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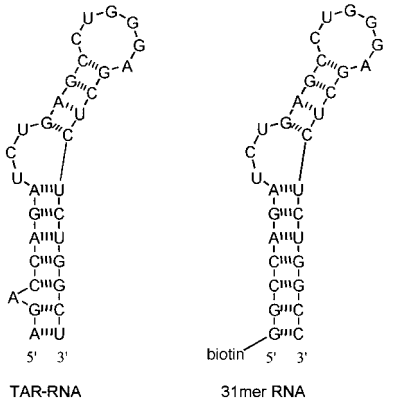
Selective Cleavage of the HIV-1 TAR-RNA with a Peptide–Cyclen Conjugate**

Katrin Michaelis and Markus Kalesse*

The hydrolysis of phosphodiesters by small molecules is of great interest since efficient catalysis of phosphodiester hydrolysis can significantly change the life cycle of cells or viruses. In order to assess the effect of metal ions in such hydrolyses we investigated the hydrolysis of metal complexes covalently linked to peptides. We chose the TAR-RNA of HIV-1 as the target for our hydrolysis studies (Scheme 1). The



Scheme 1. The TAR-RNA of HIV-1 and the 31mer RNA used in the cleavage experiments.

TAR-RNA of HIV-1 is recognized by the HIV-1 regulatory protein Tat (Scheme 2). It has been found that the binding of Tat to TAR-RNA up-regulates HIV-1 mRNA transcription.[1] Detailed spectroscopic methods have been used to investigate the Tat–TAR interactions in which arginine residues (52 or 53) make specific contacts to the bulge region of the TAR-

M E P V D P R L E P W K H P G S Q P K T A C T N C T Y C K K C
C F H C Q V C F I T K A L T I S Y G **R K K R52 R53 Q54 R R R** P
P Q G S Q T H Q V S L S K Q P T S Q S R G D P T G P K E.

Scheme 2. The Tat protein. The arginine-rich region is written in bold letters. The arginine residues Arg52 and Arg53 are responsible for the selective binding to the bulge region.

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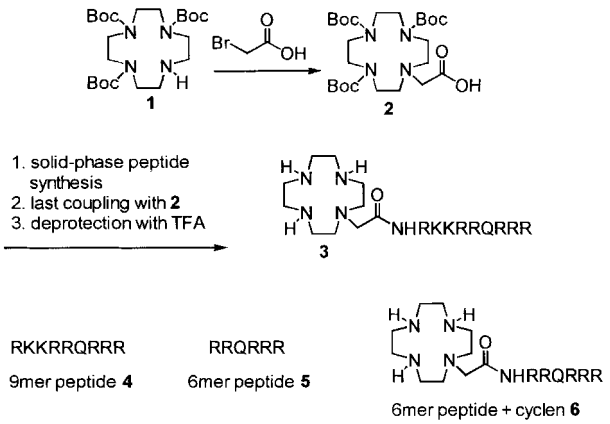
RNA.[2] It is known that the binding of the Tat protein to the TAR-RNA is a crucial event in the transcription of the viral DNA, because it stabilizes the active complex between the polymerase, the DNA template, and the nascent mRNA. When the Tat protein is not bound to the TAR-RNA premature abortion of transcription takes place.[3] Even more interesting is the fact that the arginine-rich region of the Tat protein is sufficient to induce budding of the virus in vivo. The fact that peptide fragments containing the basic region of Tat show similar TAR binding as the wild-type Tat protein[4] encouraged us to investigate the hydrolysis of this particular RNA with metal complexes bound to the arginine-rich region of the Tat protein.

Our initial idea was to use 1,4,7,10-tetraazacyclododecane (cyclen) as the metal-coordinating ligand and to conjugate it to the arginine-rich region of the Tat protein. This strategy would then allow the cleavage of the TAR-RNA by various metal ion complexes of cyclen to be examined. The arginine-rich nonamer should specifically bind to the targeted RNA and bring the metal complex in close proximity to phosphodiester bonds. As a result of this event RNA strand cleavage should occur.

The nonamer **3** with the attached cyclen moiety was synthesized by solid-phase synthesis using the Fmoc protocol (Scheme 3). As the last coupling step, the Boc-protected



Scheme 3. Synthesis of the peptides **3**–**6** by solid-phase synthesis. Boc = *tert*-butoxycarbonyl, Fmoc = fluorene-9-ylmethoxycarbonyl, TFA = trifluoroacetic acid.

cyclen acetic acid[5] (**2**) was coupled to the N-terminus of the nonapeptide with subsequent cleavage from the resin by using standard conditions (TFA) to yield the desired peptide–cyclen conjugate. The peptides were purified by semi-preparative RP-HPLC and the molecular weight was determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).[6] For the purpose of control experiments we also synthesized the corresponding nonamer peptide without the cyclen moiety (**4**) and the hexamer peptides with and without the cyclen moiety. We synthesized both hexamers **5** and **6** to probe whether the distance of the cyclen moiety from the crucial arginine residues (Arg52 and Arg53) would have an effect on the cleavage of the RNA.

The target TAR-RNA of HIV was purchased from Genset with a slight modification at the termini. The unpaired

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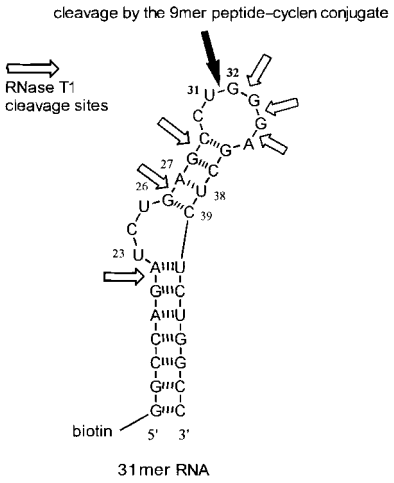
adenine residue (A17) was not incorporated and the base pair A15:U46 was changed into G15:C46. Biotin was introduced at the 5′-end as a label for detecting the cleavage fragments. We will refer to the labeled RNA as the 31mer RNA (Scheme 1). The cleavage experiments were carried out in autoclaved Eppendorf reaction vessels. In reactions with Eu^{III} or Zn^{II} the peptides were preincubated with the metal salt for 15 min at room temperature at three different pH values (pH = 6, 7.4, and 8). The 31mer RNA was added after the metal complexes had been formed. The reaction was stopped by the addition of fish sperm DNA and formamide loading buffer, and the mixture was then analyzed on a 20% denaturing polyacrylamide gel (Scheme 4). The omission of the fish sperm DNA



Scheme 4. Selective cleavage of the 31mer RNA by **3**. Arrows indicate the cleavage sites.

did not alter the cleavage results and was used to ensure that the RNA was liberated from the peptide.

Following this general protocol we also used the nonapeptide–cyclen conjugate **3** without addition of metal ions as a control experiment to see whether the observed cleavage was a result of the metal complex or if cleavage had taken place in the absence of metal ions. Very much to our surprise the nonamer–cyclen conjugate without any metal present gave selective and efficient cleavage at pH 6 and 7.4. Only a small amount of hydrolysis was observed at pH 8. These results indicate a pH-dependent cleavage mechanism in which the protonated nitrogen atoms on the cyclen residue[7] catalyze the phosphodiester cleavage. The only cleavage site was located between U31 and G32, as can be seen by comparing the lanes of the alkaline hydrolysis and the RNase T1 digestion to the cleavage experiments (Figure 1).

It was even more surprising to us that upon addition of Eu^{III} [8] or Zn^{II},[9] which were used very successfully in RNA cleavage reactions previously, the cleavage with the nonamer–cyclen conjugate diminished. The presence of 13.5 μM $Eu(NO_3)_3$ in cleavage reactions with 0.135 μM RNA and 167.2 μM peptide conjugate **3** inhibited the hydrolysis of the RNA. The presence of $Zn(ClO_4)_2$ also inhibited the RNA cleavage but only in concentrations of 1.35 mM. Interestingly both metal ions seem to interfere with the cleavage reaction in different concentrations, which could be the result of different

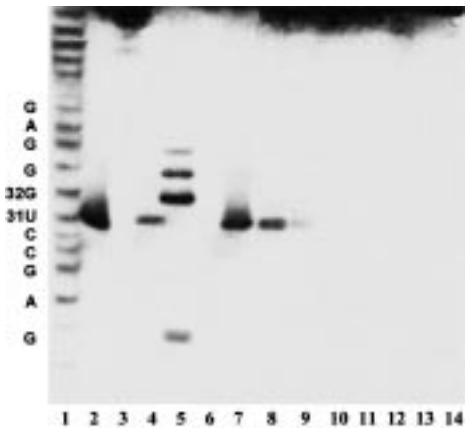


Figure 1. Polyacrylamide gel electrophoresis (PAGE) for the sequence-selective hydrolysis of the 31mer RNA (labeled with biotin at the 5′-end) by peptides at room temperature and pH 7.4. Peptide concentration: 167.2 μM. Lane 1: alkaline hydrolysis; lane 2: **3** for 3 h; lane 3: no treatment; lane 4: **3** and EDTA (0.5 mM) for 1 h; lane 5: hydrolysis by RNase T_1 (G-specific); lane 6: **4** for 1 h; lane 7: **3** for 2 h; lane 8: **3** for 1 h; lane 9: **3** for approximately 3 min; lane 10: **5** for 1 h; lane 11: **6** for 1 h; lane 12: **3** and Eu^{III} (167.2 μM) for 1 h; lane 13: **6** 167.2 μM and Eu^{III} (167.2 μM) for 1 h; lane 14: **4** 167.2 μM and Eu^{III} (167.2 μM) for 1 h.

binding constants with the RNA–peptide complex or different binding sites. It is noteworthy that Eu^{III} inhibits the cleavage in concentrations of approximately 1/10 of compound **3**. The exact mechanism for the inhibition of the cleavage reaction is still under investigation, but it is remarkable that metal ions diminish the hydrolysis of **3**. Also the control peptides without the cyclen moiety (**4**, **5**), as well as the hexamer with the cyclen moiety (**6**) gave no cleavage (lanes 6, 10, 11).[10] In contrast, compound **3** cleaves very efficiently at pH 6 and 7.4 at room temperature. Analysis of the cleavage reaction immediately after the RNA and compound **3** had been dissolved in Tris bufffer showed only a small amount of cleavage. Incubation for 1, 2 or 3 hours at room temperature resulted in increasing, but still selective, cleavage. The degree of cleavage increases with time (lanes 7–9). On the other hand, the control experiments with peptide **4**, which lacks the cyclen moiety, (lane 6) as well as the lanes with Eu^{III} present (lanes 12 and 13) show no hydrolysis.

The control experiments in the presence of EDTA (lane 4) showed that the observed hydrolysis was not catalyzed by metal contamination, and in order to show that these results were not artifacts, all experiments have been reproduced with RNA purchased from a different source and with newly synthesized peptides. The fact that no hydrolysis was observed in cleavage experiments with peptides lacking the cyclen moiety together with the fact that hydrolysis decreases at higher pH values confirms the presence of an ammonium, presumably even a bis(ammonium), species in the hydrolytic reaction. Even though it has been shown that basic amines can efficiently hydrolyze RNA, it was anticipated that the cleavage of nuclease model systems by metal ion complexes is more effective than cleavage by basic groups. The important work done by Komiyama et al.[11] shows that bis(amines) can efficiently cleave RNA by an acid/base mechanism that would increase the nucleophilicity at the 2′-hydroxyl group and at the same time increase its ability to function as a leaving

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group through interaction with the ammonium group. On the other hand Goebel et al.[12] showed that RNA cleavage can also be accomplished without the aid of an internal base. Since the cyclen moiety exists as a bis(ammonium) salt at neutral pH it can be envisioned that the remarkable hydrolysis is the result of a mechanism that involves the formation of a bis-cation on the cyclen moiety. Investigations addressing the role of the cyclen subunit and the mode of action for this cleavage are presently being performed in our laboratories.

The fact that the nonamer–cyclen conjugate cleaves selectively and very efficiently at neutral pH and room temperature and that these cleavage reactions are more efficient in the absence than in the presence of Eu^{III}, makes them a promising tool for a selective interaction with the life cycle of HIV-1.

Experimental Section

All experiments were performed in autoclaved Eppendorf reaction vessels. Extreme precaution has been taken to avoid RNase contamination. Water (Millipore quality) and all equipment had been treated with diethyl pyrocarbonate (DEPC) and then autoclaved prior to used. The RNA cleavage reaction was carried out in pH 7.4 Tris-HCl (20 mM) containing RNA (75.4 nM), peptides (167.2 μM), and NaCl (20 mM). The reaction mixture (5 μL) was incubated for 1 h at room temperature unless otherwise stated. After reaction, fish sperm DNA (1 μg) and formamide gel loading buffer (6 μL) was added to the reaction mixture. The mixture was then heated to 85 °C for 5 min and then cooled in ice. 7 μL of each sample was loaded onto a 20% denaturing polyacrylamide gel. After electrophoresis the RNA was transferred onto a positively charged nylon membrane by electro-blotting. After immobilization at 80 °C for 30 min followed by the wash protocol from Ambion, the RNA was visualized with streptavidin/alkaline phosphatase and CDP-star on Kodak film.

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[5] For the synthesis of **1**, see E. Kimura, S. Aoki, T. Koike, M. Shiro, *J. Am. Chem. Soc.* **1997**, *119*, 3068–3076. Synthesis of **2**: The Boc-protected cyclen **1** (950 mg, 1.7 mmol) and K_2CO_3 (300 mg, 2.15 mmol) were dissolved in acetonitrile (10 mL). Methyl bromoacetate (0.20 mL, 2.15 mmol) was added over 4 h at RT. The reaction mixture was stirred for 4 days, then filtered and concentrated. The crude material was dissolved in methanol/water (3/1, 12 mL) and LiOH (90 mg, 2.15 mmol) added. The reaction was stirred for 12 h, neutralized with NH_4Cl, and extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, concentrated and purified by flash chromatography (MeOH/ethyl acetate, 5/1) to yield compound **2** as a crystalline solid (0.6 g, 66%). ^1H NMR (400 MHz, $CDCl_3$): $\delta =$ 3.35–3.62 (m, 14H), 2.8–3.0 (m, 4H), 1.47 (s, 9H), 1.45 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 175.7$, 173.6, 156.15, 155.43, 80.0, 79.6, 54.21, 52.1, 49.87, 47.55, 28.59, 28.39. Synthesis of **3**: The nonapeptide was synthesized following the Fmoc technique. Each coupling step was monitored for completeness by the Kaiser Test. The coupling between the nonapeptide and cyclen residue **2** was achieved in CH_2Cl_2 with DCC, HOBT, and DMAP as the coupling reagents (DCC = dicyclohexylcarbodiimide, HOBT = 1-hydroxy-1*H*-benzotriazole, DMAP = 4-dimethylaminopyridine). The peptide–cyclen conjugate was cleaved off the resin with TFA under standard conditions. These conditions cleaved off all protecting groups including the Boc protecting groups on the cyclen. Compound **3** was purified by semi-preparative RP-HPLC and analyzed by MALDI-TOF MS.[6]

[6] Spectrometer: Kompakt Maldi 3 from Kratos. The experiments were performed in the positive-linear-high mode with α-cyanocinnamic acid as the matrix. The matrix was saturated with a mixture of MeCN/0.1% TFA (2/1). The calculated mass for compound **6** is 1552, the observed mass was 1552 and corresponds to $[M^+]$ of **6**.

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The First Synthesis of Organic Diselenolates: Application to the Synthesis of Diorganyl Diselenides**

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Dedicated to Professor Léon Ghosez on the occasion of his 65th birthday

There has been a growth in interest[1] of organoselenium chemistry over the last two decades and although several new reactions have been described, a relatively small number of

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