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A PEPTIDE-ACRIDINE CONJUGATE WITH RIBONUCLEOLYTIC ACTIVITY

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Abstract: A tetrapeptide-intercalator conjugate has been designed to function as a ribonuclease mimic. This activity is demonstrated by electrophoretic and chromatographic analysis using ribosomal RNA as the substrate.

Messenger RNA is a potential target for gene therapeutic applications. Expression of a particular protein can be inhibited by scission of the corresponding mRNA, using an antisense DNA carrying an active compound for the cleavage reaction. Several compounds have been attached to oligonucleotides for this purpose, including transition-metal complexes,[1,2] alkylation groups,[3-5] enzymes,[6-8] chemical reactive groups[9] and photosensitive groups.[10] Using imidazole or histidine to get site-selective cleavage also has been proposed.[11] A new type of catalytic molecular scissors, mimicking the active site of ribonuclease A (RNase A), is now reported.

The RNAse A catalyzed RNA hydrolysis is believed to be a two step reaction.[12] In the first step, the RNA chain is cleaved and a cyclic 2',3'-phosphoribose intermediate is obtained. In the second step, the cyclic intermediate is opened to form the ribose-3'-phosphate. Some ß-cyclodextrin complexes containing two imidazole rings have been synthesized and have been demonstrated to possess hydrolytic capability on particular RNA model compounds.[13] Based on these results, a tetrapeptide-acridine complex, His-Pro-His-Lys(2-methylacridine-9-methylene)-NH_2, has been designed as a potential ribonuclease mimic. The first three amino acids of the peptide, His-Pro-His, were designed to function as the His-119 and His-12 residues at the active site of RNAse A. In this design, the imidazole groups on the peptide would act as both acid and base. In the presumed mechanism, the first histidine would contact the phosphate group, while the other histidine would approach the 2'-hydroxyl group. Thereafter, the P-O(5') bond would

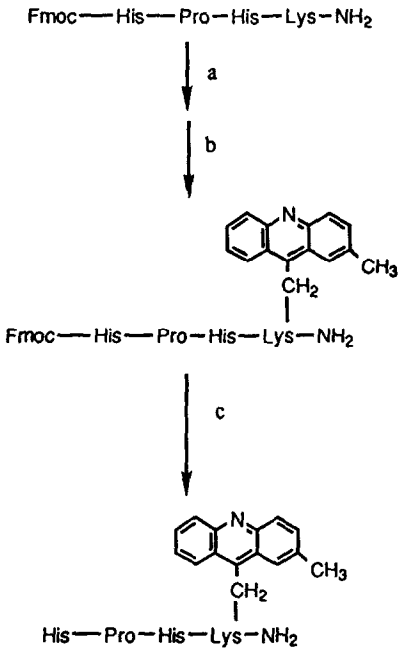

Figure 1: Synthetic scheme of His-Pro-His-Lys(Acr)-NH_2. (a) 2-methyl-9-acridinecarboxaldehyde, N,N-diisopropylethylamine, anhydrous methanol, 2 hrs.; (b) $NaCNBH_3$, 1 hr.; (c) piperidine.

be cleaved through acid-base catalysis. The placement of proline in the middle of the tripeptide would limit the conformational space of the peptide, thereby adjusting the histidine residues to the desired positions. The last amino acid, lysine, on the tetrapeptide was used as a linker to connect the intercalator, 2-methyl acridine (Acr). The intercalator was used as a carrier[14] to increase the effective concentration of the peptide catalyst on the RNA substrate. Another important role of the intercalator may be to spatially orient the peptide, thereby improving its catalytic potency. The C-terminus of the peptide was converted into the neutral amide to prevent any unwanted charge interactions.

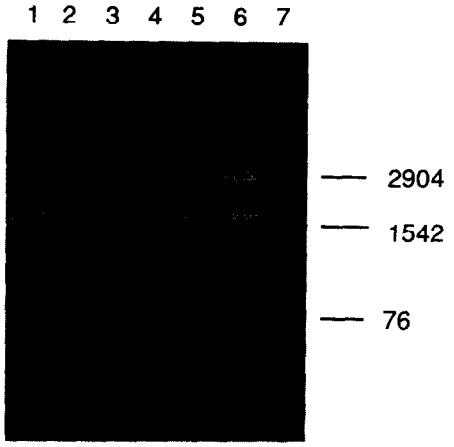

Figure 2: Cleavage of 23S (2904 bases) and 16S (1542 bases) rRNA was tested using 2-methyl-9-acridinecarboxaldehyde, His-Pro-His-Lys-NH_2 and His-Pro-His-Lys(Acr)-NH_2. To each well, 0.1 A_{260} Unit of rRNA was reacted with test compounds in PBS buffer at 37°C. The reaction mixture was denatured in a solution of 25% formamide and 9% formaldehyde at 55°C for 15 min., and then electrophoresed through a denaturing (18% formaldehyde) agarose gel (1.0 %), followed by staining with ethidium bromide. Lane 1: 200 μM of His-Pro-His-Lys-NH_2; Lanes 2 and 3: 200 μM of His-Pro-His-Lys(Acr)-NH_2; Lane 4: 200 μM of 2-methyl-9-acridinecarboxaldehyde; Lane 5: 36 hours negative control (rRNA alone); Lane 6: 0 hours negative control; Lane 7: molecular weight marker $tRNA^{Phe}$ (76 nucleotides). Samples in lane 1, 3, 4 and 5 had been incubated at 37°C for 36 hours, whereas that in lane 2 had been incubated for 18 hours.

The side-chain protecting groups on the tetrapeptide-amide were removed, but the Fmoc (9-fluorenylmethyloxycarbonyl) protecting group on the N-terminus was saved after peptide synthesis.[15] The 2-methyl-9-acridinecarboxaldehyde was attached to the lysine side chain under reductive conditions (Figure 1). The product was purified by reverse-phase HPLC, using an Fmoc-on and Fmoc-off two step purification procedure.[16]

Random cleavage of ribosomal RNA 23S and 16S (E. coli R13) was detected by two different techniques, denaturing electrophoresis and gel-permeation chromatography. Ribosomal RNA (0.1 A_{260} Unit) was mixed with 200 μM of either 2-methyl-9-acridinecarboxaldehyde, His-Pro-His-Lys-NH_2 or

His-Pro-His-Lys(Acr)-NH_2 in phosphate-buffered-saline solution (PBS) (pH 7.4). These concentrations were equivalent to a ratio of tested compound to nucleotide of 2 to 5. The reaction mixture was denatured at 65°C for 10 min., and then incubated at 37°C in the dark for 36 hours. According to denaturing agarose gel electrophoresis (Figure 2), peptide alone did not contribute significantly to rRNA cleavage. The peptide-acridine complex, however, did show considerable ribonuclease activity. After 18 hours incubation, there was still a minor amount of undegraded rRNA, but at the 36 hour time point, the rRNA was thoroughly degraded. In contrast, rRNA alone and rRNA incubated with peptide (i.e. no appended acridine) or with 2-methyl-9-acridinecarboxaldehyde (i.e. no peptide) remained intact (Figure 2).

The possibility of altered electrophoretic migration due to interactions between the peptide-acridine and rRNA was also considered. When the gel was observed under 254 nm UV light, the peptide-acridine was seen above the wells, migrating toward the cathode (data not shown). This indicated that interactions between the peptide-acridine and the rRNA had been eliminated under denaturing and high voltage conditions.

Table 1: Gel-permeation analysis of rRNA cleavage.

Tested compound	Difference in retention time (min.)
His-Pro-His-Lys-NH_2	0.2
His-Pro-His-Lys(Acr)-NH_2	3.4
2-methyl-9-acridinecarboxaldehyde	0.3
36 hrs. Control	0.3

These results were confirmed by gel-permeation chromatography using a denaturing eluent.[17] The starting rRNA gave a broad peak at 19.6 min; the 16S and 23S rRNA cannot be separated by this column. Although the column did not give exceptional resolution, the molecular weight changes could be easily observed by peak shifts to longer retention times. The differences in retention times are given in Table 1. In the control experiment, a slight peak shift was found (i.e. 0.3 min.). It is not known whether this is due to a small amount of non-specific degradation or represents a conformational change. Samples treated with 2-methyl-9-acridinecarboxaldehyde and the peptide without acridine also displayed 0.2 to 0.3 min. shift, indicating no ribonuclease activity. The peptide-acridine conjugate, however, produced a 3.4 min shift in the chromatrogram, indicating significant rRNA cleavage and confirming the electrophoresis results.

The mechanism of cleavage is still under investigation. The hydrolytic activity of the peptide-acridine conjugate was found to increase with decreasing pH and to be optimal at about pH 6.0. This is contrary to studies using imidazole buffer and metal ion chelation complexes, in which hydrolytic activity was found to increase steadily above pH 7 and to be maximal at pH 7.8.[12,18] Accordingly, trace metal ion impurities chelated by the peptide-acridine compounds might not be involved in hydrolytic cleavage.

In conclusion, we have successfully mimicked the enzyme ribonuclease using a tetrapeptide-acridine complex. Random cleavage of rRNA was achieved under physiological temperature and buffer conditions. This type of compound is a potential candidate for site-selective oligonucleotide cleavage.

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15. The peptide was synthesized by standard Fmoc chemistry on a peptide synthesizer. The amino acids were coupled to the PAL^{TM} support by BOP and HOBt. Side chain protecting groups of lysine and histidine, Boc and trityl groups, were removed by TFA at the same time when the peptide was cleaved from the solid support. The products were confirmed by amino acid analysis and peptide sequencing.
16. Vydac 218TP1010, reverse-phase C-18 column (1.0x25 cm) was used in HPLC purification. Flow rate was 3 mL/min. Solution A contained 0.1% TFA and solution B contained 70% acetonitrile, 20% isopropyl alcohol, 10% buffer A. The gradient was: 0-5 min 0% B, 5-10 min. 0-10% B and 10-50 min. 10-50% B. The retention times of peptide-acridine complex with and without the Fmoc group were 42 min. and 21 min., respectively.
17. Column (1.0 x 45 cm) was packed with Sephacryl S-400 HR. Elution buffer was 1.5 M NaCl in 50% of 20 mM sodium phosphate buffer and 50% of formamide, pH adjusted to 7.0. Flow rate was 1 mL/min. The UV detector was set at 270 nm. After the reaction, the mixture was denatured with elution buffer at 55°C for 10 min., and then applied directly onto the Sephacryl column.
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