
Chemical reactivity of *E. coli* 5S RNA *in situ* in the 50S ribosomal subunits

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ABSTRACT

E. coli 50S ribosomal subunits were reacted with monoperphthalic acid under conditions in which non-base paired adenines are modified to their 1-N-oxides. 5S RNA was isolated from such chemically reacted subunits and the two modified adenines were identified as A₇₃ and A₉₉. The modified 5S RNA, when used in reconstitution of 50S subunits, yielded particles with reduced biological activity (50%). The results are discussed with respect to a recently proposed three-dimensional structure for 5S RNA, the interaction of the RNA with proteins E-L5, E-L18 and E-L25 and previously proposed interactions of 5S RNA with tRNA, 16S and 23S ribosomal RNAs.

INTRODUCTION

The large subunits of pro- and eukaryotic ribosomes contain one molecule of 5S RNA (1,2). From sequence analysis it is known that these small ribosomal RNAs are 120 nucleotides long and, in general, do not contain modified nucleotides (3). Although a large number of 5S RNA sequences have been determined their secondary and tertiary structure remains unknown (2).

Prokaryotic ribosomes require 5S RNA for their biological activity (4,5). 50S ribosomal subunits reconstituted in the absence of 5S RNA show significantly reduced capacities in the enzymatic binding of aminoacyl-tRNA to the ribosomal A-site. This observation is in accordance with a previous hypothesis which suggested that the T-Ψ-C-G sequence which is strongly conserved in loop IV of tRNAs interacts with the constant C-G-A-A sequence of 5S RNAs (6,7). The proposal that the T-Ψ-C-G sequence in tRNAs is involved in binding to the A-site of the ribosome is further supported by experiments in which this tRNA fragment was shown to inhibit the nonenzymatic (8,9) and enzymatic (10) bind-

ing of tRNAs to the ribosome. Further evidence for this is derived from the observation that T- Ψ -C-G can replace deacylated tRNA in catalyzing pppGpp and ppGpp synthesis in a ribosome and stringent factor dependent system (11).

The evidence for the 5S RNA-tRNA interaction on the ribosome is, however, indirect. It has been observed that the tetranucleotide T- Ψ -C-G or its synthetic analog U-U-C-G binds to a specific E. coli 5S RNA protein complex and not to the free 5S RNA (12). Modification of the 5S RNA, when part of the 50S ribosomal subunit, with monoperphthalic acid results in the modification of two non base paired adenines. 5S RNA thus modified is still capable of interacting with its binding proteins and is incorporated into the 50S ribosomal subunit, however, the ribosomal particles thus formed have 50% reduced activity in protein synthesis. Since the modified 5S RNA-protein complex is now unable to bind T- Ψ -C-G and U-U-C-G, this suggests that the two modified adenines are on the surface of the 50S ribosomal subunit and that they may be involved in the binding of these tetranucleotides.

This paper describes the results of our studies on the analysis of the A residues which have been modified in the 5S RNA by monoperphthalic acid. We show that these two A residues are not the tandem A's present in the CGAA sequence but are A₇₃ and A₉₉. These results are consistent with recent findings (13,14) that ribosomes reconstituted with 5S RNAs, which have been enzymatically cleaved within or in the vicinity of C-G-A-A sequence still possess substantial activity in a poly(U) directed protein synthesis system.

MATERIALS AND METHODS

(a) Ribosomes, reconstitutions and 5S RNA

E. coli (strain A 19) and B. stearothermophilus (strain 799) cells were grown as previously described (15). 70S ribosomes and their 50S and 30S ribosomal subunits were isolated by zonal centrifugation and analyzed for their capacity to synthesize polyphenylalanine in a poly(U) directed, cell free system (16).

Chemically modified E. coli 5S RNA was checked for incorporation and biological activity in B. stearothermophilus 50S recon-

stituted ribosomes (17). E. coli 5S RNA-protein complexes were reconstituted, isolated and characterized as previously described (18). The three 5S RNA binding proteins E-L5, E-L18 and E-L25 were found to bind to normal and N-oxidized E. coli 5S RNA. Isolation procedure for normal and modified 5S RNAs from their 50S ribosomal subunits were as previously published (19). 1mg E. coli 5S RNA (M.W. 40,778) when dissolved in 1ml buffer exhibited 19.2 A_{260} units at 23°C (22).

(b) N-oxidation of E. coli 50S ribosomal subunits

E. coli 50S subunits were N-oxidized with monoperphthalic acid and the 5S RNA isolated as previously described (12). The reaction was carried out in the dark at room temperature in a total volume of 12.0ml which consisted of 6.0ml 50S subunits (10mg per ml, corresponding to 160 A_{260} units per ml) in TMAI buffer, 0.120ml 1.0M phosphate buffer, pH 7.0, 0.06ml 1.0M $MgCl_2$, 1.0ml 1.0M monoperphthalic acid and 5.8ml water. TMAI buffer contained 0.01M Tris-HCl, pH 7.8, 0.01M $MgCl_2$, 0.03M NH_4Cl and 0.006M β -mercaptoethanol. After 5, 15 and 45 minutes 4.0ml of the reaction mixture were diluted with 20ml TMAI and dialysed against 5 litres of the same buffer at 0°C for 6 hrs. After phenol extraction of the 50S ribosomes the 5S RNA was isolated by Sephadex G-100 chromatography (19) and the extent of N-oxidation estimated as described (20,21).

(c) Equilibrium dialysis

Synthesis of 3H labeled U-U-C-G, equilibrium dialysis experiments and calculations of association constants were carried out as published (12,22,23).

(d) Sequence analysis of 5S RNA and identification of N-oxidized adenines in 5S RNA

5S RNA isolated from untreated and monoperphthalic acid treated 50S ribosomal subunits were digested with pancreatic RNase and the oligonucleotides present in such digests were labeled with ^{32}P at their 5'-end as previously described (24,25). The 5'-(^{32}P)-labeled oligonucleotides were separated by fingerprinting (26) and their sequences were established by partial digestion with snake venom phosphodiesterase followed by analysis of the partial digests by two dimensional homochromatography (25,27).

RESULTS

(a) Chemical modification and biological activity of 5S RNA

E. coli 50S ribosomal subunits were treated with monoperphthalic acid under the conditions outlined in Materials and Methods. After incubations of 5, 15 and 45 minutes the reaction was stopped and 5S RNA isolated by phenol extraction and Sephadex G-100 chromatography. The extent of N-oxidation of the 5S RNA was estimated by determining the ratio of absorption at 232 to 259nm and comparing these values with previously established standard curves (20,21). The results are summarized in Table I and indicate that after 5, 15 and 45 minutes 0.9, 1.5 and 2.0 adenines per 5S RNA molecule were converted to their 1-N-oxides.

Reconstitution of B. stearothermophilus 50S subunits with the N-oxidized E. coli 5S RNA yielded ribosomal particles with significantly reduced biological activities (Table I). The 5S RNA modified for 45 minutes containing two adenine 1-N-oxides per molecule produced ribosomal particles with 48% activity in a poly(U) directed polyphenylalanine system. Polyacrylamide gel electrophoresis of the phenol extracted RNA from the reconstituted subunits showed that all modified 5S RNAs were quantita-

Table I
Extent of 5S RNA modification and its biological activity

Time of modification (minutes)	Number of adenine 1-N-oxides per 5S RNA	Biological activity (%)
0	0	100
5	0.9	70
15	1.5	60
45	2.0	48

E. coli 50S ribosomal subunits were modified with monoperphthalic acid, the 5S RNA isolated, the extent of adenine modification determined and their biological activity analysed in a poly(U) directed polyphenylalanine system after incorporation of the 5S RNA in B. stearothermophilus 50S ribosomes. For details see Materials and Methods. 100% biological activity (20moles phenylalanine polymerized per mole 50S ribosomes) corresponds to B. stearothermophilus 50S ribosomal subunits reconstituted with unmodified E. coli 5S RNA.

tively incorporated into the 50S subunit (data not shown).

(b) Oligonucleotide binding to *E. coli* 5S RNA and 5S RNA ν protein complexes

Equilibrium dialysis binding studies of ^3H -labeled U-U-C-G to *E. coli* 5S RNA and the *E. coli* 5S RNA ν L5-L18-L25 protein complex showed that the tetranucleotide binds to 5S RNA only when the latter is in the form of a 5S RNA-protein complex (Table II). The observed association constant (K) was 17,800 L/mol.

When these experiments were repeated with *E. coli* 5S RNA modified for 45 minutes (two adenine 1-N-oxides per 5S RNA), the tetranucleotide was unable to bind to either free 5S RNA or 5S RNA complexed with proteins (Table II). Thus, the modification of two adenines in 5S RNA affects the U-U-C-G binding site in the RNA protein complex.

(c) Identification of the modified adenine residues in 5S RNA

Figure 1 shows the fingerprints obtained from complete pancreatic RNase digests of 5S RNA isolated from untreated and mono-perphthalic acid treated 50S ribosomal subunits: Panel A shows the pattern obtained from untreated 5S RNA, panels B, C and D show the patterns obtained after treatment with mono-perphthalic acid for 5, 15 and 45 minutes respectively. The fingerprints are

Table II
Binding of U-U-C-G to 5S RNA and modified 5S RNA and their protein complexes

<u><i>E. coli</i></u> <u>5S RNA</u>	<u><i>E. coli</i></u> <u>proteins</u> <u>bound</u>	U-U-C-G association constant K (L/mol)
unmodified	-	2300
unmodified	L5-L18-L25	17800
modified	-	2150
modified	L5-L18-L25	2450

Equilibrium dialysis experiments were performed as described under Materials and Methods. Each measurement of the association constant represents the average of at least three independent experiments. The modified *E. coli* 5S RNA used in this experiment was obtained from 50S subunits reacted for 45 minutes with mono-perphthalic acid and contained two adenine 1-N-oxides per molecule.

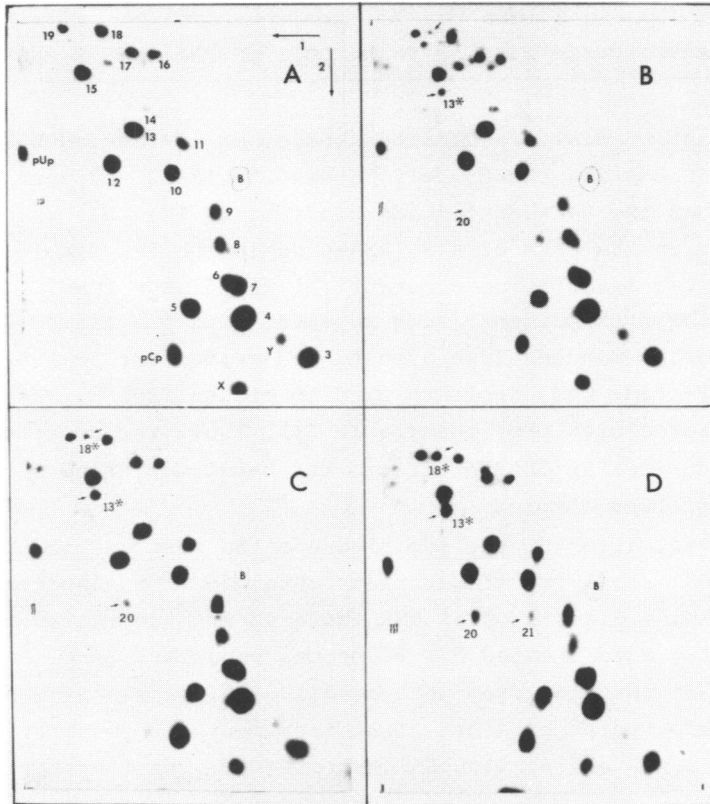


Figure 1: Fingerprints obtained from pancreatic RNase digests of 5S RNA isolated from *E. coli* 50S ribosomal subunits which had been treated with monoperphthalic acid for A, 0min., B, 5min., C, 15min. and D, 45min. New spots appearing during the time course of reaction are indicated by arrows. Numbering of the spots in untreated RNA is according to Brownlee et al. (7).

essentially identical except that two new major oligonucleotides (labeled 13* and 18*) and one minor product (labeled 20*) appear after prolonged exposure to monoperphthalic acid. One additional minor product (spot 21) was present only in the 5S RNA isolated after 45min. treatment. This was not further studied.

As shown below the spot labeled 13* is derived from 13 and that labeled 18* is derived from 18.

The sequence and molar yields of all the oligonucleotides in

the four fingerprints (Fig. 1 A-D) are indicated in Table III. Identification of sequences was based mostly on their location on the the fingerprint (7) and 5'-end group analyses; several of these identifications were also confirmed by partial digestion with snake venom phosphodiesterase followed by analysis by two-dimensional homochromatography (25). The two oligonucleotides whose yield decreases with time of exposure to monoperphthalic acid are 13 (from 1.2mole to 0.18mole) and 18 (from 0.87mole to

Table III
Modification of *E. coli* 5S RNA with monoperphthalic acid

Spot No.	Sequence	5S RNA (theoretical)	5S RNA (control)	5S RNA (5Min.)	5S RNA (15Min.)	5S RNA (45Min.)
3	AC	1	2.0	2.4	1.4	1.8
4	GC	7	6.4	7.2	9.1	9.2
5	AU	3	2.8	2.9	2.9	3.2
6,7	GAC,AGC	1+2	3.6	3.8	3.5	3.1
8	GAAC	1	1.0	1.6	1.0	1.0
9	GAAAC	1	0.9	0.9	1.6	1.6
10	GGC	2	2.1	1.8	2.3	2.7
11	AGGC	1	0.9	0.9	0.9	1.1
12	GU	2.5	3.6	4.8	3.4	4.0
13	GAU	1	1.3	1.4	0.7	0.3
14	AGU	1	1.6	1.8	1.7	1.9
15	GGU	3	3.0	2.9	3.5	3.5
16	AGGGAAC	1	0.7	0.7	0.6	0.6
17	AGAAGU	1	0.8	1.0	1.1	1.0
18	GAGAGU	1	0.9	0.9	0.8	0.5
19	GGGGU	1	0.5	0.4	0.6	0.7
13*	GA*U	-	-	0.3	0.5	1.2
18*	GA*GAGU	-	-	-	0.2	0.5
20*	A*U	-	-	-	0.2	0.6

Oligonucleotide Sequences and their Molar Yields in Fingerprints (Fig. 1, A-D) of pancreatic RNase digests of *E. coli* 5S RNA isolated from 50S ribosomal subunits treated for A, 0min., B, 5min., C, 15min. and D, 45min. with monoperphthalic acid. Spot numbers are taken from Figure 1. Molar yields of oligonucleotides were estimated by Cerenkov counting of excised fingerprint spots. The molar yields of spots 13 and 14 were calculated from the ratio of G to A 5' end groups in material from spots 13 + 14.

0.51mole). The oligonucleotides that appear during the time course of exposure to monoperphthalic acid follow the reverse kinetics, those of 13* being inversely proportional to 13, and of 18* to 18.

Figure 2 shows the two-dimensional homochromatographic pattern of partial snake venom phosphodiesterase digests on spots 13* and 18*, and of spot 18 from untreated sample. Spot 13* is

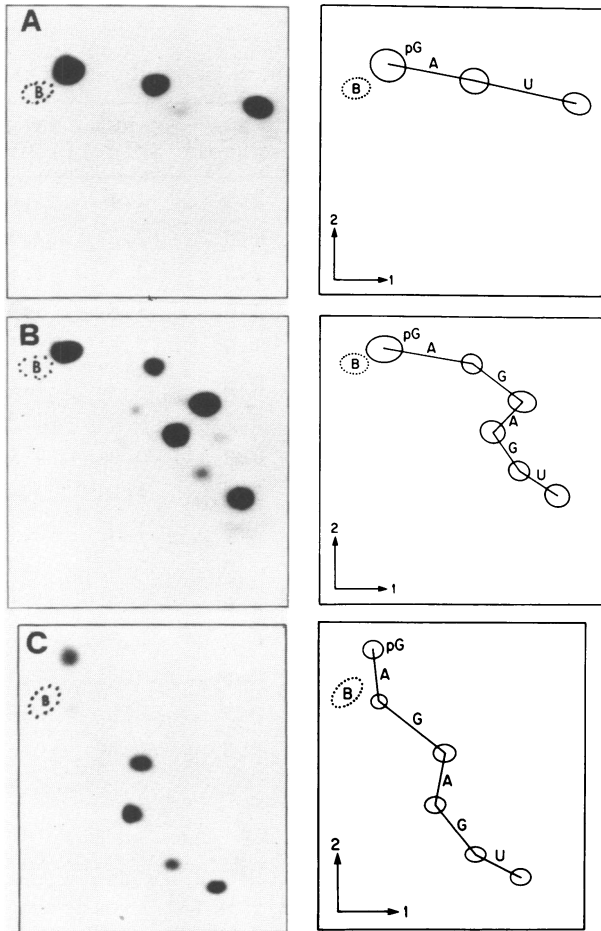


Figure 2:
Two-dimensional homochromatographic analysis of partial snake venom phosphodiesterase digests on spots 13*, 18* and 18 of Figure 1. A, 13*, B, 18* and C, 18.

characterized as ^{32}P G-A*-U (A*, adenosine-1-N-oxide) and spot 18* as ^{32}P G-A*-G-A-G-U. From a comparison of the patterns obtained from ^{32}P G-A*-G-A-G-U (Fig. 2B) and ^{32}P G-A-G-A-G-U (Fig. 2C) it is seen that the mobility shift due to pA* is quite different from that of pA or of any one of the other three commonly occurring ribonucleotides. This mobility shift is essentially like that of pG in the first dimension (electrophoresis on cellulose acetate at pH 3.5) but is more like that of a pyrimidine nucleotide in the second dimension (homochromatography). Based on the position of spot 20* on the fingerprint, we infer it to be ^{32}P A*-U.

Since pancreatic RNase digestion of *E. coli* 5S RNA yields only one mole each of ^{32}P G-A-U and ^{32}P G-A-G-A-G-U (Table III), the identification of ^{32}P G-A*-U and ^{32}P G-A*-G-A-G-U, as the major products of modification of 5S RNA, allows us to identify the A residues which are the primary targets of monoperphthalic acid reaction as A₇₃ and A₉₉.

DISCUSSION

Using in vitro labeling methods (24,25) we have identified the two A residues of *E. coli* 5S RNA which are most accessible in the 50S ribosomal subunit to chemical modification by monoperphthalic acid as A₇₃ and A₉₉. These two A residues are not the tandem As present within the sequence C-G-A-A₄₆-, which has been postulated to interact with the constant T-Ψ-C-G-sequence of tRNAs during the binding of aminoacyl-tRNAs to the ribosomal A site, but are among those nucleotides which are strictly conserved in all prokaryotic 5S RNAs (3,28,33). Since the modified 5S RNA analyzed was generated by reaction of 50S ribosomal subunits with monoperphthalic acid, it implies that A₇₃ and A₉₉ are exposed in the ribosomal subunit. In this context it is interesting to note from the analysis of spot 18* (Table III) that A₉₉ is accessible to modification by monoperphthalic acid while A₁₀₁ is totally unreactive.

The answer to the question why the two modified adenines prevent the binding of UUCG to C₄₃GAA₄₆ of the 5S RNA in the protein complex, may possibly be found in so far not well understood "long range" structural effects. Such "long range" structural ef-

fect was recently demonstrated for E. coli 5S RNA in which a transition from the C in position 92 to a U was described (34).

The N-oxidation results are at variance with recent slow-tritium-exchange studies in which G₄₁, A-C₃₅ and A-A-C₁₁₀ of 5S RNA were most accessible within the 70S ribosome (29). The differences observed may be due to the fact that in the latter study 70S ribosomes, instead of 50S ribosomal subunits were analyzed, or that in the N-oxidation and in slow-tritium-exchange studies two different kinds of "accessibility" are tested.

Since A₇₃ was quantitatively modified in the 5S RNA and A₉₉ to only about 50%, this suggests that A₇₃ is more accessible than A₉₉ in the 50S ribosomal unit. The question of whether one or both of these modifications lead to a reduction in the biological activity of the 5S RNA cannot be clearly answered at the moment. Since the N-oxidized 5S RNA-protein complex has quantitatively lost its ability to bind the tetranucleotide U-U-C-G- it is tempting to speculate that modification of A₇₃ is mainly responsible for this loss in activity.

The molecular basis by which modification of A₇₃ and A₉₉ results in a loss of the biological activity of E. coli 5S RNA is unknown. Some of the possibilities may be discussed in conjunction with a recently proposed three-dimensional structure model for E. coli 5S RNA (Figure 3). It is of interest to note that the two modified adenines, which are 26 nucleotides apart in the primary structure, are only 10-15 Å apart in the three-dimensional model, and on the surface. It is known that the 1-N-oxides of adenines tend to destack polynucleotides, thus, the modification may interfere with the precise interaction of the 5S RNA binding proteins (Figure 3) with 5S RNA, and thereby lead to a reduction in biological activity.

Other alternatives, for example the modified adenine residues may interfere with proposed base paired interactions involving 5S RNA and 23S RNA (30,31) and/or 16S RNA (32) are also possible (Figure 3). The N-oxidation results presented here are also in agreement with the proposed 16S RNA (32) and 23S RNA (31) interactions involving nucleotides 90 to 100 and 69 to 77 respectively of the 5S RNA. It is interesting that in both types of interaction A and A are "looped out" and therefore expected to be

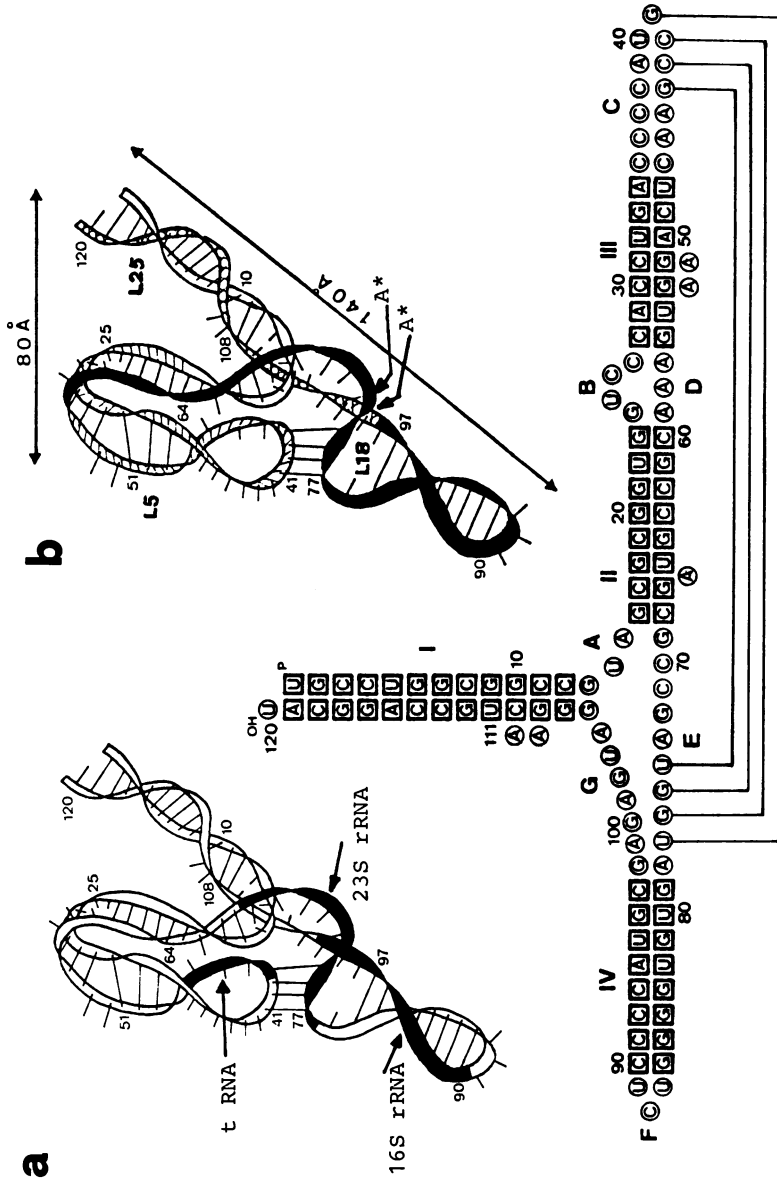


Figure 3: Three-dimensional 5S RNA structural model for *E. coli* 5S RNA (28,33). (a) shows previously proposed interaction sites for tRNA, 16S rRNA and 23S rRNA on *E. coli* 5S RNA and (b) the binding sites for the 5S RNA binding proteins E-L5, E-L18 and E-L25. A* denotes the two adenines modified by monoperphthalic acid to their 1-N-oxides when part of the 50S ribosomal subunit. For more details see Discussion.

especially reactive. Because of the above mentioned destacking effect of N-oxidized adenines it would again be possible to envision a negative influence of the modified adenines on 5S RNA and on its interaction with either 16S or 23S rRNAs.

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§On the occasion of his 60th birthday, we would like to dedicate this paper to Professor Friedrich Cramer

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