin-induced cleavage of DNA is also manifested in DNA methylation. Enzymatic methylation of cytosine residues at position 5 diminishes the extent of cleavage in the vicinity of such sites. The protective effect is observed even at a distance of 14 nucleotides from the methylation site for DNA duplexes having the lengths from 100 to 400 base pairs.[179] This may be associated with local changes in the DNA structure, since it is known that methylation of cytidine facilitates the B–Z transition, the major conformational change in DNA.[180] Further studies with poly(dG)·poly(dC) and poly(dG)·poly(dC^{Me}) revealed that the B–Z transition induced by increasing the salt concentration strongly inhibited the cleavage of these polynucleotides by bleomycin. This finding indicates that the Z-form of DNA is conformationally unprofitable for the manifestation of the antibiotic activity. Additional evidence can be derived from circular dichroism data. In the presence of bleomycin, the B–Z transition needs a far greater ionic strength for its realisation which indicates that bleomycin predominantly binds to and stabilises the B-form of DNA.[123]

Methylation of cytidine has a different inhibiting action on DNA cleavage by various bleomycins. Demethylbleomycin A_2 and bleomycin B_2 are less sensitive to the presence of 5-methylcytidine than bleomycin A_2 and bleomycin BAPP. At the same time, methylation of adenosine at the N(6) atom diminishes the DNA cleavage by the above-mentioned bleomycins.[123] In another study, a self-complementary dodecanucleotide methylated at one of its cytidine residues was used as a target. Long et al.[181] showed that the efficiency of oxidation of methylated and nonmethylated oligonucleotides appeared to be the same, but the ratio of liberated free bases and their propenal derivatives was different. In case of the methylated dodecanucleotide, the amount of

liberated free bases was 1.5 times as great as that with the nonmethylated one; hence, the 4′-radical tended to be more susceptible to hydroxylation than to the interaction with molecular oxygen.

The effect of the polynucleotide secondary structure on the efficiency and specificity of the bleomycin-induced cleavage is especially pronounced in the case of cleavage of RNA.

3. Cleavage of RNA by bleomycin

Early studies dating back to the mid 1970s repeatedly demonstrated that RNA was resistant to the action of bleomycin.[34,182,183] In RNA–DNA hybrids, the cleavage affected only the DNA chain.[159,184,185] Cleavage of DNA by bleomycin was not inhibited even by a great excess of RNA.[62–65,186]

This generally accepted viewpoint was dispelled in 1989 when it was demonstrated that approximately three molecules of Fe(III)Blm bind to one molecule of yeast phenylalanine tRNA with an equilibrium binding constant of 0.86×10^5 dm^3 mol^{-1}. The cleavage occurred at three separate sites with low efficiency [5%–10% for 0.3 mmol dm^{-3} Fe(II)Blm and 3 mmol dm^{-3} tRNA as calculated per nucleotide] and resulted in the formation of free adenine and uracyl.[61] This pioneering study was followed by a number of publications[65,187–195] dealing with various aspects of bleomycin interaction with RNA, particularly with a comparison of RNA and DNA cleavage patterns. In both cases the cleavage occurs due to oxidation of the sugar–phosphate backbone [191] with iron ions and molecular oxygen (or peroxy compounds) as necessary cofactors.[191,194] The presence of reducing agents, such as ascorbate or 2-mercaptoethanol favours the RNA cleavage. A chromatographic analysis of oligonucleotide degradation products and data on electrophoretic mobility in polyacrylamide gel of degradation products of long-chain RNA testify to the formation, at the cleavage site, of 3′-phosphoglycollate and 5′-phosphate.[191,194] However, in contrast with DNA, the cleavage of RNA results in the liberation of free bases alone.[61,65,191,194] In all probability, the detachment of the H(4′) atom of ribose triggers a chain of events resulting in the cleavage of the RNA chain.[190,192,194]

The specificity and efficiency of RNA cleavage by bleomycin differ essentially from those for DNA. Firstly, RNA cleavage is more selective at a lesser number of sites. Secondly, the efficiency of the bleomycin-induced cleavage varies considerably for different RNA molecules. Some RNAs are cleaved even by micromolar concentrations of bleomycin, whereas other RNAs are resistant to even very high (0.3 mmol dm^{-3}) concentrations of the antibiotic.[61,194]

Bleomycin splits both single- and double-stranded fragments in RNA. However, the cleavage is especially efficient in those parts of the RNA molecule where the single-stranded structure transforms into the double-stranded one and vice versa. Interestingly, the sequence specificity typical of DNA cleavage is not observed in this case. The available data suggest that the bleomycin molecule predominantly cleaves GU sites in RNA;[65,188–190,191,193,194] however, by no means all these sites undergo cleavage, while the intensity of cleavage for each GU site may vary considerably. It may thus be concluded that the specificity of RNA cleavage by bleomycin depends not so much on the primary sequence but is rather determined by peculiarities of the secondary or tertiary structure of the polymer molecule. The resistance of some RNA molecules to the effect of bleomycin and the lability of other molecules indicate a crucial role of certain spatial structures in the realization of effective cleavage.

The sensitivity of bleomycin to the structure of the substrate to be cleaved is likely to be manifested in its interaction with RNA. As has been mentioned, bleomycin preferentially cleaves the B-form of DNA. It is known also that the A-form of the double helix exists in RNA. The difference in sizes between the major and minor grooves and the conformation of the carbohydrate residue may be the reason for 'non-recognition' and, as a consequence, of 'non-cleavage' of RNA. Nevertheless, at definite sites, e.g., at the sites of the helix transition from the double-stranded to the single-stranded structure and vice versa, structures may be formed that represent suitable targets for the cleavage by bleomycin. These structures may resemble the B-form of DNA. An important role in this process is played by the size of the minor groove and the position of the 4′ hydrogen atom in it. It may also be assumed that such sites favour stronger binding of the antibiotic, presumably due to the possibility of intercalation of the bithiazole residue.[71,177] The low incidence of such sites accounts for the low number of RNA cleavage sites, which results in higher selectivity in RNA cleavage, than for DNA.[194]

A spectacular proof of the significance of the spatial structure in effective cleavage of the double helix is given in the study by Holmes and Hecht.[193] These authors showed that DNA, which represented a copy of histidine tRNA of *Bacillus subtilis*, had one major cleavage site that coincided with the single cleavage site for this tRNA.

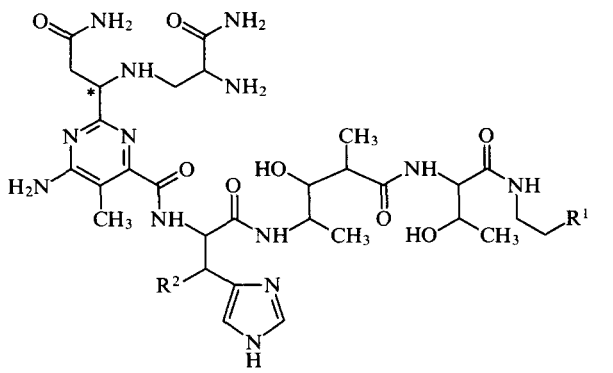
4. The role of domains in determining the specificity of bleomycin action in DNA cleavage

Immediately after the discovery of sequence-specific DNA cleavage by bleomycin, a question arose as to the nature of this specificity. By then, it had already been established that the C-terminal fragment of the antibiotic molecule is responsible for the binding to DNA.[38,39,47] The fact that various bleomycins cleave DNA in a similar way [196,197] suggests that the terminal amine does not contribute at all to the specificity of bleomycin action. For this reason, the preferential cleavage of certain DNA sequences was attributed to the specificity of bithiazole residue binding, particularly during intercalation.[164] However, an increasing body of experimental evidence argues against this hypothesis.

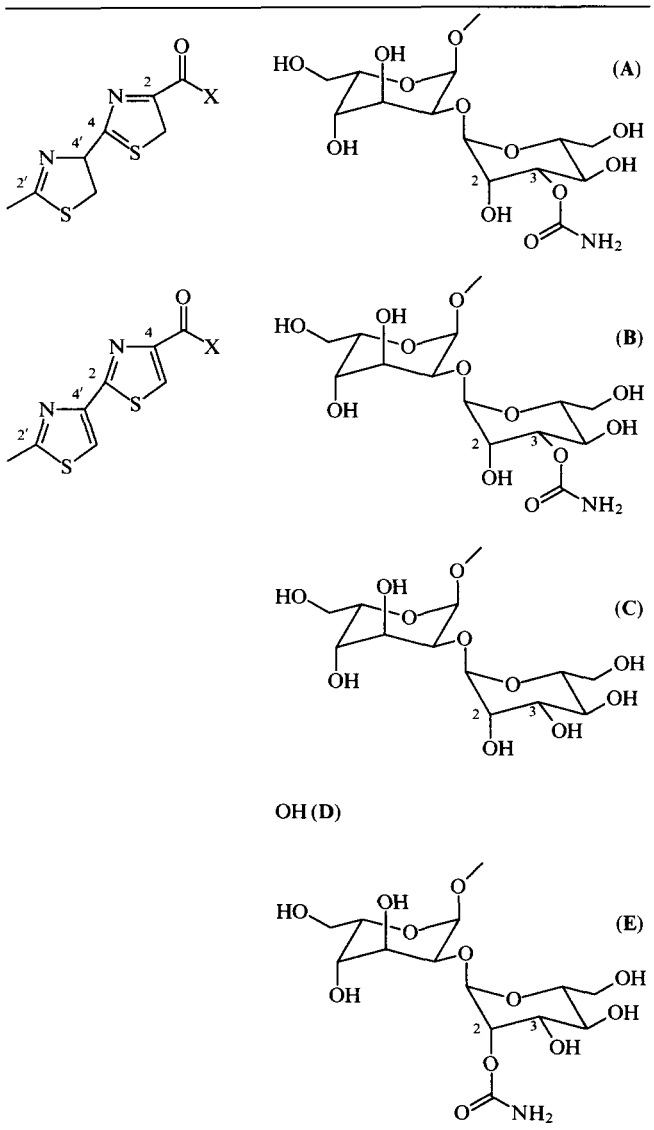
Thus, phleomycin (**B**) having one reduced bond in the bithiazole residue [101] and, correspondingly, incapable of intercalation, displays the same site specificity.[48,167,197] Bleomycin with a phototransformed bithiazole residue (the 4,4′ bond between the thiazole rings instead of the 2,4′ bond) (**A**) has a site specificity that is practically identical with that of natural bleomycin.[198,199]

On the other hand, according to the most recent data, a certain role in sequence specificity of DNA cleavage is played by the metal-binding domain. Thus, cleavage of the self-complementary dodecanucleotide CGCTTTAAAGCG by bleomycin occurs at GC sequences (underlined), the C(3) residue being cleaved to the greatest extent.[119] Removal of the disaccharide residue (deglycobleomycin, **D**) or the carbamoyl group of D-mannose (decarbamoylbleomycin, **C**) from the bleomycin molecule does not influence the sequence specificity of cleavage of this oligonucleotide. However, the predominant site for such cleavage is residue C(11).[200,201] According to the data reported by Oppenheimer et al.,[202] the carbamoyl group of D-mannose may become involved in the chelation of the iron ion in the metal-binding domain of bleomycin. Its removal may affect the coordination sphere of the metal as follows from NMR data.[203] Hence, the change of the site of preferential cleavage of the dodecanucleotide seems to be due to the alterations in the metal-binding domains of deglyco- and decarbamoylbleomycin.

Experimental proof for the dependence of the geometry of the metal-binding domain on the sequence specificity of DNA cleavage has been presented.[204] The degradation of three restriction fragments of DNA under the action of various bleomycin derivatives has been studied. Bleomycin B_2, deglycobleomycin A_2 (**D**), and isobleomycin A_2 (**E**) having a carbamoyl group at position 2 of D-mannose [205] (in the case of natural bleomycin this group is at position 3) displayed a sequence specificity identical with that of bleomycin A_2. At the same time, in the case of epibleomycin A_2, which differs from bleomycin A_2 in the configuration at the chiral carbon atom of the substituent at position 2 of the pyrimidine ring (marked with an asterisk) and, consequently, in the orientation of the propionamide residue relative to the metal-binding domain,[206] the mode of DNA cleavage changed significantly. Epibleomycin A_2 effectively cleaved guanosine at sequence TG, this cleavage mode being



R¹ R²



(A) (B) (C) OH (D) (E)

non-typical of natural bleomycin. The percentage of cleaved CT and GG sequences was also higher in this case; sequences AT, TT, TC, and GC were cleaved to a lesser extent. Decarbamoylbleomycin A_2 (C) did not cleave sequences AA, AC, or CT at all, though they are weakly cleaved by bleomycin A_2. With bleomycinic acid, not only the cleavage of DNA was by 35% weaker than with bleomycin A_2 but the sequence specificity of the cleavage also changed. The main cleavable sequences were GT and GA (but not GT and GC); for CT, the percentage of cleavage was also relatively high.[204]

The nature of the chelated metal (Cu, Co, Mn) has also certain influence on the specificity of the antibiotic action.[59,207-209] In the presence of dithiothreitol, the Cu(II)Blm complex can effectively cleave DNA, the site specificity for such cleavage being different from that for Fe(II)Blm. The difference in the specificity of DNA cleavage by the Fe(II)- and Cu(II)-complexes of bleomycin was especially apparent when the 127-meric double-stranded fragment of SV40 DNA was used.[207] Unfortunately, no data on the efficiency of cleavage of specific dinucleotide sequences were given. It is interesting to note that the cleavage of DNA by the Cu(II)–deglycobleomycin complex is more effective than by the Cu(II)–decarba-moylbleomycin complex, whereas for Fe(II)-dependent cleavage an inverse dependence is observed.[200] Noteworthy, the isobleomycin A_2–Fe(II) complex effectively cleaves DNA,[204] whereas the isobleomycin A_2–Cu(II) complex is fully inactive.[207]

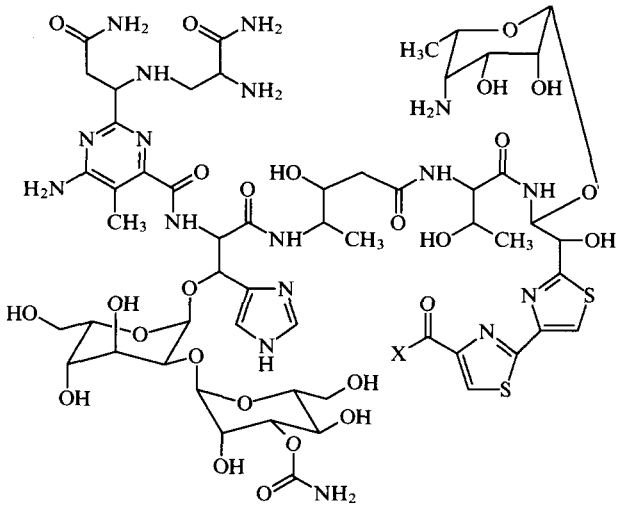
Upon irradiation with UV-light, the specificity of DNA cleavage by bleomycin complexes containing trivalent cobalt differs from that by Fe-containing bleomycin complexes.[59,208] A statistical analysis of sequence specificity of Co(III)–bleomycin complexes is also absent in these studies. What is evident from these experiments, is that sequences GT and GA are preferably cleaved, while sequence GC is cleaved less effectively.

Valuable information on the nature of sequence specificity of the bleomycin action can be derived from the studies of bleomycins with a significantly modified structure. One such work [210] describes the synthesis of deglycodemethylbleomycin A_2 analogues (see below) carrying glycine residues $(Gly)_n$ (where $n = 0-2$ and 4) instead of threonine, and gives an analysis of their cleaving capacity. It turned out that the sequence specificity of such analogues differs only slightly from that of the natural antibiotic. However, the efficiency of cleavage was much lower than with the natural deglycodemethylbleomycin A_2. Thus, the analogue with $n = 0$ hardly cleaved DNA, whereas the level of DNA degradation by the analogues with $n = 1$ and 2 possessing the maximum efficiency was comparable with that characteristic of the natural antibiotic at 10–20-fold higher concentrations.[188,210]

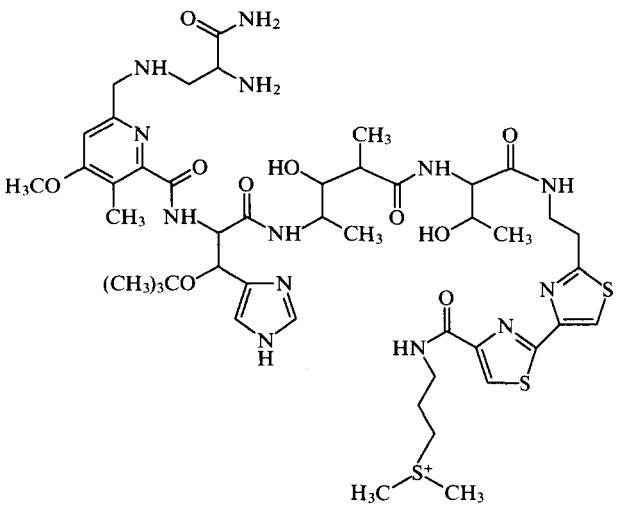
It was thus concluded that the metal-binding domain plays a decisive role in the sequence specificity of DNA cleavage. However, this definite conclusion is appropriate only in case of a mechanistic approach to the consideration of the bleomycin molecule as was the case in the late 1970s: the C-terminal fragment specifically binds to DNA and the change in the length of the spacer between this fragment and the cleaving (metal-binding) domain would alter the specificity of cleavage. If this does not take place, the situation is reversed, namely, the metal-binding domain determines the specificity of cleavage, and the C-terminal fragment defines the affinity for DNA.

As noted above, bleomycin is highly sensitive to the structure of the substrate to be cleaved. High selectivity of oxidation of C(4′) of deoxyribose evidences the fine adjustment of the bleomycin molecule to the double helix conformation. It may thus be assumed that any alteration in the spacer leads to an unprofitable (with regard to cleavage) positioning of the metal-binding domain, resulting in self-degradation (but not oxidation) of DNA. Alternatively, instead of additional stabilisation of binding due to electrostatic interactions, the unfavourable localisation of the metal-binding domain may decrease the affinity of the modified antibiotic for the polymer which can also be the reason for the low cleavage efficiency. And, finally, in all previous speculations the threonine residue was regarded only as a constitutive component of the spacer, which does not take part in binding or determining the sequence specificity of the cleavage. However, one must not rule out the possibility that the free OH group can form intra- or intermolecular hydrogen bonds and thus contribute to the sequence specificity of the bleomycin action.

It is known that the bleomycin analogue tallysomycin [211] having additional OH groups in the aliphatic chain at position 2′ of the bithiazole residue and the aminosugar tallose attached to one of those OH groups induce the formation of single-strand scissions less effectively than bleomycin and differ from the latter in sequence specificity.[173,212,213] Thus, the cleavage of supercoiled DNA of bacteriophage PM2 by tallysomycin proceeds 5–10 times



Tallysomycin

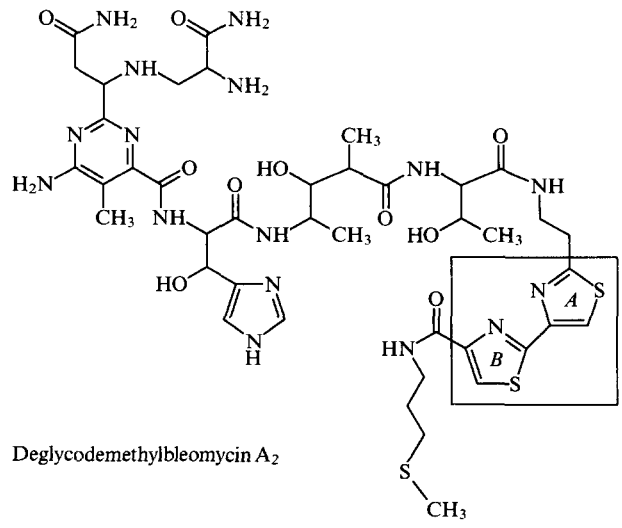


Blm-PYLM(6)

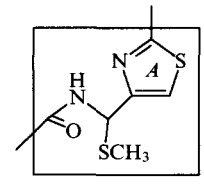
less effectively than that by bleomycin.[53,214] Furthermore, tallysomycin cleaves GA sequences far more effectively,[166,167,197] GT and GA sequences being preferential for this antibiotic.[168] This finding points to the role of the bleomycin C-terminal fragment in determining the specificity and efficiency of DNA cleavage.

Convincing evidence of the ability of the bleomycin C-terminal fragment to determine sequence specificity has been given in the studies carried out by Japanese investigators.[215,216] These authors performed the chemical synthesis of a bleomycin metal-binding domain analogue, in which the 4-aminopyrimidine ring was substituted by methoxypyridine, the disaccharide residue was substituted by a *tert*-butyl group, and the carbamoylmethyl residue in the 2-substituent of pyrimidine was missing. In the presence of iron ions, this compound activated molecular oxygen and oxidised low molecular weight organic substrates with an efficiency comparable with that of the natural antibiotic.[215,216] Attachment to this compound of the C-terminal fragment of bleomycin A_2 [Blm-PYLM(6)] was accompanied by the appearance of the DNA-cleaving activity[217,218] despite significant changes in the metal-binding domain. These studies received further development, culminating in the synthesis of a molecule comprising the metal-binding domain, 4-aminopentanoic acid as a spacer and a distamycin A residue instead of the C-terminal tripeptide S.[219] This synthetic molecule effectively cleaved DNA (relative to natural bleomycin), the maximum degree of cleavage being observed in the regions enriched with AT sequences, to which distamycin A binds especially strongly.[220] No specificity to GT and GC sequences was observed, which is characteristic of bleomycin-induced cleavage.

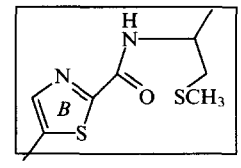
Studies by Hamamichi et al.[221] provided valuable information as to whether the C- or the N-terminal fragment of the antibiotic molecule determines the sequence specificity of bleomycin action. To this end, the authors synthesised and assayed DNA-cleaving agents (analogues of natural deglycodemethylbleomycin A_2) containing, instead of one of the thiazole rings (*A* or *B*), its biosynthetic precursor, *S*-methyl-L-cysteine).[222]



Deglycodemethylbleomycin A_2



Bleomycin monothiazole A



Bleomycin monothiazole B

It turned out that such analogues cleave supercoiled covalently closed and linear double-stranded DNA less effectively than natural deglycobleomycin A_2. No discernible sequence specificity was observed in the cleavage of 5′- or 3′-labelled linear double-stranded DNA. Thus, the synthetic analogues cleave DNA statistically in contrast with sequence-specific cleavage by the natural antibiotic. This study is a sound argument in favour of the determining role of the bithiazole residue in sequence specificity of the bleomycin action, the bi- (but not mono-) thiazole fragment being the crucial factor.[222]

Another recent study of the role of bleomycin N- and C-terminal fragments in the recognition of definite DNA sequences[223] concerned the synthesis of molecules containing both DNA-binding and DNA-cleaving groups. As the DNA-binding group the C-terminal fragment of bleomycin A_2, or demethylbleomycin A_2 carrying a bithiazole residue and a terminal amine, was used. The DNA-cleaving group, EDTA (ethylenediaminetetraacetic acid), was attached to the bithiazole residue by an amide bond. If the bithiazole fragment in the bleomycin molecule is the critical factor determining the direction of the antibiotic action, such molecules will cleave DNA in the vicinity of guanine – pyrimidine sequences. However, no appreciable sequence specificity of DNA cleavage characteristic of bleomycin was noted in these model compounds.[223] One serious pitfall of this study is that its authors did not provide any experimental proof for the binding of their synthetic model

compounds to DNA. This is an important circumstance if one takes into consideration that synthetic compounds cleave DNA far less effectively (approximately 10-fold) than natural bleomycin. The efficiency of DNA cleavage by EDTA-containing compounds is comparable with that for EDTA. It can thus be assumed that at the concentration of the analogues of 100 μmol dm^{-3} that had been used to demonstrate the sequence specificity of the antibiotic action, the degradation of DNA is predominantly effected by diffusing ·OH radicals generated by the reagent molecules not bound to DNA. In this case, the conclusion made by the authors in favour of the determining role of the bleomycin N-terminal fragment in sequence specificity appears to lack sufficient experimental justification.

Yet another detail worth noting is important for the correct understanding of the nature of sequence specificity. According to Otsuka et al.,[219] cleavage by a bleomycin analogue containing distamycin A occurred at sites enriched with AT sequences, i.e., at sites responsible for the binding of distamycin A. This provides evidence of the spatial proximity of the metal-binding domain to the DNA-binding fragment in the bleomycin molecule. Molecular models for this compound suggest the existence of a bend in the residue linking distamycin A and a metal-binding site providing for such cleavage. Presumably, a similar situation takes place in the binding and cleavage of DNA by natural bleomycin; in this case, site-specific recognition of DNA may be effected by both C- and N-terminal fragments of the antibiotic molecule.

However, the data available do not provide any unambiguous conclusion on the nature of the sequence-specific effect of bleomycin. Many of these data and, particularly, the model of binding in the minor groove of DNA, point to the important role of the bleomycin C-terminal fragment in the specific recognition of DNA. Nevertheless, one should not rule out that the C-terminal fragment alone cannot induce either the sequence-specific binding or cleavage of DNA. There are strong grounds for believing that the metal-binding domain plays an important role in preferential recognition of the dinucleotide sequence to be cleaved. It is not excluded that an important role in this process is played by the matching of the sizes of the metal-binding domain and the minor groove. Apparently, the residues of threonine and 4-amino-3-oxy-2-methylpentanoic acid providing correct spatial orientation of N- and C-terminal fragments, also contribute to the specificity of the bleomycin action. It follows from these data that adequate interpretation of the nature of such site specificity is possible if one takes into consideration all the three structural elements, such as affinity for the cleavage site provided predominantly by the C-terminal fragment, microvariations in DNA structure determining the size of the minor groove and the optimal position of the deoxyribose C(4′)–H bond for oxidation by the iron hydroperoxide complex as well as the possibility of fixing the orientation of the metal-binding domain needed for the attack through the connecting group and/or due to the interaction of the bleomycin N-terminal fragment with the double helix of DNA. To this can be added the necessity of penetration of the oxygen molecule to the chelated iron ion provided by the disaccharide which may form a hydrophobic pocket and the important role of the reducing agent in generating 'activated bleomycin'. The elucidation of these factors will indoubtedly shed more light on the nature of site specificity of bleomycin.

5. Correlation between the affinity and efficiency of cleavage

Cleavage of DNA by bleomycin is preceded by the binding of the antibiotic to the polymer. This raises the following questions: Do cleavage and binding sites coincide? How many base pairs does bleomycin cover upon binding? Is the cleavage efficiency determined exclusively by the bleomycin affinity for the given site or are other factors also involved? Some recent results provide answers to these questions. In particular, studies [126, 224–226] describe DNA cleavage by different endonucleases in the absence and in the presence of bleomycin and phleomycin complexes with Co(III). The mode of DNA cleavage by DNAase I and micrococcal nuclease changed considerably in the presence of bleomycin, showing a distinct protective effect at definite regions on the radiograms. These regions comprised, as a rule, a site for the cleavage by the Fe(II)Blm complex. In this case, one bleomycin molecule 'covered' from 3 to 5 adjacent base pairs from the endonuclease action [126, 225] which agreed well the earlier observations.[124] Nightingale and Fox [126] even made an attempt to validate, on the basis of nuclease hydrolysis inhibition data, the orientation of arrangement of the bleomycin molecule at the binding-cleavage site 5′-...ACGT...-3′, stipulating that one antibiotic molecule 'covers' three base pairs. The authors assumed that bleomycin encompasses sequence 5′-CGT-3′, i.e., its molecule is positioned at the 5′-end of the chain relative to the base to be cleaved. Another interesting finding of this study is that the sites with alternating $(AT)_n$ sequences, where $n = 3$, are cleaved by bleomycin with almost the same efficiency as GT sequences. At the same time, the Co(III)Blm complex does not protect these sites from the DNAase I action. In other words, these sites are characterized by efficient cleavage at a relatively weak binding. At the $(AT)_n$ sites, the cleavage affected only the AT (but not TA) sequences. $(AAT)_n \cdot (ATT)_n$, $(TTA)_n \cdot (TAA)_n$, $A_n \cdot T_n$ and some individual AT sites did not practically undergo cleavage. It was assumed that the observed phenomenon is due to an unusual alternating conformation of the B-form in sequences $(AT)_n$, where the turn of the helix at 5′-TA-3′ is greater than at 5′-AT-3′.[227] It is probable also that changes in the normal B-form of DNA are due to the low ionic strength of the buffer solution used for the cleavage.[126, 225, 226]

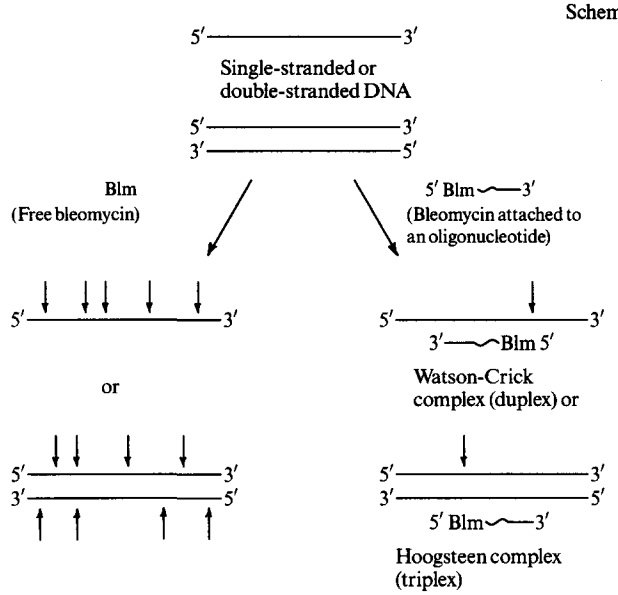
Thus, there exists a certain dependence between the strong binding of bleomycin to DNA and the efficient cleavage of the latter. An important factor is the presence of guanine in the nucleotide sequence. The efficiency of cleavage depends, to a large extent, on the local structure of the double helix which must be in conformation B (but not Z or A). Obviously, the cleavage is more efficient in alternating purine–pyrimidine sequences as can be evidenced from preferential cleavage of guanine–pyrimidine (relative to guanine–purine) and thymine–purine–pyrimidine (relative to adenine–purine–pyrimidine) sequences, and efficient cleavage of $(AT)_n$. Summing up, one may conclude that the efficiency of cleavage depends not only on the strength of binding but also on DNA structure. Therefore, the cleavage of DNA by bleomycin can be used as a sensitive method for determining microvariations in the double helix structure.

IV. Bleomycin derivatives of oligonucleotides. Synthesis and site-specific cleavage of DNA

As noted above (see Section I), owing to its relatively low toxicity, bleomycin has found wide use in treating squamous tumours both separately or in combination with other chemotherapeutic drugs. Nevertheless, the application of this antibiotic is restricted due to its damaging action on certain tissues, in which the content of a bleomycin-inactivating enzyme is low. One of the most common side effects produced by this antibiotic is pulmonary fibrosis.[13, 15, 228] Among other ways to enhance the specificity of therapeutic actions of this drug is to increase its selectivity towards specific nucleic acids. The inherent specificity of natural bleomycin towards dinucleotide sequences prevents it from distinguishing various nucleic acid molecules or different sites of the same macromolecule. The enhancement of specificity of the bleomycin action can be achieved through its attachment to the oligonucleotide exemplified in Scheme 3. In this scheme, the sites of hypothetical cleavage of the DNA chain are marked by arrows. This approach, which was first suggested in the 1960s [229, 230] and is widely used now [231–235] permits spatial approximation of bleomycin and the preselected nucleic acid region and thus ensures its directed chemical modification. Such an approach is now widely used in routine studies.[231–235]

A broad spectrum of reactive oligonucleotide derivatives have been created by now that carry alkylating or photoreactive groups, platinum complexes capable of coordinating target heterocycles,

Scheme 3



and groups catalysing oxidative degradation of target nucleic acids.[231, 235] The latter can chelate transient metals; their interaction with molecular oxygen can catalytically generate active oxygen-containing species which degrade DNA. There exist natural compounds (among those is the antitumour antibiotic bleomycin) which cause destruction of DNA in the same way. From the data presented in Sections I – III, one may conclude that oligonucleotides carrying the residue of this antibiotic should be effective site-specific reagents able to 'switch-off' the function of definite genes. However, taking into account the complex nature of bleomycin, which has several nucleophilic centres, special efforts will be necessary in order to obtain its oligonucleotide derivatives.

1. Synthesis and properties of bleomycin derivatives of mono- and oligonucleotides

When constructing bleomycin derivatives of oligonucleotides suitable for site-specific degradation of target DNA, it is necessary to preserve both the ability of the antibiotic to cleave the DNA of an oligonucleotide fragment to form complementary complexes.

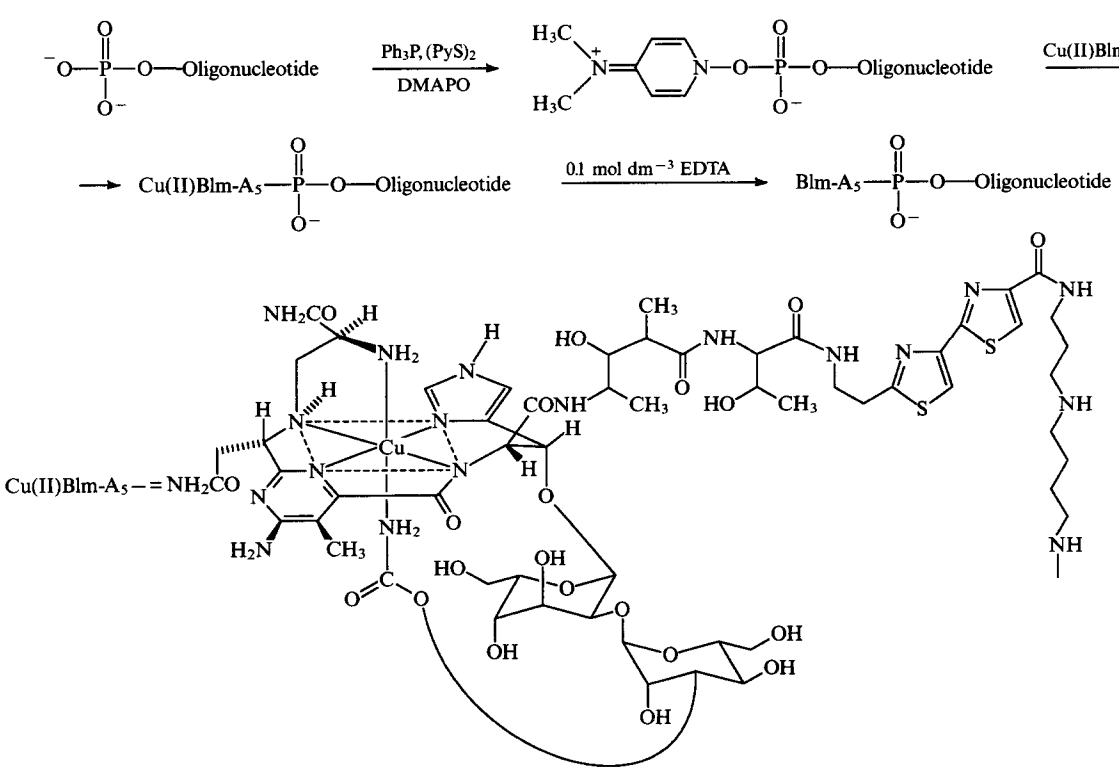
To meet these requirements, the antibiotic was attached to an oligonucleotide at the 5′- or 3′-terminal phosphate group preactivated with triphenylphosphine-2,2′-dipyridyl disulfide in the presence of 4-*N*,*N*-dimethylaminopyridine-1-oxide (DMAPO) (Scheme 4). It is known that the phosphate group in the thus obtained zwitterion derivative readily reacts with aliphatic amino groups.[236] Bleomycin A_5 contains several such groups. The amino groups in the metal-binding domain essential for the DNA-cleaving activity of the antibiotic were protected by chelation with the copper ion, thus directing the attachment to the amino groups of the spermidine residue at the C-terminal fragment of the bleomycin molecule. The successful use of the copper-containing bleomycin complex for selective acylation of the C-terminal amine was documented in earlier studies.[237] In order to avoid the attachment of two oligonucleotide molecules to one antibiotic molecule, the latter was taken in a 10-fold excess.

Analysis of electronic absorption spectra revealed the presence in the synthetic compounds of both the oligonucleotide fragment and the bleomycin residue, the contribution of each component to the total spectrum being commensurate with the binding of one antibiotic molecule to one oligonucleotide molecule.[238, 239]

The structure of bleomycin – oligonucleotide derivatives was finally established in ^{13}C NMR studies.[240] It was found that the linkage of the terminal phosphate group of the oligonucleotide involves the spermidine primary amino group in bleomycin A_5 (see Scheme 4).

As has been mentioned above (see Table 1), bleomycin can form relatively stable complexes ($K_b = 10^5$ dm^3 mol^{-1}) with double-stranded DNA. Moreover, being bound to DNA, this antibiotic stabilises the structure of the double helix. Thus, the melting temperature for calf thymus DNA in the presence of bleomycin

Scheme 4



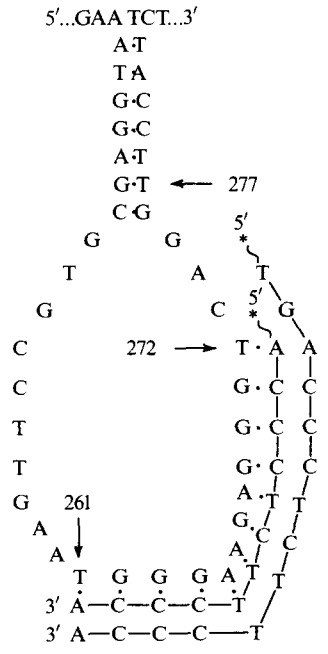
rises by 4 °C.[104] Attachment of bleomycin to an oligonucleotide should stabilise the complementary complex formed thereby. Indeed, the melting temperature for a complex of tetradecanucleotide pd(TGTTTGGCGAAGGA) with the bleomycin derivative of the complementary heptanucleotide, Blm-A_5 – pd(CCAAACA), is 33 °C which is 11 °C higher than that for the analogous complex formed by the original heptanucleotide.[238,239] This is an important point in the realisation of the targeted complementary action, since the efficiency of this action on DNA depends, in a large measure, on the stability of the complementary complex formed by target DNA and the reagent.[241]

The next step in this direction was to investigate the DNA-cleaving activity of bleomycin derivatives of oligonucleotides.

2. Site-specific cleavage of a single-stranded fragment of DNA by bleomycin derivatives of oligonucleotides

In a study by Sergeev et al.,[242] a 302-meric DNA fragment representing a copy of the RNA fragment of the tick-borne encephalitis virus was used to examine the site specificity of oligonucleotide derivatives of bleomycin. Two oligonucleotide reagents carrying a bleomycin A_5 residue at their 5′-ends were complementary to sequence 261 – 274 which is predominantly located in the single-stranded hairpin (the Blm-A_5 residue attached to 5′-phosphate is marked by an asterisk) (Scheme 5).[243,244]

Scheme 5



Cleavage of the 302-meric DNA target was studied using polyacrylamide gel electrophoresis. This approach allows one to separate the degradation products from the original DNA target and to determine (with an accuracy of one base) the site where the target has been cleaved. The latter is achieved by comparing electrophoretic mobilities of degradation products of the target by the reagent with electrophoretic mobilities of the products of statistical degradation of the target at one or two types of bases using the procedure described by Maxam and Gilbert in 1979.[162]

The results of electrophoretic analysis suggest that under conditions of complementary complex formation, both oligonucleotide reagents induce scissions at the GT sequences of the target closest to the antibiotic residue, i.e., at T(272) and T(277) (Fig. 2). The cleavage proceeds with high efficiency and selectivity. By illustration, when the shorter, 12-meric reagent is used at a concentration of 1×10^{-5} mol dm^{-3}, direct breaks (without an additional alkaline treatment, Scheme 2, pathway *a*) occur at sequences T(272) and T(277) in 70% of the DNA target.

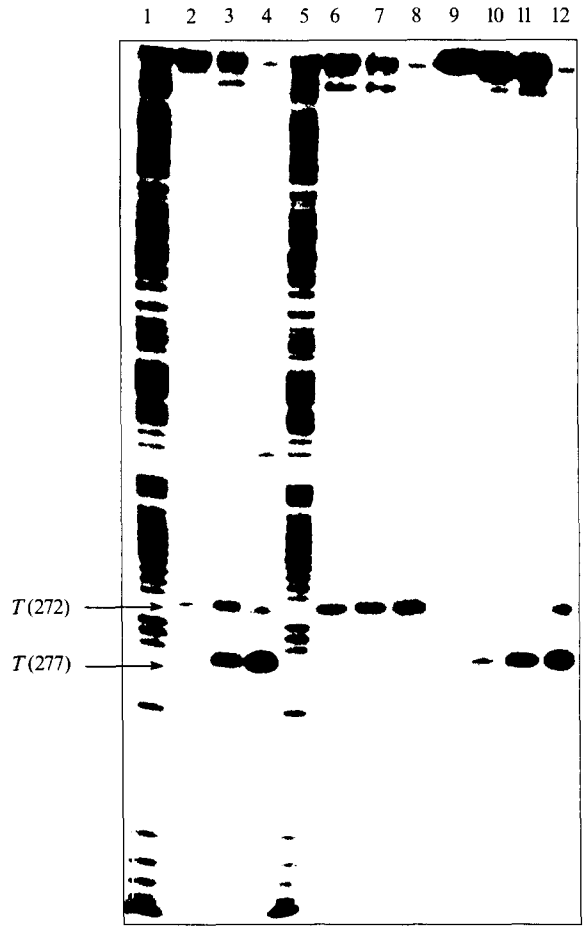


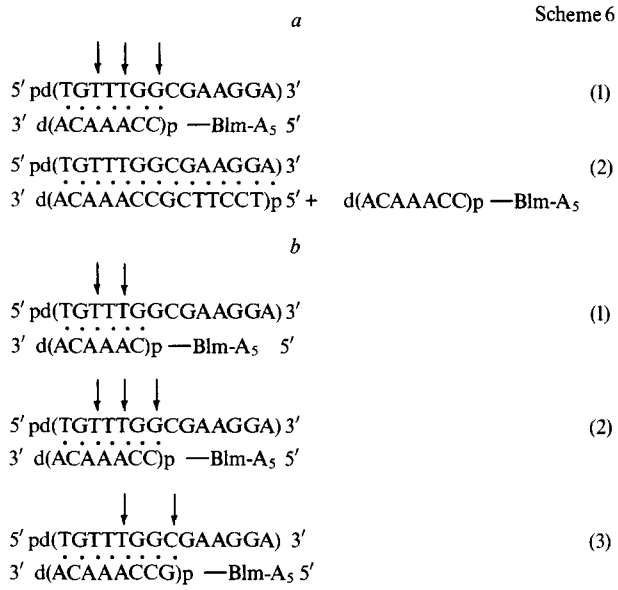
Figure 2. The results of analysis of degradation products of a 302-meric 3′-^{32}P-labelled DNA target by 12- and 14-meric oligonucleotide reagents. Lanes 1 and 5, is statistical cleavage of the DNA target at purine residues;[162] lanes 2 – 4, is the 12-meric reagent (1×10^{-7}, 1×10^{-6} and 1×10^{-5} mol dm^{-3}, respectively); lanes 6 – 8, is cleavage by a 14-meric reagent (5×10^{-6} mol dm^{-3}) for 1, 3 and 6 h, respectively; lane 9, is the DNA target incubated in the absence of the reagents; lanes 10 – 12, is cleavage by a 12-meric reagent (5×10^{-6} mol dm^{-3}) for 1, 3 and 6 h, respectively.

This cleavage is determined by site-specific binding of the oligonucleotide fragment of the reagents to the complementary sequence of the DNA fragment. This becomes obvious when analysing the DNA cleavage by bleomycin and its oligonucleotide derivative whose nucleotide sequence is devoid of the complementary binding site at the preselected fragment. It was shown that (i) both reagents cleave the DNA target with lower efficiency than do complementary reagents and at multiple positions and (ii) T(272) and T(277) are not the preferential sites for the cleavage.[242]

Evidence for the necessity of complementary complex formation for effective degradation of the DNA target is presented in Scheme 6 (the main cleavage sites are marked by arrows).[239] The 14-meric DNA target is effectively cleaved by the 7-meric oligonucleotide reagent Blm-A_5 – pd(CCAAACA) [Scheme 6*a* (1)]. In the presence of a tetranucleotide complementary to the target, this reagent loses its ability to form a complementary complex with the target and does not practically cleave it [Scheme 6*a* (2)].

Complementary oligonucleotide reagents of various lengths are characterised by different positional directionality of cleavage. Thus, for a 6-meric reagent, the main cleavage sites are T(5) and T(3) [Scheme 6*b* (1)]; those for a 7-meric reagent are G(7), T(5), and T(3) [Scheme 6*b* (2)], while those for a 8-meric reagent are C(8) and T(5) [Scheme 6*b* (3)]. It is interesting to note that when 7- and 8-meric reagents are used, the target is directly cleaved at the

Scheme 6



nucleotide closest to bleomycin which is involved in the formation of a complementary complex with the reagent (at residues G(7) and C(8), respectively). In the case of a 6-meric reagent, the adjacent nucleotide [residue G(6)] does not undergo cleavage, while residue T(5) is cleaved. Nucleotide G(6) remains intact both when 7- and 8-meric reagents are used (Scheme 6 *b*).[239] Apparently, under these conditions, the specificity determined by the moiety of the oligonucleotide reagents is superimposed on the specificity of the antibiotic itself, for which the cleavage at sequence TG is not typical (see Section III).

Thus, the alteration in the length of the oligonucleotide moiety of the reagent changes the site-direction of the DNA cleavage, indicating a site-specific mode of action of the covalently attached bleomycin residue.

3. Degradation of a double-stranded DNA target by bleomycin derivatives of oligonucleotides within the triplex structure

One of the versions of the complementary targeted modification is the use of reactive oligonucleotide derivatives capable of attacking selected sites of a double-stranded DNA by forming a ternary complex.[235,247] Oligonucleotides containing alkylating[246,247] or photoreactive groups,[248] as well as groups with a redox mechanism of action,[249,250] have been used. The bleomycin A_5 residue, which has demonstrated effective cleavage of a DNA target within a duplex structure was assayed as the reactive group.[251]

The DNA target for the study of the oligonucleotide reagent action within the ternary complex was selected in accordance with the following demands. First, the target had to contain a region of homopurine and homopyrimidine sequences necessary for the ternary complex formation. Second, a stable ternary complex should be formed at neutral pH values, since bleomycin effectively cleaves DNA in the pH range of 7 – 9.[37,62,63] If these conditions are fulfilled, stable ternary T·AT complexes are formed.[252,253] Third, the fragment involved in the formation of the complex with the reagent must be flanked with sequences which could serve as targets for the action of the bleomycin residue during the complex formation. Since bleomycin predominantly cleaves DNA at sequences GT, GC, AT, and GA at deoxyribose, the second nucleotide (Section III), a 30-meric double-stranded target (Scheme 7) was selected (the cleavage sites are marked by arrows whose length is proportional to the cleavage intensity).

Under conditions of ternary complex formation, 5′- and 3′-bleomycin derivatives of the 16-meric oligothymidylate induce the cleavage of the target. Both reagents cleave DNA at both chains in the vicinity of the antibiotic residue upon binding of the third chain

Scheme 7



(reagent), which is parallel to purine-rich (triplex I) and antiparallel to pyrimidine-rich (triplex II) sequences. This is in agreement with the literature data[249,250] for the cleavage of double-stranded DNA by EDTA- and 1,10-phenanthroline-containing oligonucleotide reagents which form a ternary complex with DNA.

Cleavage of a pyrimidine-rich chain by a 3′-bleomycin derivative of hexadecathymidylate occurs both in the vicinity of the 5′-end of the oligo(T)-track due to the formation of a ternary complex and around the 3′-end of the chain (Scheme 7). The latter cleavage occurs at sites C(30), C(28) and T(26) of the GC and GT sequences that are preferential for the binding and cleavage by the bleomycin itself (Section III). At a 5-fold excess of the reagent (relative to the target), this cleavage may be due to the ability of the bleomycin residue to bind to a double-stranded DNA without the formation of a triplex by the oligonucleotide moiety of the reagent. To check this hypothesis, a DNA target was cleaved by a 5′-bleomycin derivative of a 16-meric oligonucleotide, Blm-A_5 – pd(CAGCGGGATGCAACAC), which a priori is incapable to form a ternary complex with the target. In this case, the purine-rich chain is cleaved only insignificantly (by 6%), whereas the pyrimidine-rich chain, as in the case of bleomycin derivatives of hexadecathymidylate, is cleaved at the 3′-end at sites C(30), C(28), and T(26) (16%). This suggests that the action of a 5-fold excess of 5′- and 3′-bleomycin derivatives of hexadecathymidylate on the double-stranded DNA target can be both site-specific due to the targeted attachment of the oligonucleotide in the ternary complex formation and nonspecific when the bleomycin residue binds to double-stranded DNA.

Comparing the total extent of cleavage of the double-stranded DNA target by bleomycin-containing reagents (Table 2), one may conclude that for both 5′- and 3′-bleomycin derivatives of hexadecathymidylate, site-specific cleavage associated with the ternary complex formation prevails over nonspecific ones.

Table 2. Total extent of cleavage of a 30-meric double-stranded DNA target by bleomycin derivatives of oligonucleotides.

Reagent	Total extent of cleavage (%)	
	Purine-rich chain	Pyrimidine-rich chain
Blm-A_5 – p(dT)$_{16}$	25	47
(dT)$_{16}$p – Blm-A_5	35	36
Blm-A_5 – pd(CAGCGGGATGCAACAC)[a]	6	16

[a] Does not form a ternary complex with the DNA target.

4. Catalytic degradation of a DNA target by an oligonucleotide derivative of bleomycin

The advantage of applying groups with a redox-type mechanism of action [Fe(III) – EDTA, metalloporphyrins or Cu(II) – 1,10-phenanthroline] over alkylating or photoreactivate groups for the site-specific DNA modification is their ability to produce catalytically active oxygen-containing species and thus exert manyfold action on the DNA target.[254 – 256] The glycopeptide antibiotic bleomycin is an illustrious representative of compounds with a redox mechanism of action. Cyclic oxidation – reduction of the iron ion chelated in the metal-binding domain of bleomycin by molecular oxygen and some reducing agent results in multiple production of 'activated bleomycin' and, as a consequence, in catalytic oxidation of organic substrates.[56, 257, 258] In the process of oxygen reduction, bleomycin acts as an enzyme with the iron ion as a cofactor.[56, 259] Recent studies[260] provide evidence that in the presence of ascorbate one molecule of bleomycin can catalyse the reduction of 4.7 molecules of oxygen. The main advantage of bleomycin as a reactive group for the site-specific cleavage of DNA is its ability to oxidise catalytically not only low molecular weight substrates but also DNA.[201, 261]

It has already been noted that all the redox groups used for site-specific modification of DNA can reduce molecular oxygen catalytically. However, the capability of these groups to cause multiple degradation of DNA within the structure of the oligonucleotide reagent has not yet been realised. One of the reasons for this is an accidental damage to nucleotide residues by reactive oxygen-containing species generated by these reagents. The analysis by chromatomass spectrometry revealed the presence of a greater number of modified bases (compared to the number of direct cleavages) after treatment of DNA with Fe(III) – EDTA and Cu(II) – 1,10-phenanthroline complexes.[262, 263] Similarly, a great deal of modified bases (primarily, guanine) are formed upon treatment of DNA with metalloporphyrins.[256]

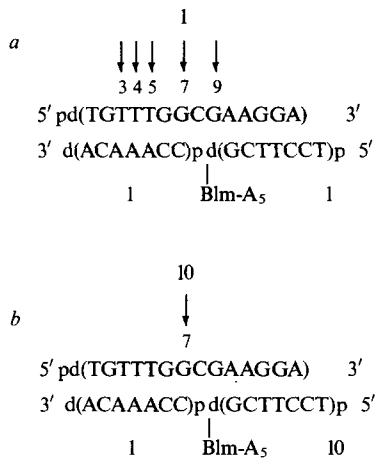
Modification of bases does not lead to direct cleavage of the DNA chain. Moreover, many modified bases, particularly 8-hydroxyguanine and 8-hydroxyadenine, the major products of $^{\cdot}OH$ radical interaction with purine heterocycles, are stable under alkaline conditions and even treatment with piperidine does not result in the cleavage of the sugar – phosphate backbone at these sites. Such hidden modifications mask the catalytic activity of the above-mentioned reagents.

The mechanism of effective degradation of DNA by bleomycin differs essentially from that by redox groups. Under physiological conditions, bleomycin-induced selective oxidation of the C(4′) atom of deoxyribose leads with equal probability either to direct cleavage of the DNA chain or to the formation of a base-labile site (Section III). For this antibiotic, modification of nucleotides is only a minor reaction.[160, 161] Thus, the yield of the major side product of the reaction, 8-hydroxyguanosine, constitutes 2% – 5% of the amount of oxidised deoxyribose.[160] Thus, bleomycin oxidises nucleotide residues with a far greater selectivity than its redox analogues.

Bleomycin does not form covalent linkages with DNA following oxidation of deoxyribose but it can bind to a new site in DNA, and after reduction of the iron ion in the active centre it can again oxidise the sugar – phosphate backbone. Analysis of extended DNA revealed that one antibiotic molecule stimulates the formation of several molecules of free heterocyclic bases or propenal derivatives.[261] The ability of one bleomycin molecule to induce degradation of several DNA molecules has been demonstrated for oligonucleotides used as targets.[201]

In order to examine the ability of the bleomycin residue within the structure of the oligonucleotide conjugate to cleave DNA catalytically, a model with an equimolar target : reagent ratio (Scheme 8 *a*) and 10-fold excess of the target relative to the reagent (Scheme 8 *b*) was used. The cleavage sites in the scheme are marked by arrows.[264]

Scheme 8



It is known that bleomycin more effectively cleaves double-stranded DNA than single-stranded DNA.[167, 265 – 267] This circumstance considered, a model comprising a 14-meric DNA target, a 5′-bleomycin derivative of a heptanucleotide, and a heptanucleotide complementary to the 3′-end of the target was chosen.

The catalytic activity of the bleomycin residue covalently bound to the oligonucleotide, can be manifested (depending on the experimental conditions) in a damage to several DNA target molecules by one reagent molecule or in repeated attacks on the same target by the complementary complex.

a. Repeated cleavage of a 14-meric DNA target by an oligonucleotide derivative of bleomycin

Interaction of an oligonucleotide derivative of bleomycin with the tetradecanucleotide at their equimolar ratio (Scheme 8 *a*) results in a practically complete (80%) degradation of the target. This is accompanied by the formation of a broad range of degradation products and modification of sites T(3) – T(5) remote from the site of covalent attachment of the antibiotic. At the same time, with a 10-fold excess of the target, the modification predominantly occurs at G(7) (Scheme 8 *b*). This result can be interpreted in terms of catalytic properties of the bleomycin residue, which under conditions of an equimolar reagent : target ratio can be manifested in repeated action on the DNA target. Analysis of the time dependence of the tetradecanucleotide degradation at 20 °C revealed that the target is primarily attacked at position G(7) (Fig. 3). This is followed by accumulation of shorter fragments formed upon the destruction of sites T(5), T(4) and T(3). The amount of the degradation product at position G(7) does not increase any longer but decreases with time. A similar situation is observed when the cleavage occurs at position G(9). These findings provide direct evidence in favour of repeated exposure of the DNA target to the antibiotic action.

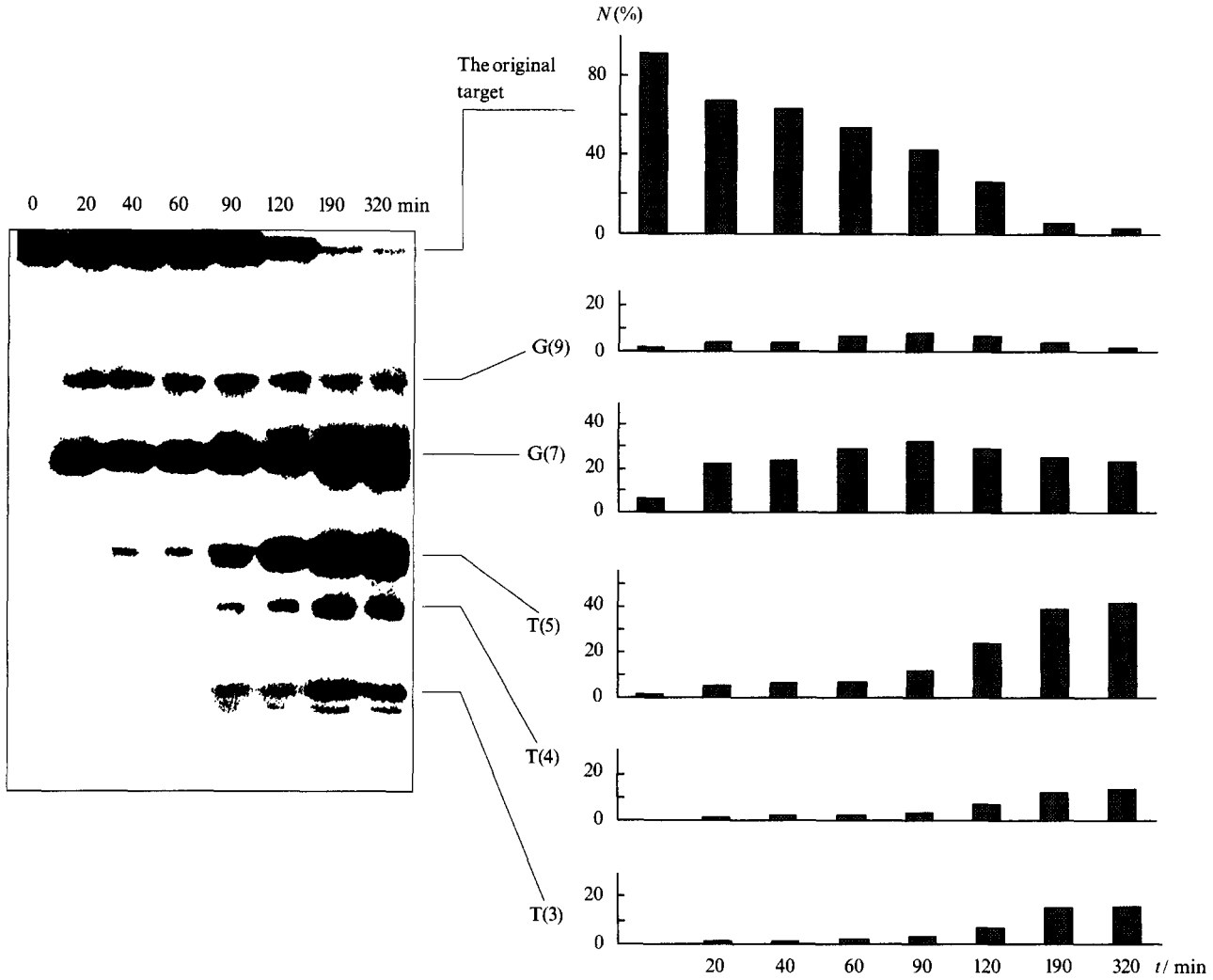


Figure 3. Time dependence of the cleavage of the 14-meric DNA target by the oligonucleotide reagent (see Scheme 8 *a*) (electrophoretic and densitometric data). Oligonucleotide concentration is 1×10^{-5} mol dm^{-3}; N is the concentration of the product in the reaction mixture.

b. Degradation of several target molecules by one reagent molecule

In order to manifest the catalytic activity, the oligonucleotide derivative of bleomycin has to attack one target molecule after another. This will require an excess of the target and selection of conditions enabling both ready dissociation of the complementary reagent – target complex and, simultaneously, the stability needed for efficient modification. Fig. 4 illustrates the cleavage of the DNA target by the reagent at different temperatures and under conditions when the reagent concentration is 10 times less than that of the target and the heptanucleotide. The cleavage of the target is a maximum in the temperature range of 25 – 35 °C which includes the melting temperature for the reagent – target complex ($T_{m.p.}$ = 33 °C). Under these conditions, the reagent readily dissociates from the complementary complex, thereby enabling its transfer from one target molecule to another. At the same time, about half of the reagent molecules remain within the complementary complex. An increase in temperature sharply decreases the degree of cleavage, apparently, due to the decrease in the concentration of the complementary reagent – target complex. Lowering of temperature also diminishes the degree of the target cleavage, which can be attributed to nonoptimal conditions for the manifestation by the reagent of its catalytic activity. It has been established that at temperatures around 30 °C, about 20% of the target undergoes direct cleavage, while subsequent treatment with piperidine reveal an additional 10% of sites that are labile under alkaline conditions.[264]

Degradation of about 30% of the tetradecanucleotide by the oligonucleotide reagent and in the presence of a 10-fold excess of the target testifies to the fact that one reagent molecule can attack, on average, three target molecules. Restricted efficiency of the reagent action in the presence of a sufficient amount of iron ions,

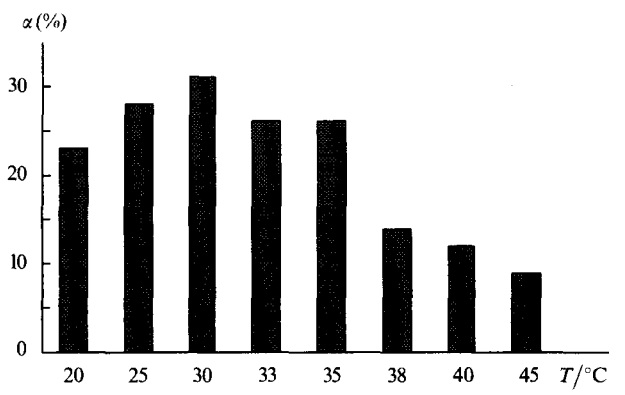


Figure 4. The total extent of cleavage (α) of 14-meric target DNA (1×10^{-5} mol dm^{-3}) by the oligonucleotide reagent (1×10^{-6} mol dm^{-3}) in the presence of heptanucleotide (1×10^{-5} mol dm^{-3}) (see Scheme 8 *b*) at different temperatures, according to densitometric analytical data.

reducing agents, and molecular oxygen may be attributed either to the degradation of its oligonucleotide moiety or to the degradation of the antibiotic itself. To elucidate possible reasons for the restriction of the catalytic activity, synthesis of the 5′-^{32}P-labelled reagent and cleavage of the nonlabelled target DNA were carried out under conditions of 10-fold target excess (Scheme 8 *b*). Electrophoretic analysis data indicate that catalytic degradation of the target is accompanied by significant degradation of the oligonucleotide reagent.[264] Removal of the bleomycin residue from the reagent after termination of the reaction gave rise to a nonmodified oligonucleotide. Hence, the restriction of the reagent catalytic activity is associated with the destruction of bleomycin itself.

Thus, chemical attachment of the antibiotic to the oligonucleotide makes it possible to direct it to a preselected site of one of nucleic acids and thus to increase markedly the selectivity of its action on all these acids. The method described of covalent attachment of bleomycin to the oligonucleotide[238,239] makes it possible to preserve not only the ability of the antibiotic to cleave DNA, but also its capacity for catalytic cleavage characteristic of free bleomycin.

References

1. H Umezawa, K Maeda, T Takeushi, Y Okami *J. Antibiot.* **19** 200 (1966)
2. H Umezawa, Y Suhora, T Takita, K Maeda *J. Antibiot.* **19** 210 (1966)
3. M Ishizuka, H Takayama, T Takeushi, H Umezawa *J. Antibiot.* **20** 15 (1967)
4. T Ishikawa, A Matsuda, K Yamamoto, M Tsubosaki, T Kaihara, K Sakamoto, H Umezawa *J. Antibiot.* **20** 149 (1967)
5. H Umezawa, M Ishizuka, K Maeda, T Takeushi *Cancer* **20** 891 (1967)
6. M Suzuki, K Nagai, H Yamaki, N Tanaka, H Umezawa *J. Antibiot.* **21** 379 (1968)
7. H Umezawa, M Ishizuka, K Kimura, J Iwanaga, T Takeushi *J. Antibiot.* **21** 592 (1968)
8. M Takeushi, T Yamamoto *J. Antibiot.* **21** 631 (1968)
9. T Ichikawa, in *Progress in Antimicrobial and Anticancer Chemotherapy (Proceedings of the 6th International Congress of Chemotherapy)* (Tokyo: University of Tokyo Press, 1970) Vol. 2, p. 288
10. M B Mosher, R C DeConti, J R Bertino *Cancer* **30** 56 (1972)
11. R A Rudders *Blood* **40** 317 (1972)
12. B I Sikis, M Rozencweig, S K Carter (Eds) *Bleomycin Chemotherapy* (Orlando: Academic Press, 1985)
13. R H Blum, S K Carter, K Agre *Cancer* **31** 903 (1973)
14. S S Cohen, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 267
15. S T Crooke, W T Bradner *J. Med.* **7** 333 (1976)
16. H Umezawa, in *Antibiotics. Mechanism of Action of Antimicrobial and Antitumor Agents* Vol. 3 (Eds J W Corcoran, F E Hahn) (Berlin: Springer, 1975) p. 21
17. W E G Muller, R K Zahn, in *Progress in Nucleic Acid Research and Molecular Biology* Vol. 20 (Ed. W E Cohn) (London: Academic Press, 1977) p. 21
18. H Umezawa *Lloydia* **40** 67 (1977)
19. S M Hecht, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 1
20. H Umezawa *Cancer Treat. Rep.*, **68** 137 (1984)
21. M Watanabe, Y Takabe, T Katsumata *J. Antibiot.* **26** 417 (1973)
22. Z I Yamazaki, W E G Muller, R K Zahn *Biochim. Biophys. Acta* **308** 412 (1973)
23. G P Sartiano, A Winkelstein, W Lynch, S S Boggs *J. Antibiot.* **26** 437 (1973)
24. R S Bornstein, D A Hungerford, G Haller, P F Engstrom, J W Yarbro *Cancer Res.* **31** 2004 (1971)
25. T Onishi, K Shimada, Y Takagi *Biochim. Biophys. Acta* **312** 248 (1973)
26. M Miyaki, T Kitayama, T Ono *J. Antibiot.* **27** 647 (1974)
27. T Takita, Y Myraoka, T Yoshioka, A Fujii, K Maeda, H Umezawa *J. Antibiot.* **25** 755 (1972)
28. T Takita, Y Myraoka, T Nakatani, A Fujii, Y Umezawa, H Naganawa, H Umezawa *J. Antibiot.* **31** 801 (1978)
29. T Takita, Y Umezawa, S-I Saito, H Morishima, H Naganawa, H Umezawa, T Tsuchiya, T Miyake, S Kageyama, S Umezawa, Y Myraoka, M Suzuki, M Otsuka, M Narita, S Kobayashi, M Ohno *Tetrahedron Lett.* **23** 521 (1982)
30. K Nagai, H Yamaki, H Suzuki, N Tanaka, H Umezawa *Biochim. Biophys. Acta* **179** 165 (1969)
31. K Nagai, H Suzuki, N Tanaka, H Umezawa *J. Antibiot.* **22** 569 (1969)
32. K Nagai, H Suzuki, N Tanaka, H Umezawa *J. Antibiot.* **22** 624 (1969)
33. H Suzuki, K Nagai, H Yamaki, N Tanaka, H Umezawa *J. Antibiot.* **22** 446 (1969)
34. H Suzuki , K Nagai, E Akutsu, H Yamaki, N Tanaka, H Umezawa *J. Antibiot.* **23** 473 (1970)
35. E A Sausville, J Peisach, S B Horwitz *Biochem. Biophys. Res. Commun.* **73** 814 (1976)
36. E A Sausville, J Peisach, S B Horwitz *Biochemistry* **17** 2740 (1978)
37. E A Sausville, R W Stein, J Peisach, S B Horwitz *Biochemistry* **17** 2746 (1978)
38. M Chien, A P Grollman, S B Horwitz *Biochemistry* **16** 3641 (1977)
39. T Takita, Y Myraoka, T Nakatani, A Fujii, Y Iitaka, H Umezawa *J. Antibiot.* **31** 1073 (1978)
40. J D Glickson, R P Pillai, T T Sakai *Proc. Natl. Acad. Sci. USA* **78** 2967 (1981)
41. D H Petering, R W Byrnes, W E Antholine *Chem.-Biol. Interact.* **73** 133 (1990)
42. J C Dabrowiak, F T Greenaway, W E Longo, M van Husen, S T Crooke *Biochim. Biophys. Acta* **517** 517 (1978)
43. J C Dabrowiak, F T Greenaway, F S Santillo, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 137
44. J C Dabrowiak *J. Inorg. Biochem.* **13** 317 (1980)
45. Y Sugiura, T Suzuki, M Otsuka, S Kobayashi, M Ohno, T Takita, H Umezawa *J. Biol. Chem.* **258** 1328 (1983)
46. A Kittaka, Y Sugano, M Otsuka, S Kobayashi, M Ohno, Y Sugiura, H Umezawa *Tetrahedron Lett.* **27** 3631 (1986)
47. L F Povirk, M Hogan, N Dattagupta *Biochemistry* **18** 96 (1979)
48. L F Povirk, M Hogan, N Dattagupta, M Buechner *Biochemistry* **20** 665 (1981)
49. S N Roy, G A Orr, C F Brewer, S B Horwitz *Cancer Res.* **41** 4471 (1981)
50. J P Albertini, A Garnier-Suillerot *Biochemistry* **21** 6777 (1982)
51. J P Albertini, A Garnier-Suillerot *Biochemistry* **23** 47 (1984)
52. H Hiroaki, T Nakayama, M Ikehara, S Uesugi *Chem. Pharm. Bull.* **39** 2780 (1991)
53. J E Strong, S T Crooke *Cancer Res.* **38** 3322 (1978)
54. C-H Huang, L Galvan, S T Crooke *Biochemistry* **19** 1761 (1980)
55. D L Boger, S L Colletti, T Honda, R F Menezes *J. Am. Chem. Soc.* **116** 5607 (1994)
56. W J Caspary, D A Lanzo, C Niziak *Biochemistry* **21** 334 (1982)
57. H Kasai, H Naganawa, T Takita, H Umezawa *J. Antibiot.* **31** 1316 (1978)
58. T T Sakai, J M Riordan, J D Glickson *Biochemistry* **21** 805 (1982)
59. C-H Chang, C F Meares *Biochemistry* **23** 2268 (1984)
60. T T Sakai, J M Riordan, T E Booth, J D Glickson *J. Med. Chem.* **24** 279 (1981)
61. R S Magliozzo, S Peisach, M R Ciriolo *Mol. Pharmacol.* **35** 428 (1989)
62. H Umezawa, H Asakura, K Oda, S Hori *J. Antibiot.* **26** 521 (1973)
63. H Asakura, S Hori, H Umezawa *J. Antibiot.* **28** 537 (1975)
64. M Hori, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 195
65. B J Carter, E de Vroom, E S Long, G A van der Marel, I H van Boom, S M Hecht *Proc. Natl. Acad. Sci. USA* **87** 9373 (1990)
66. J Stubbe, J W Kozarich *Chem. Rev.* **87** 1107 (1987)
67. D M Chen, T T Sakai, J D Glickson, D J Patel *Biochem. Biophys. Res. Commun.* **92** 197 (1980)
68. R P Pillai, N R Krishna, T T Sakai, J D Glickson *Biochem. Biophys. Res. Commun.* **97** 270 (1980)

69. T T Sakai, J M Riordan, N G Kumar, F J Haberle, G A Elgavish, J D Glickson, A Levy *J. Biomol. Struc. Dyn.* **1** 809 (1983)
70. T T Sakai, J M Riordan, J D Glickson *Biochim. Biophys. Acta* **758** 176 (1983)
71. T E Booth, T T Sakai, J D Glickson *Biochemistry* **22** 4211 (1983)
72. M P Gamcsik, J D Glickson, G Zon *J. Biomol. Struc. Dyn.* **7** 1117 (1990)
73. H Urata, Y Ueda, Y Usami, M Akagi *J. Am. Chem. Soc.* **115** 7135 (1993)
74. W Wu, D E Vanderwall, J Stubbe, J W Kozarich, C J Turner *J. Am. Chem. Soc.* **116** 10843 (1994)
75. R A Manderville, J F Ellena, S M Hecht *J. Am. Chem. Soc.* **116** 10851 (1994)
76. J Kross, W D Henner, W A Haseltine, L Rodriguez, M D Levin, S M Hecht *Biochemistry* **21** 3711 (1982)
77. L M Fisher, R Kuroda, T T Sakai *Biochemistry* **24** 3199 (1985)
78. R E Kilkuskie, H Suguna, B Yellin, N Murugesan, S M Hecht *J. Am. Chem. Soc.* **107** 260 (1985)
79. E Farinas, J D Tan, N Baidya, P K Mascharak *J. Am. Chem. Soc.* **115** 2996 (1993)
80. R J Guajardo, S E Hudson, S J Brown, P K Mascharak *J. Am. Chem. Soc.* **115** 7971 (1993)
81. D L Boger, T Honda, R F Menezes, S L Colletti *J. Am. Chem. Soc.* **116** 5631 (1994)
82. Y Muraoka, A Fujii, T Yoshioka, T Takita, H Umezawa *J. Antibiot.* **30** 178 (1977)
83. Y Sugiura, K Ishizu, K Miyoshi *J. Antibiot.* **32** 453 (1979)
84. D E Berry, L-H Chang, S M Hecht *Biochemistry* **24** 3207 (1985)
85. M J Levy, S M Hecht *Biochemistry* **27** 2647 (1988)
86. W E Antholine, D H Petering, L A Saryan, C E Brown *Proc. Natl. Acad. Sci. USA* **78** 7517 (1981)
87. W E Antholine, D H Petering *Biochem. Biophys. Res. Commun.* **91** 528 (1979)
88. Y Sugiura, K Ishizu *J. Inorg. Biochem.* **11** 171 (1979)
89. R M Burger, J Peisach, S B Horwitz *J. Biol. Chem.* **256** 11636 (1981)
90. Y Sugiura *J. Antibiot.* **31** 1206 (1978)
91. Y Sugiura *J. Am. Chem. Soc.* **102** 5216 (1980)
92. M Chikara, W E Antholine, D H Petering *J. Biol. Chem.* **264** 21478 (1989)
93. Y Iitaka, H Nakamura, T Nakatani, Y Myraoka, A Fujii, T Takita, H Umezawa *J. Antibiot.* **31** 1070 (1978)
94. M A J Akkerman, E W J F Neijman, S S Wijmenga, C W Hilbers, W Bermel *J. Am. Chem. Soc.* **112** 7462 (1990)
95. H Shields, C McGlumphy, P J Hamrick *Biochim. Biophys. Acta* **697** 113 (1982)
96. M Chikira, T Sato, W E Antholine, D H Petering *J. Biol. Chem.* **266** 2859 (1991)
97. H Shields, C McGlumphy *Biochim. Biophys. Acta* **800** 277 (1984)
98. G Koyama, H Nakamura, Y Myraoka, T Takita, K Maeda, H Umezawa, Y Iitaka *Tetrahedron Lett.* 4635 (1968)
99. S Neidle, Z Abraham *CRC Crit. Rev. Biochem.* **17** 73 (1984)
100. M J Waring *Annu. Rev. Biochem.* **50** 159 (1981)
101. T Takita, Y Myraoka, A Fujii, H Itoh, K Maeda, H Umezawa *J. Antibiot.* **25** 197 (1972)
102. S Y Lin, A P Grollman *Biochemistry* **20** 7589 (1981)
103. K J Miller, M Lauer, W Caloccia *Biopolymer* **24** 913 (1985)
104. J-P Henichart, J-L Bernier, N Helbecque, R Houssin *Nucleic Acids Res.* **13** 6703 (1985)
105. K H Langley, M R Patel, M J Fournier, in *Biomedical Application of Laser Light Scattering* (Eds D B Sattelle, W I Lee, B R Ware) (Amsterdam: Elsevier, Biomedical Press, 1982) p. 37
106. S-J Hong, L H Piette *Cancer Res.* **36** 1159 (1976)
107. J-L Bernier, J-P Henichart, J P Catteau *Anal. Biochem.* **117** 12 (1981)
108. T R Krugh, F N Wittlin, S P Cramer *Biopolymers* **14** 197 (1975)
109. D J Patel *Acc. Chem. Res.* **12** 118 (1979)
110. J Feigon, W A Denny, W Leupin, D R Kearns *J. Med. Chem.* **27** 450 (1984)
111. W D Wilson, R L Jones *Nucleic Acids Res.* **10** 1399 (1982)
112. K Wuthrich, in *NMR of Proteins and Nucleic Acids* (New York: Wiley, 1986) p. 3
113. H Murakami, H Mori, S Taira *J. Theor. Biol.* **59** 1 (1976)
114. E J Gabbay, P D Adawadkar, W D Wilson *Biochemistry* **15** 146 (1976)
115. R Kuroda, S Neidle, J M Riordan, T T Sakai *Nucleic Acids Res.* **10** 4753 (1982)
116. J M Riordan, T T Sakai *J. Med. Chem.* **26** 884 (1983)
117. R E Dickerson, in *Implication for Carcinogenesis and Risk Assessment in Basic Life Sciences* Vol. 38 (Eds M G Simic, L Grossman, A C Upton) (New York: Plenum, 1986) p. 245
118. Y Sugiura, T Suzuki *J. Biol. Chem.* **257** 10544 (1982)
119. H Sugiyama, R E Kilkuskie, S M Hecht, G A van der Marel, J H van Boom *J. Am. Chem. Soc.* **107** 7765 (1985)
120. K Yamamoto, S Kawanishi *Biochem. Biophys. Res. Commun.* **183** 292 (1992)
121. H M Sobell, S C Jain *J. Mol. Biol.* **68** 21 (1972)
122. T Suzuki, J Kuwahara, Y Sugiura *Biochem. Biophys. Res. Commun.* **117** 916 (1983)
123. R P Hertzberg, M J Caranfa, S M Hecht *Biochemistry* **27** 3164 (1988)
124. J Kuwahara, Y Sugiura *Proc. Natl. Acad. Sci. USA* **85** 2459 (1988)
125. R L Petrusek, G L Anderson, T F Garner, Q L Fannin, D J Kaplan, S G Zimmer, L H Hurley *Biochemistry* **20** 1111 (1981)
126. K P Nightingale, K R Fox *Nucleic Acids Res.* **21** 2549 (1993)
127. H Murakami, H Mori, S Taira *J. Theor. Biol.* **42** 443 (1973)
128. T Onishi, H Iwata, Y Takagi *J. Biochem.* **77** 745 (1975)
129. R Ishida, T Takahashi *Biochem. Biophys. Res. Commun.* **66** 1432 (1975)
130. J W Lown, S-K Sim *Biochem. Biophys. Res. Commun.* **77** 1150 (1977)
131. J W Lown, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 184
132. Y Sugiura *J. Am. Chem. Soc.* **102** 5208 (1980)
133. Y Sugiura, T Kikushi *J. Antibiot.* **31** 1310 (1978)
134. L W Oberley, G R Buettner *FEBS Lett.* **97** 47 (1979)
135. J W Lown, A V Joashua *Biochem. Pharmacol.* **29** 521 (1979)
136. L Galvan, C-H Huang, A W Prestayko, J T Stout, J E Evans, S T Crooke *Cancer Res.* **41** 5103 (1981)
137. L O Rodriguez, S M Hecht *Biochem. Biophys. Res. Commun.* **104** 1470 (1982)
138. R M Burger, J Peisach, W E Blumberg, S B Horwitz *J. Biol. Chem.* **254** 10906 (1979)
139. H Kuramoshi, K Takahashi, T Takita, H Umezawa *J. Antibiot.* **34** 576 (1981)
140. G Prativel, J Bernadou, B Meunier *Biochem. Pharmacol.* **38** 133 (1989)
141. A Natrajan, S M Hecht, G A van der Marel, J H van Boom *J. Am. Chem. Soc.* **112** 3397 (1990)
142. A Natrajan, S M Hecht, G A van der Marel, J H van Boom *J. Am. Chem. Soc.* **112** 4532 (1990)
143. J W Sam, X-J Tang, J Peisach *J. Am. Chem. Soc.* **116** 5250 (1994)
144. S M Hecht *Acc. Chem. Res.* **19** 383 (1986)
145. D G Knorre, O S Fedorova, E I Frolova *Usp. Khim.* **62** 70 (1993) [*Russ. Chem. Rev.* **62** 65 (1993)]
146. L Giloni, M Takeshita, F Johnson, C Iden, A P Grollman *J. Biol. Chem.* **256** 8608 (1981)
147. J C Wu, J W Kozarich, J Stubbe *J. Biol. Chem.* **258** 4694 (1983)
148. J C Wu, J W Kozarich, J Stubbe *Biochemistry* **24** 7562 (1985)
149. J W Kozarich, L J Worth, B L Frank, D A Christner, D E Vanderwall, J Stubbe *Science* **245** 1396 (1989)
150. L J Worth, B L Frank, D A Christner, M J Absalon, J Stubbe, J W Kozarich *Biochemistry* **32** 2601 (1993)
151. S Uesugi, T Shida, M Ikehara, Y Kobayashi, Y Kyogoku *Nucleic Acids Res.* **12** 1581 (1984)
152. N Murugesan, C Xu, G M Ehrenfeld, H Sugiyama, R E Kilkuskie, L O Rodriguez, L-H Chang, S M Hecht *Biochemistry* **24** 5735 (1985)
153. L E Rabow, G H McGall, J Stubbe, J W Kozarich *J. Am. Chem. Soc.* **112** 3203 (1990)
154. J C Wu, J W Kozarich, J Stubbe *Biochemistry* **24** 7569 (1985)
155. H Sugiyama, C Xu, N Murugesan, S M Hecht *J. Am. Chem. Soc.* **107** 4104 (1985)
156. H Sugiyama, C Xu, N Murugesan, S M Hecht *Biochemistry* **27** 58 (1988)

157. L E Rabow, J Stubbe, J W Kozarich *J. Am. Chem. Soc.* **112** 3196 (1990)
158. R M Burger, J Peisach, S B Horwitz *J. Biol. Chem.* **257** 3372 (1982)
159. M J Absalon, C R Krishnamoorthy, J W Kozarich, J Stubbe *Nucleic Acids Res.* **20** 4179 (1992)
160. E Gajewski, O I Aruoma, M Dizdaroglu, B Halliwell *Biochemistry* **30** 2444 (1991)
161. K Kohda, H Kasai, T Ogawa, T Suzuki, Y Kawazoe *Chem. Pharm. Bull.* **37** 1028 (1989)
162. A M Maxam, W Gilbert *Proc. Natl. Acad. Sci. USA* **74** 560 (1977)
163. A D D'Andrea, W A Haseltine *Proc. Natl. Acad. Sci. USA* **76** 3608 (1979)
164. M Takeshita, A P Grollman, E Ohtsubo, H Ohtsubo *Proc. Natl. Acad. Sci. USA* **76** 5983 (1979)
165. L F Povirk, W Kohnlein, F Hutchinson *Biochim. Biophys. Acta* **521** 126 (1978)
166. C K Mirabelli, W G Beattie, C-H Huang, A W Prestayko, S T Crooke *Cancer Res.* **42** 1399 (1982)
167. J Kross, D W Henner, S M Hecht, W A Haseltine *Biochemistry* **21** 4310 (1982)
168. C K Mirabelli, A Ting, C-H Huang, S Mong, S T Crooke *Cancer Res.* **42** 2779 (1982)
169. V Murray, R F Martin *Nucleic Acids Res.* **13** 1467 (1985)
170. R S Lloyd, C W Haidle, D L Robberson *Biochemistry* **17** 1890 (1978)
171. R S Lloyd, C W Haidle, D L Robberson *Gene* **7** 289 (1979)
172. R S Lloyd, C W Haidle, D L Robberson, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 222
173. C K Mirabelli, C-H Huang, S T Crooke *Biochemistry* **22** 300 (1983)
174. J E Grimwade, E B Cason, T A Beerman *Nucleic Acids Res.* **15** 6315 (1987)
175. P K Mascharak, Y Sugiura, J Kuwahara, T Suzuki, S J Lippard *Proc. Natl. Acad. Sci. USA* **80** 6795 (1983)
176. B Gold, V Dange, M A Moore, A Eastman, G A van der Marel, J H van Boom, S M Hecht *J. Am. Chem. Soc.* **110** 2347 (1988)
177. L D Williams, I H Goldberg *Biochemistry* **27** 3004 (1988)
178. V Murray, L Tan, J Matthews, R F Martin *J. Biol. Chem.* **263** 12854 (1988)
179. R P Hertzberg, M J Caranfa, S M Hecht *Biochemistry* **24** 5285 (1985)
180. M Bene, G Felsenfeld *Proc. Natl. Acad. Sci. USA* **78** 1619 (1981)
181. E C Long, S M Hecht, G A van der Marel, J H van Boom *J. Am. Chem. Soc.* **112** 5272 (1990)
182. W E G Muller, Z-I Yamazaki, H J Breter, R K Zahn *Eur. J. Biochem.* **31** 518 (1972)
183. M T Kuo, L T Auger, G F Saunders, C W Haidle *Cancer Res.* **37** 1345 (1977)
184. C W Haidle, J Bearden, Jr *Biochem. Biophys. Res. Commun.* **65** 815 (1975)
185. C R Krishnamoorthy, D E Vanderwall, J W Kozarich, J Stubbe *J. Am. Chem. Soc.* **110** 2008 (1988)
186. C W Haidle, M T Kuo, K K Weiss *Biochem. Pharmacol.* **21** 3308 (1972)
187. B J Carter, K S Reddy, S M Hecht *Tetrahedron* **47** 2463 (1991)
188. B J Carter, C E Holmes, R B van Atta, V Dange, S M Hecht *Nucleosides Nucleotides* **10** 215 (1991)
189. A Huttenhhofer, S Hudson, H F Noller, P K Mascharak *J. Biol. Chem.* **267** 24471 (1992)
190. M J Absalon, C R Krishnamoorthy, G McGall, J W Kozarich, J Stubbe *Nucleic Acids Res.* **20** 4179 (1992)
191. C E Holmes, B J Carter, S M Hecht *Biochemistry* **32** 4293 (1993)
192. R J Duff, E de Vroom, A Geluk, S M Hecht *J. Am. Chem. Soc.* **115** 3350 (1993)
193. C E Holmes, S M Hecht *J. Biol. Chem.* **268** 25909 (1993)
194. S M Hecht *Bioconjugate Chem.* **5** 513 (1994)
195. M A Morgan, S M Hecht *Biochemistry* **33** 10286 (1994)
196. M Takeshita, A P Grollman, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 207
197. M Takeshita, L S Kappen, A P Grollman, M Eisenberg, I H Goldberg *Biochemistry* **20** 7599 (1981)
198. T Morii, T Matsuura, I Saito, T Suzuki, J Kuwahara, Y Sugiura *J. Am. Chem. Soc.* **108** 7089 (1986)
199. T Morii, I Saito, T Matsuura, J Kuwahara, Y Sugiura *J. Am. Chem. Soc.* **109** 938 (1987)
200. H Sugiyama, G M Ehrenfeld, J B Shipley, R E Kilkuskie, L-H Chang, S M Hecht *J. Nat. Prod.* **48** 869 (1985)
201. H Sugiyama, R E Kilkuskie, L-H Chang, L-T Ma, S M Hecht *J. Am. Chem. Soc.* **108** 3852 (1986)
202. N J Oppenheimer, L O Rodrigues, S M Hecht *Proc. Natl. Acad. Sci. USA* **76** 5616 (1979)
203. N J Oppenheimer, C Chang, L-H Chang, G Ehrenfeld, L O Rodrigues, S M Hecht *J. Biol. Chem.* **257** 1606 (1982)
204. J B Shipley, S M Hecht *Chem. Res. Toxicol.* **1** 25 (1988)
205. Y Nakayama, M Kunishima, S Omoto, T Takita, H Umezawa *J. Antibiot.* **29** 400 (1973)
206. M Kunishima, T Fujii, Y Nakayama, T Takita, H Umezawa *J. Antibiot.* **29** 853 (1976)
207. G M Ehrenfeld, J B Shipley, D S Heimbrook, H Sugiyama, E C Long, J H van Boom, G A van der Marel, N J Oppenheimer, S M Hecht *Biochemistry* **26** 931 (1987)
208. M J McLean, A Dar, M J Waring *J. Mol. Recognit.* **1** 184 (1989)
209. T Suzuki, J Kuwahara, M Goto, Y Sugiura *Biochim. Biophys. Acta* **824** 330 (1985)
210. B J Carter, V S Murty, K S Reddy, S-N Wang, S M Hecht *J. Biol. Chem.* **265** 4193 (1990)
211. M Konishi, K Saito, K Numata, T Tsuno, K Asama, H Tsukuira, T Naito, H Kawagushi *J. Antibiot.* **30** 789 (1977)
212. C K Mirabelli, S Mong, C-H Huang, S T Crooke *Biochem. Biophys. Res. Commun.* **91** 871 (1979)
213. C K Mirabelli, C-H Huang, A W Prestayko, S T Crooke *Cancer Chemother. Pharmacol.* **8** 57 (1982)
214. C K Mirabelli, S Mong, C-H Huang, S T Crooke *Cancer Res.* **40** 4173 (1980)
215. Y Sugano, A Kittaka, M Otsuka, M Ohno, Y Sugiura, H Umezawa *Tetrahedron Lett.* **27** 3635 (1986)
216. A Kittaka, Y Sugano, M Otsuka, M Ohno *Tetrahedron* **44** 2811 (1988)
217. M Otsuka, A Kittaka, M Ohno, T Suzuki, J Kuwahara, Y Sugiura, H Umezawa *Tetrahedron Lett.* **27** 3639 (1986)
218. A Kittaka, Y Sugano, M Otsuka, M Ohno *Tetrahedron* **44** 2821 (1988)
219. M Otsuka, T Masuda, A Haupt, M Ohno, T Shiraki, Y Sugiura, K Maeda *J. Am. Chem. Soc.* **112** 838 (1990)
220. M L Kopka, C Yoon, D Goodsell, P Pjura, R E Dickerson *Proc. Natl. Acad. Sci. USA* **82** 1376 (1985)
221. N Hamamichi, A Natrajan, S M Hecht *J. Am. Chem. Soc.* **114** 6278 (1992)
222. A Fujii, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 75
223. S A Kane, A Natrajan, S M Hecht *J. Biol. Chem.* **269** 10899 (1994)
224. K R Fox, G W Grigg, M J Waring *Biochem. J.* **243** 847 (1987)
225. K R Fox *Anti-Cancer Drug Des.* **5** 99 (1990)
226. K P Nightingale, K R Fox *Biochem. J.* **284** 929 (1992)
227. A Klug, A Jack, O Kennard, Z Shakked, T Steitz *J. Mol. Biol.* **131** 669 (1979)
228. I H Raisfeld, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 324
229. A M Belikova, V F Zarytova, N I Grineva *Tetrahedron Lett.* 3557 (1967)
230. N I Grineva, in *Afinnaya Modifikatsiya Biopolimerov* (Affine Modification of Biopolymers) (Novosibirsk: Nauka, 1983) p. 187
231. D G Knorre, V V Vlassov, V F Zarytova, A V Lebedev *Sov. Sci. Rev.* **12** 271 (1989)
232. J Goodchild *Bioconjugate Chem.* **1** 165 (1990)
233. C Helene, J-J Toulme *Biochim. Biophys. Acta.* **1049** 99 (1990)
234. E Wickstrom (Ed.)*Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS* (New York: Wiley-Liss, 1991)
235. D G Knorre, V V Vlassov, V F Zarytova, A V Lebedev, O S Fedorova, in *Design and Targeted Reactions of Oligonucleotide Derivatives* (Boca Raton, FL: CRC Press, 1994)
236. T S Godovikova, V F Zarytova, T V Mal'tseva, L M Khalimskaya *Bioorg. Khim.* **15** 1246 (1989)

237. T Takita, A Fujii, T Fukuoka, H Umezawa *J. Antibiot.* **26** 252 (1973)
238. T S Godovikova, V F Zarytova, D S Sergeev *Bioorg. Khim.* **17** 1193 (1991)
239. V F Zarytova, D S Sergeyev, T S Godovikova *Bioconjugate Chem.* **4** 189 (1993)
240. D S Sergeev, A Yu Denisov, V F Zarytova *Bioorg. Khim.* **22** 695 (1996)
241. G G Karpova, L O Kozlova, N P Pichko *Izv. Sib. Otd. Akad. Nauk, Ser. Khim. Nauk* (3) 96 (1983)
242. D S Sergeyev, V F Zarytova, S V Mamaev, T S Godovikova, V V Vlassov *Antisense Res. Dev.* **2** 235 (1992)
243. V V Vlassov, V F Zarytova, I V Kutiavin, S V Mamaev, M A Podyminogin *Nucleic Acids Res.* **14** 4065 (1986)
244. E B Brosalina, V V Vlasov, I V Kutyavin, S V Mamaev, A G Pletnev *Bioorg. Khim.* **12** 240 (1986)
245. C Helene *Curr. Opinion Biotechnol.* **4** 29 (1993)
246. O S Fedorova, D G Knorre, L M Podust, V F Zarytova *FEBS Lett.* **228** 273 (1988)
247. T J Povsic, S A Strobel, P B Dervan *J. Am. Chem. Soc.* **114** 5941 (1992)
248. M Takasugi, A Guendouz, M Chassignol, J L Decout, J Lhomme, N T Thuong, C Helene *Proc. Natl. Acad. Sci. USA* **88** 5602 (1991)
249. S A Strobel, P B Dervan *Science* **249** 73 (1990)
250. J-C Francois, T Saison-Behmoaras, C Barbier, M Chassignol, N T Thuong, C Helene *Proc. Natl. Acad. Sci. USA* **86** 9702 (1989)
251. D S Sergeev, T S Godovikova, V F Zarytova *Bioorg. Khim.* **21** 188 (1995)
252. S Arnott, E Selsing *J. Mol. Biol.* **88** 509 (1974)
253. D S Pilch, C Levenson, R H Shafer *Proc. Natl. Acad. Sci. USA* **87** 1942 (1990)
254. L A Basile, J K Barton, in *Metal Ions in Biological System* Vol. 25 (Eds H Sigel, A Sigel) (New York: Marcel Dekker, 1989) p. 31
255. P Hertzberg, P B Dervan *Biochemistry* **23** 3934 (1984)
256. R B van Atta, J Bernadou, B Meunier, S M Hecht *Biochemistry* **29** 4783 (1990)
257. N Murugesan, G M Ehrenfeld, S M Hecht *J. Biol. Chem.* **257** 8600 (1982)
258. N Murugesan, S M Hecht *J. Am. Chem. Soc.* **107** 493 (1985)
259. W J Caspary, C Niziak, D A Lanzo, R Friedman, N R Bachur *Mol. Pharmacol.* **16** 256 (1979)
260. G R Buettner, P L Moseley *Biochemistry* **31** 9784 (1992)
261. L F Povirk *Biochemistry* **18** 3989 (1979)
262. O I Aruoma, B Halliwell, E Gajewski, M Dizdaroglu *J. Biol. Chem.* **264** 20509 (1989)
263. M Dizdaroglu, O I Aruoma, B Halliwell *Biochemistry* **29** 8447 (1990)
264. D S Sergeev, T S Godovikova, V F Zarytova *Bioorg. Khim.* **22** 55 (1996)
265. H Umezawa, in *Bleomycin: Chemical, Biochemical, and Biological Aspects* (Ed. S M Hecht) (New York: Springer, 1979) p. 15
266. K Ueda, S Kobayashi, H Sakai, T Komano *J. Biol. Chem.* **260** 5804 (1985)
267. H Sugiyama, T Sera, Y Dannoue, R Marumoto, I Saito *J. Am. Chem. Soc.* **113** 2290 (1991)