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A new RNA-RNA crosslinking reagent and its application to ribosomal 5S RNA

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ABSTRACT

The synthesis of a new RNA specific bifunctional crosslinking reagent, 1.4-phenyl-diglyoxal, is described which reacts exclusively with guanosines.

The properties of the crosslinked products enabled us to develop a straightforward method for identifying the reacted nucleotides.

Results obtained with ribosomal 5S RNA of Escherichia coli demonstrate the formation of an intramolecular crosslink between guanosine-2 and guanosine-112 in the stem region.

INTRODUCTION

Crosslinking of macromolecules with chemical reagents of defined length and specificity is a direct method for investigating structure (e.g. 1-3). Several reagents have been employed for crosslinking protein-protein or protein-RNA components. However, apart from the highly reactive and unspecific sulfur mustards (4), the only reagents used for RNA-RNA crosslinking are photosensitive (e.g. 5-7). All of these reagents render localisation of RNA attachment points very difficult because (i) the reagents are not base-specific and (ii) the nucleotides are irreversibly modified. One base-specific reagent has been described, very recently, for crosslinking RNA to DNA (8). However, the extreme chemical conditions required for activating the reagent render it unsuitable for RNA topographical studies.

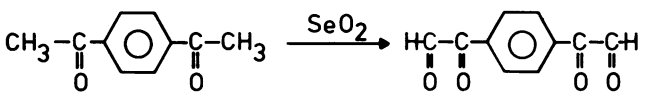
We describe here the synthesis and use of the reagent 1,4-phenyl-diglyoxal which is both guanosine specific, and readily dissociable, such that the crosslinked nucleotides can be identified by a simple RNA sequencing procedure. Modification studies on the native and denatured conformations of 5S RNA with guano-

sine-specific kethoxal, identified 8 to 12 guanosines that could be modified in each conformation (9). Therefore, 5S RNA was selected for intramolecular crosslinking studies with the new reagent.

MATERIALS AND METHODS

Synthesis of 1.4-phenyl-diglyoxal (PDG)

1.4-phenyl-diglyoxal was prepared according to the following scheme:



1.4-Diacetyl-benzene **1.4-Phenyl-diglyoxal (PDG)**

1.62 g (10 mM) 1,4-diacetylbenzene and 2.22 g (20 mM) SeO_2 were suspended in 10 ml dioxan. 0.2 ml water was added and the mixture was refluxed for 6 hr and cooled overnight. The suspension was diluted with 10 ml hot dioxan and filtered. The yellow filtrate was evaporated, dissolved in 50 ml water, boiled for 10 min, filtered again and boiled for 2 min with charcoal to remove traces of selenium. The filtrate was cooled and the crystals that formed were recrystallised three times from 5% acetic acid and then from water.

Characterisation of the new compound

The product was free from starting material and migrated as a single spot on thin-layer chromatography in solvent systems: A. acetone and acetylacetate (1:1) saturated with water. R_f = 0.84. B. butanol and acetic acid (1:1). R_f = 0.82. It decomposed at 129-130°C.

The ultraviolet spectra, in Fig. 1, show characteristics typical of phenyl glyoxal compounds (10), namely both the red shift of the phenyl residue and the disappearance of the carbonyl shoulder, at high pH's. Like other glyoxal compounds PDG reacted exclusively with guanosine (11).

Reaction of PDG with ^{32}P 5S RNA

^{32}P 5S RNA from Escherichia coli MRE 600 was fractionated

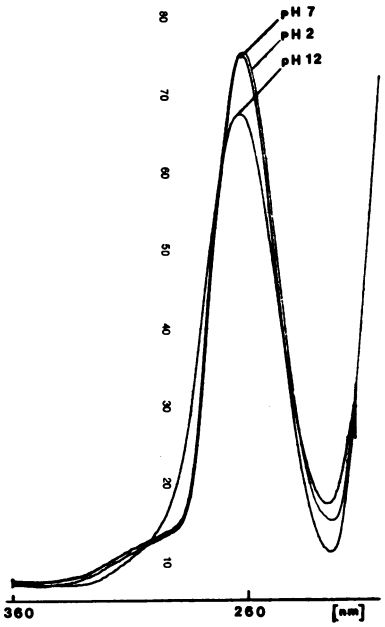


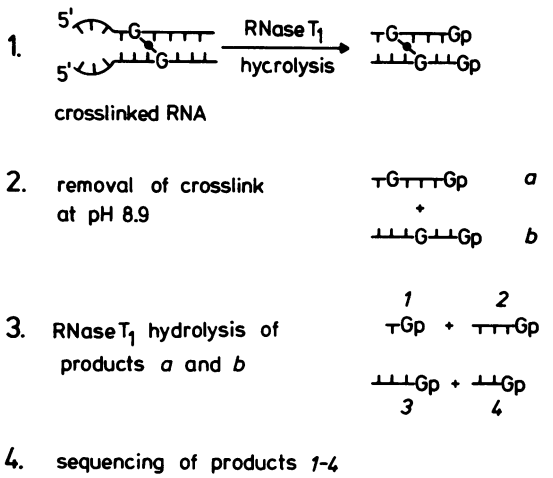
Figure 1: Ultraviolet spectra of PDG at different pH values.

on a LiCl-dodecylsulphate sucrose gradient as described by Fellner (12) and separated from tRNA electrophoretically by the method of Aubert et al. (13). The 5S RNA was renatured according to Aubert et al. (14). The denatured conformation was also prepared as described earlier (13,14). Each conformation was shown to be stable, under the chemical crosslinking conditions, by an electrophoretic method (14).

In a typical crosslinking experiment 0.3 A_{260} units (10^7 cpm) of ^{32}P 5S RNA in 70 mM Na cacodylate, pH 7.2, 20 mM $MgCl_2$, and 0.3 M KCl was reacted with 2.5 mM reagent in a total volume of 0.5 ml for 5-6 hr at 4°C.

Principle of the analysis of the crosslinked nucleotides

The following properties of the crosslinked products were exploited in the analyses. (i) The reaction is guanosine specific, (ii) the phosphodiester bond between the modified guanosine and the adjacent 3'-nucleotide is resistant to RNase T_1 hydrolysis and (iii) the chemical crosslink is stable at neutral pH, in the presence of borate, but can be removed by treatment at pH 8.9 for 2 hr at 37°C. When a cross-link is formed, at least two pairs



of oligonucleotides are produced, in equimolar amounts, after the second RNase T_1 cleavage. The analyses were performed in 4 steps as shown in the scheme.

Analysis procedure

After the reaction the 5S RNA samples were precipitated with 2 volumes ethanol, three times, to remove excess reagent. The pellet was lyophilised from 0.2 ml water, dissolved in 20 µl of 20 mM Tris-borate, pH 7.4, 8 mM EDTA and digested with 50 units RNase T_1 for 60 min at 37°C. The sample was adjusted to 8 M urea and 20% sucrose and loaded on a 15 to 20% compound polyacrylamide gel in 90 mM Tris-borate, pH 7.4, 3 mM EDTA, 8 M urea (40 x 20 x 0.2 cm). Electrophoresis was continued for 20 hr at 4°C and 30 mA.

The higher molecular weight bands were excised from the gel and dialysed 3 hr against 8 M urea containing bromophenol blue. The gel pieces were applied to a second gel (22% acrylamide, 8 M urea) and repurified at 40 mA for 16 hr in 90 mM Tris-borate, pH 7.4, 3 mM EDTA. The oligonucleotides were electrophoresed from the gel piece onto DE-paper (10) and they were eluted from the DE-paper with 30% triethylammonium carbonate at pH 9 when the crosslink was completely dissociated. The samples were divided. One half was applied, directly, to DE-paper (40 cm x 1 m) as a 1 cm line and the other half was again digested with 0.1 mg/ml RNase T_1 for 30 min at 37°C prior to DE-paper electrophoresis.

A total T_1 RNase hydrolysate of 5S RNA was run as a marker. The oligonucleotides were separated at 100 mA for 16 hr using a procedure similar to that described earlier (15), and detected by autoradiography.

RESULTS

Bifunctionality of the reagent

The capacity of the reagent to react with two guanosines was examined. It was incubated with ^{3}H-guanosine and tRNA which is known to contain accessible guanosines (e.g. 16). The results in Fig. 2 demonstrate that at the highest reagent concentration tested, 5 moles of ^{3}H-guanosine were crosslinked per mole of tRNA.

Chemical crosslinking of 5S RNA

The native and denatured conformations of 5S RNA were reacted as described in Methods and then digested with RNase T_1. The products were fractionated electrophoretically as shown in Fig. 3. A series of relatively high molecular weight bands were present in the crosslinked native 5S RNA sample that were not present in the untreated control sample (Fig. 3A). Only three weak extra bands were detected in the denatured form (Fig. 3C).

Each of the bands was analysed, from both the native and denatured samples, using the one-dimensional separation system illustrated in Fig. 4. Almost all of the oligonucleotides are resolved by this procedure and are numbered according to an earlier

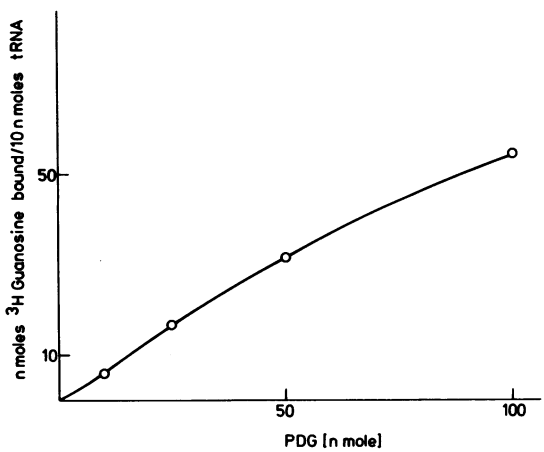


Figure 2: Crosslinking of ^{3}H-guanosine to tRNA. 2.8 µM ^{3}H guanosine and 10 nM tRNA were reacted with increasing amounts of PDG for 5 hr at 20°C in 50 mM Tris-borate, pH 7.4. The products were separated by descending paper chromatography in the solvent butanol:acetic acid:water (5:2:3).

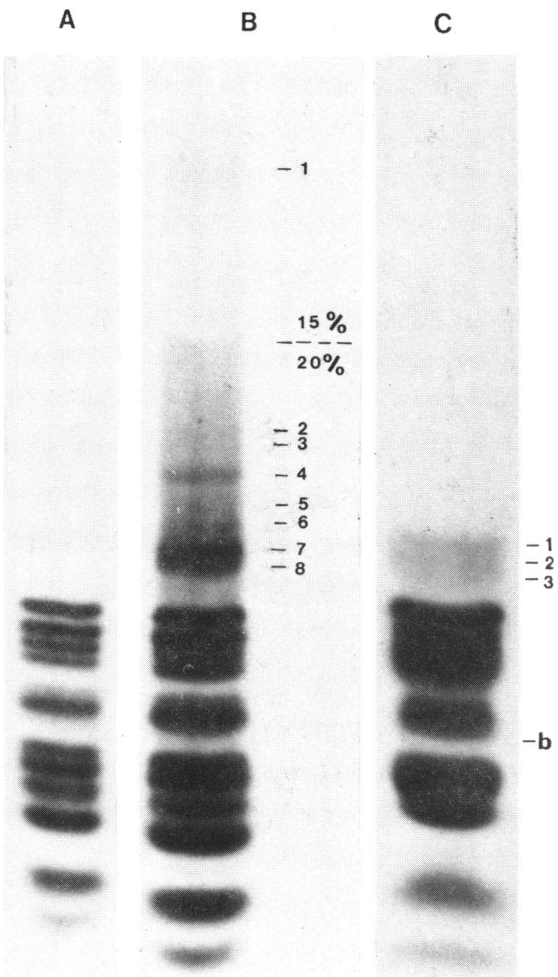


Figure 3: Autoradiogram of the gel fractionation products of 5S RNA after modification and RNase T_1 hydrolysis. A. Unmodified 5S RNA. B. Native 5S RNA after reaction with PDG, and C. denatured 5S RNA after reaction with PDG. The gel in C was not from the same experiment as those in A and B. However, it was also run with a control sample that was indistinguishable from that in A. The origin corresponds to the top of the autoradiogram and b denotes the position of the bromophenol blue marker.

used convention (17). The small oligonucleotides 1 to 5 and 10 (UAG) are redundant in the 5S RNA sequence (17) but this did not complicate the present analyses.

One criterion for the formation of a crosslink, namely the presence of four oligonucleotides in approximately equal amounts, after the second RNase T_1 hydrolysis, was satisfied for bands 1, 2, 6 and 7. After purification in a second gel, the most homogeneous product was from band 2 which contained oligonucleotides 7, 9, 12 and 18 (Fig. 4B). All of the analyses for this product are shown in Fig. 4 and the sequence data are summarised in Table 1.

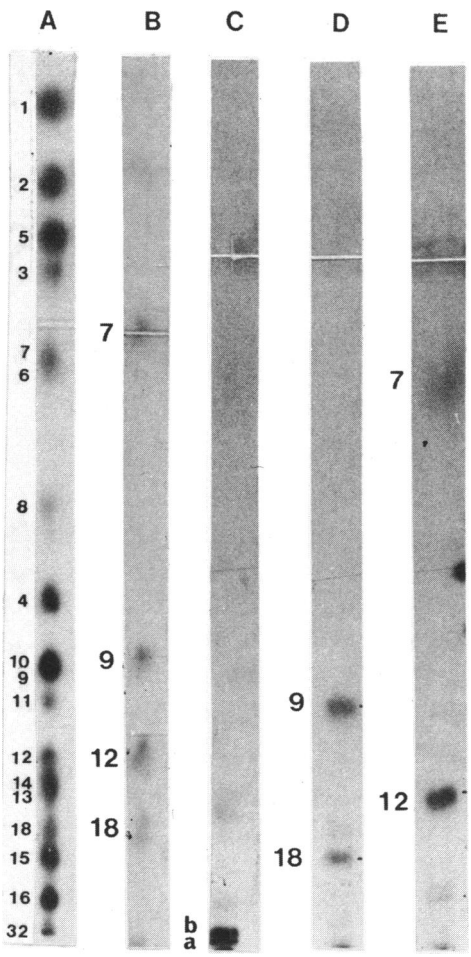


Figure 4: Autoradiogram of oligonucleotides from band 2 in Fig. 3B. A. RNase T_1 digest of 5S RNA. B. Products after removal of the crosslink and RNase T_1 hydrolysis. C. Products after removing the crosslink with no RNase T_1 treatment. D. RNase T_1 hydrolysis products from band a. E. RNase T_1 hydrolysis products from band b. The oligonucleotides were separated on DE-paper. They are numbered according to the system of Brownlee et al. (17).

The electrophoretic mobility of band 2 in Fig. 3B is consistent with a size of 15 nucleotides. Therefore, it can be excluded that modified but non cross-linked oligonucleotides were co-migrating. Moreover, the modified oligonucleotide $AAC\overset{112}{U}GCCAG$ is also found in the much faster migrating band 7.

The yield of the crosslinked product in band 2 was generally 3 to 5%. It could be argued, therefore, that the crosslink occurs in a partly denatured population of the 5S RNA sample which is present in small amounts even in carefully renatured 5S RNA samples. However, no corresponding band was found in the denatured 5S RNA sample demonstrating that the crosslink occurs in the

Table 1: The identities of the crosslinked nucleotides in band 2 of Fig. 3B that were analysed on DE-paper in Fig. 4.

products after removal of the reagent	oligonucleotides after RNase T_1 treatment	nucleotide sequence	crosslinked guanosines
a	18	pUG_p	$pUG^{2}CCUG$
	9	$CCUG_p$	
b	12	AACUGp	$AACUGCCAG^{112}$
	7	CCAGp	

native 5S RNA conformation (Fig. 3C). Bands 1 to 3 from the denatured 5S RNA (Fig. 3C) were all mono-addition products.

Finally, the possibility that an intermolecular, rather than an intramolecular crosslink, had been formed could also be excluded. Modified 5S RNA samples were run in 10% gels, prior to RNase T_1 digestion, as described in Methods, and no dimers or higher aggregates were detected.

There is tentative evidence for other crosslinks in addition to G^{2}-G^{112}, namely between G^{24}-G^{86} (in band 1) and possibly G^{41}-G^{75}, and G^{41}-G^{83} (in bands 6 and 7, respectively). However, the latter bands were contaminated with other products and we have been unable to eliminate the possibility, so far, that modified but non-crosslinked oligonucleotides were co-migrating. There is also the possibility that some crosslinks have gone undetected. For example, if a crosslink occurs to a guanosine residue with an adjacent unmodified guanosine on its 3'-side it might be difficult to detect. The lower bands in Fig. 3B (and 3C), namely those migrating ahead of band 8, were examined for the presence of very small crosslinked products but none were detected.

DISCUSSION

The crosslink reaction within the double-helical stem region of the 5S RNA, demonstrates an interesting property of the PDG reagent. Whereas kethoxal and similar aliphatic dicarbonyl reagents only react with non base-paired guanosines, the present

reagent is able, due to its aromatic character, to intercalate into double-helical RNA regions producing crosslinks of neighbouring guanosines.

The crosslink of G^2-G^{112} was, nevertheless, unexpected since if the stem region of 5S RNA is double-helical, and there is strong evidence to support this, including sequence conservation and oligonucleotide binding studies (18), and chemical modification results (9), then the G^2 and G^{112} would be 5 base-pairs apart. A three dimensional model building study (see Fig. 5) demonstrated, however, that a crosslink between G^2 and G^{112} was possible with the small reagent used because the guanosines would be in direct neighbourhood across the large groove of a double-helix. It does require, though, that the two bases unstack and protrude out of the helix (Fig. 5). This mode of action may be facilitated in the stem region of the 5S RNA by flexibility or breathing around the 3'- and 5'-ends of the 5S RNA (G^2) and at the G-U base-pair adjacent to G^{112}.

Although the identified crosslink occurred in a double-he-

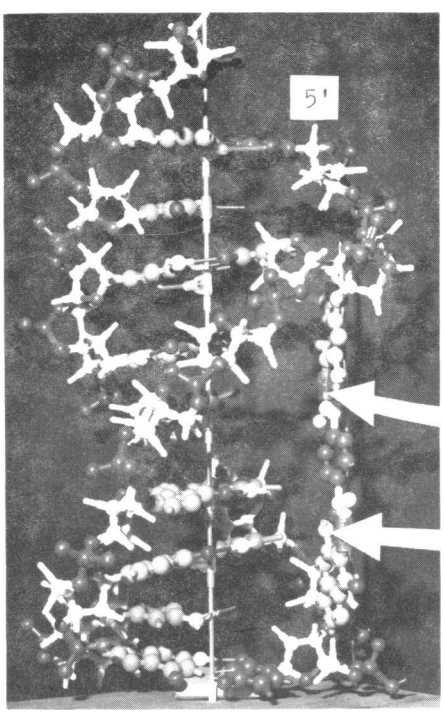


Figure 5: Three dimensional model of the 5S RNA stem region with G^2 and G^{112} crosslinked by PDG. Arrows indicate the N-1 nitrogens of the guanosine residues that are crosslinked. The arrows are 15 Å apart which corresponds to the length of the PDG reagent. The model was built assuming a minimum of distortion around the crosslinked guanosines.

lical region of the RNA, most of the modifications, and the other putative crosslinks in bands 1, 6 and 7 of Fig. 3B, occurred at non base-paired guanosines that were also modified by kethoxal (9). For example. the major modification product was band 8 containing the oligonucleotide ACCCCAUGCCG and G^{41} is one of the most accessible unpaired guanosines in 5S RNA (e.g. 9, 15). Therefore, the reagent, derivatives thereof, and the methodology described have considerable potential for topographical investigations of larger and more complex RNA structures.

The analysis of intramolecular RNA crosslinks is complicated by the presence of large amounts of mono-addition products which can lead to ambiguities in the interpretation. The above outlined method is no exception because the low molecular weight hydrolysis products are poorly resolved in polyacrylamide gels (see Fig. 3B). This particular problem could be circumvented by selectively removing modified but non-crosslinked oligonucleotides prior to gel fractionation, possibly by coupling the mono-addition products to an affinity support carrying covalently bound guanosines. Attempts to achieve this and to confirm,or refute,the putative crosslinks in bands 1, 6 and 7 of Fig. 3B are in progress.

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