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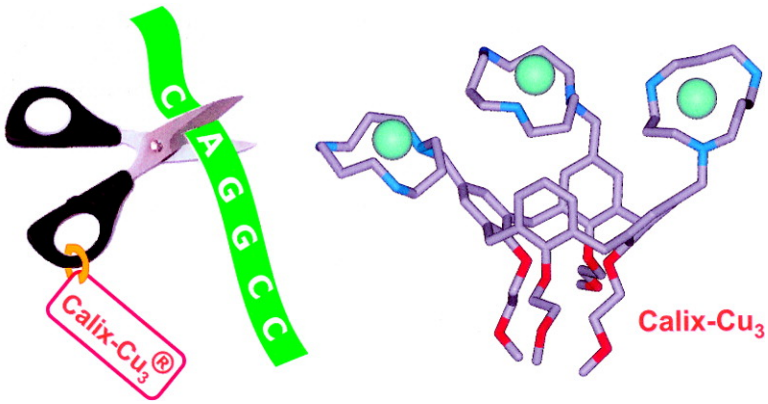

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Efficient and Selective Cleavage of RNA Oligonucleotides by Calix[4]arene-Based Synthetic Metallonucleases

Roberta Cacciapaglia,[†] Alessandro Casnati,[‡] Luigi Mandolini,*[,†] Alessio Peracchi,*[,#] David N. Reinhoudt,*[,§] Riccardo Salvio,[†] Andrea Sartori,[§] and Rocco Ungaro*[,‡]

Contribution from the Dipartimento di Chimica and IMC − CNR Sezione Meccanismi di Reazione, Università La Sapienza, Box 34 - Roma 62, 00185 Roma, Italy, Dipartimento di Chimica Organica e Industriale and Dipartimento di Biochimica e Biologia Molecolare Università degli Studi di Parma, V.le G. P. Usberti 17/A, 43100 Parma, Italy, and Laboratory of Supramolecular Chemistry and Technology, MESA+ − Research Institute, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

Received May 24, 2007; E-mail: luigi.mandolini@uniroma1.it; rocco.ungaro@unipr.it

Abstract: Di- and trinuclear copper(II) complexes of [12]aneN$_3$ macrocycles anchored at the upper rim of *cone* calix[4]arenes in 1,2-, 1,3-, and 1,2,3-positions were investigated as cleaving agents of 6-, 7-, and 17-meric oligoribonucleotides. A kinetic investigation of the cleavage reactions was carried out using gel electrophoresis to separate and analyze reactants and products having a radioactive phosphate label in the terminal 5′-position. The degree of cooperation was assessed on the basis of a comparison with rates of cleavage by mononuclear controls. A remarkable selectivity of cleavage of the CpA phosphodiester bond was observed for all metal complexes, in sharp contrast with the UpU and UpG selectivity previously observed in the cleavage of diribonucleoside monophosphates by the same metal complexes. The highest rate acceleration, brought about in the cleavage of the 5′-pCpA bond in hexanucleotide **9** by 50 μM trinuclear complex **5**−Cu$_3$ (water solution, pH 7.4, 50 °C), amounts to 5×10^5-fold, as based on the estimated background reactivity of the CpA dimer. Selectivity in the cleavage of oligoribonucleotides by copper(II) complexes closely resembles that experienced by ribonuclease A and by a number of metal-independent RNase A mimicks. The possible role of the dianionic phosphate at the 5′-terminal positions as a primary anchoring site for the metal catalyst is discussed.

Introduction

RNA transesterification reactions are fundamental and ubiquitous processes in biochemistry. In living organisms, RNA is cleaved by a diverse array of ribonucleases (RNases) for purposes that include RNA processing and maturation, regulation of gene expression, host-cell defense, and nucleotide turnover.[1] The majority of natural ribonucleases are proteins, but an important subset is constituted by RNA enzymes (ribozymes).[2] Prototypical examples of protein RNases are ribonuclease A and ribonuclease T1, whose mechanisms of action rely on acid−base catalysis operated by amino acid side chains.[3] However, other protein enzymes and several ribozymes employ divalent metal ions as cofactors in their catalytic mechanisms.[4] Natural RNases show the exceptional catalytic efficiency (cleavage rates exceeding the rate of spontaneous RNA cleavage by $>10^8$-fold) and substrate-selectivity peculiar to biocatalysts. In order to be selectively cleaved, RNA molecules must contain specific sequences and/or possess specific structural features. Ribozymes in general achieve selectivity by sequence complementarity, i.e., by base-pairing to specific sequences in their substrates.[2] Protein enzymes, lacking such possibility, achieve selectivity by appropriate organization of amino acid side chains in their substrate-binding sites.

The development of artificial ribonucleases capable of mimicking the action of natural RNases[5,6] is a long-standing goal of bioorganic chemistry, with implications for biotechnology and medicine. Small molecules able to cleave RNA efficiently, in a nondegradative manner, and with high levels of selectivity will offer many applications for the design of structural probes and perhaps the development of novel therapeutics.

[†] Università di Roma.
[‡] Università di Parma, DCOI.
[#] Università di Parma, DBBM.
[§] University of Twente.

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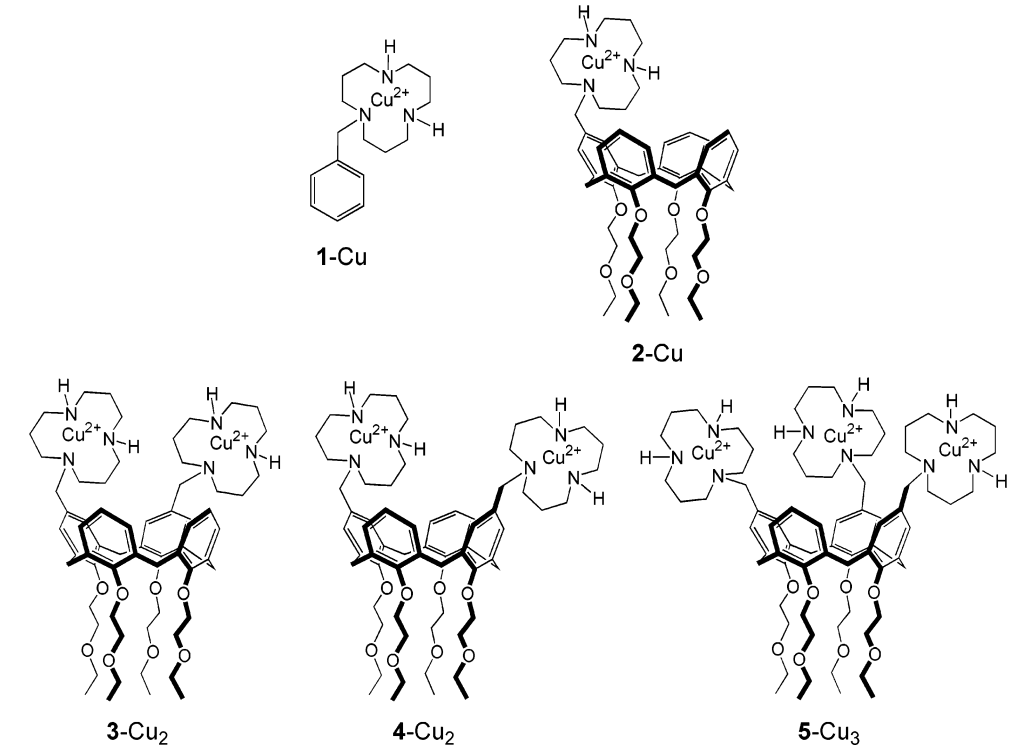
Chart 1



Among the chemical models of natural ribonucleases a special place is occupied by artificial metallonucleases[6] where one or more metal ions (usually lanthanides or transition metals) complexed to suitable synthetic ligands perform their catalytic function in the cleavage of phosphodiester bonds. With few exceptions[6a,b,7−9] synthetic metallonucleases as such are characterized by low base or sequence selectivity, the latter being in general achieved upon conjugation of the active catalysts to antisense oligonucleotides, peptides, or small molecules which act as RNA recognition agents.[5a,b,d,f,o,10−12]

We have recently reported on the synthesis of a series of water-soluble copper(II) complexes of calix[4]arene-based nitrogen ligands (Chart 1) and on their catalytic activity of diribonucleoside monophosphate (*NpN′*) cleavage.[6a] In particular, the bimetallic **3**−Cu_2 and the trimetallic **5**−Cu_3 catalysts showed a remarkable base selectivity for the cleavage of substrates having a uracil base at the 5′-hydroxyl terminus, namely for UpU and UpG. These results prompted us to test our copper(II) complexes toward longer oligoribonucleotides, which are of higher biological interest but have seldom been used as substrates of synthetic metallonucleases.[13]

Here we report on the catalytic activity of the bimetallic complexes **3**−Cu_2 and **4**−Cu_2 and of the trimetallic complex **5**−Cu_3 in the cleavage of ribonucleotides **6**−**12**[14] in water, pH 7.4, at 50 °C. The activity of the monometallic complexes **1**−Cu and **2**−Cu was also investigated for comparison purposes.

Results

The activity of copper(II) complexes based on [12]aneN_3 (Chart 1) in the cleavage of 0.05−0.5 nM oligoribonucleotides **6**−**12** was investigated in water (15 mM 1,4-piperazinediethane sulfonic acid (PIPES) buffer, pH 7.4, $I = 0.07$, 50 μM sodium dodecyl sulfate (SDS), 50 °C). To avoid contamination by natural nucleases, all of the laboratory equipment which came into contact with solutions of oligoribonucleotides, calixarenes, and buffer were sterilized and used only once. Experiments were

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(14) It is highly improbable that these oligomers adopt any regular secondary structure at 50 °C, in view of their reduced size. Algorithms commonly used to compute the stability of RNA structures, such as RNAfold and sFold, predict compounds **6**−**12** to be devoid of secondary structures.

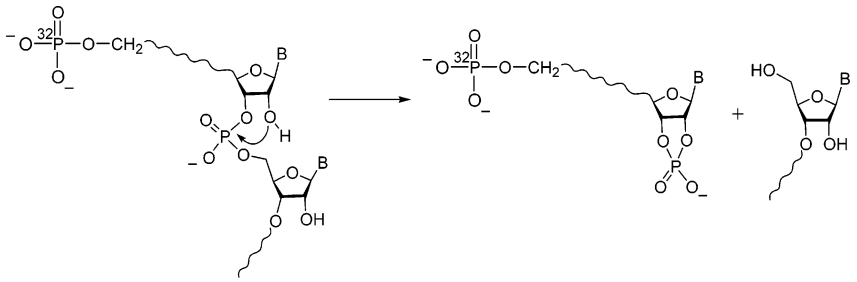

Figure 1. Transesterification of a 5′-^{32}P-labeled ribonucleotide by intramolecular attack of the 2′-OH. (B = nucleobase).

carried out in order to ascertain the possible presence of contaminating nucleases. The most reactive substrates (incubated at 50 °C in 15 mM PIPES, 50 μM SDS, in the absence and presence of 150 μM $CuCl_2$) did not show any cleavage after 20 h. Furthermore, experiments at 25 °C in the presence of **5**−Cu_3 resulted in rates of cleavage lower than those determined at 50 °C, thereby arguing against the presence of contaminating nucleases which should be at least partially denaturated at the latter temperature.

The oligoribonucleotide cleavage was followed by gel electrophoresis. To allow detection of the substrate and of the cleavage products, a radioactive phosphate (^{32}P) was introduced into the 5′-terminal position by reaction with *γ-*ATP* and *T4* polynucleotide kinase (*T4 PNK*). Product formation was followed by analyzing on the gel small aliquots of the reaction mixture quenched at proper time intervals. Detectable fragments are only those containing the 5′-^{32}P-end-label, whereas other fragments not containing any radioactive labeling are lost (Figure 1). Identification of the cleavage products was carried out by comparison with the products obtained in the aspecific hydrolysis of the oligonucleotides in 0.1 M NaOH. The basic hydrolysis gives two labeled fragments for each phosphate cleavage, one with a terminal 2′,3′-cyclic phosphate coming from transesterification and the other with a terminal acyclic 2′- or 3′-phosphate produced by hydrolysis of the cyclic phosphate. The two isomeric acyclic phosphates move on the gel slightly more rapidly than the cyclic phosphate.[15] In the experiments carried out in the presence of calix[4]arene−copper(II) complexes, only the fragments corresponding to the 2′,3′-cyclic phosphate were observed in all cases. This indicates that formation of the cyclic phosphate via intramolecular attack of 2′-OH (Figure 1) is not followed by hydrolysis under the conditions of the cleavage experiments. The bands on the gel corresponding to reactants and products were quantitatively analyzed to evaluate the mole fractions at different time intervals over a total reaction time of 4−6 h. Typical gel electrophoretic autoradiograms are shown in Figures 2, 4, and 6. Time−concentration data of the fastest reactions were subjected to time-course kinetic analysis by means of appropriate integrated rate equations to obtain catalytic pseudo-first-order rate constant k_{obs} (s^{-1}). An initial rate method was applied to the slowest reactions, for which the reaction percent in the 4−6 h time window was low. For the sake of convenience, we shall report first on oligoribonucleotides **6**−**8**, then on oligoribonucleotides **9**−**11**, and finally on the heptadecamer oligoribonucleotide **12**. The metal complex concentration in the cleavage of **6**−**11** was 50 μM, and in the cleavage of **12** it was 10 μM.[16]

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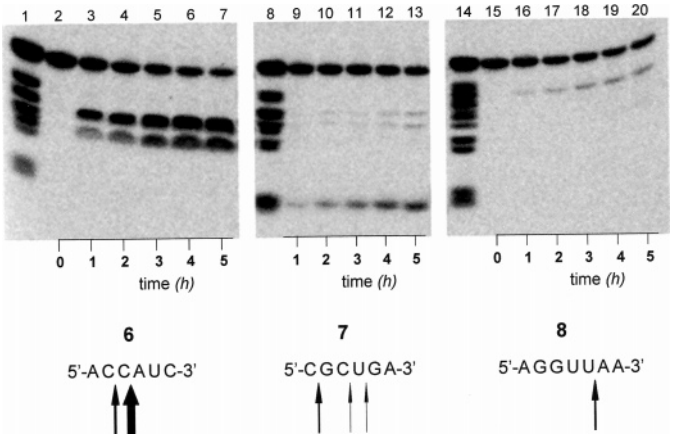
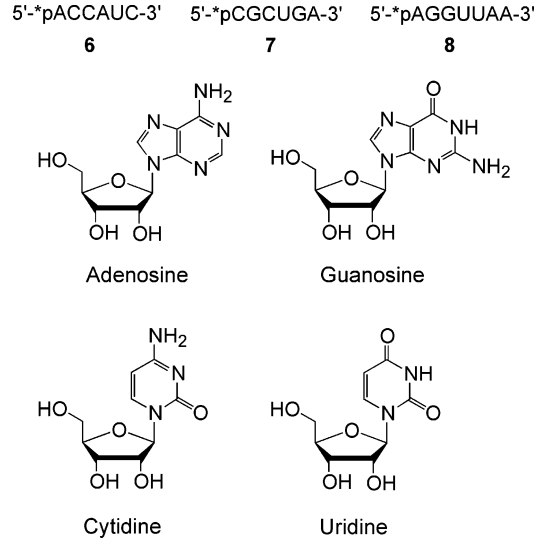


Figure 2. Gel electrophoretic autoradiograms corresponding to the cleavage of oligoribonucleotides **6**−**8** promoted by 50 μM **5**−Cu_3. Lanes 1, 8, and 14 show the aspecific hydrolysis in 0.1 M NaOH.

Cleavage of 6−8. We first investigated the cleavage of oligoribonucleotides **6**−**8** in which all of the 16 possible diribonucleotide combinations are present, thereby enabling a preliminary screening of the efficiency and selectivity of the cleaving agents.



The gel electrophoretic autoradiograms related to the reactions of the three oligoribonucleotides in the presence of 50 μM trimetallic complex **5**−Cu_3 (Figure 2)[17] show that cleavage of **6** is much faster than cleavage of **7** and **8**. In the cleavage of **6**, which takes place in the CpA and CpC bonds (Figure 2, left-hand panel), the disappearance of the band corresponding to the reactant is accompanied by the appearance of the bands

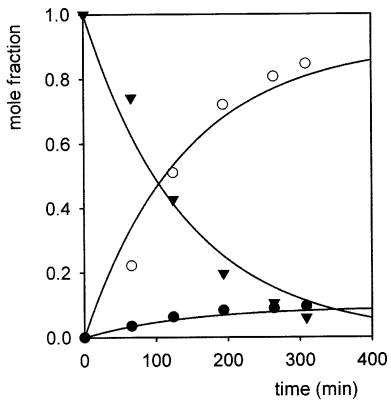
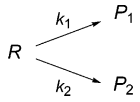


Figure 3. Reaction of 5′-p*ACCAUC (**6**) promoted by 50 μM **5**−Cu_3. Mole fractions of reactant (▼), and of fragments 5′-p*ACC (○; CpA cleavage), and 5′-p*AC (●; CpC cleavage) vs time.

Scheme 1. Parallel First-Order Reactions



corresponding to the ACC and AC fragments. Plots of the mole fractions against time are reported in Figure 3. Data points show a reasonable adherence to exponential curves based on the same first-order rate constant $k = 1.2 \times 10^{-4}$ s^{-1}, which is consistent with a reaction scheme in which a reactant *R* undergoes two parallel first-order reactions leading to products P_1 and P_2 (Scheme 1 and eqs 1 and 2,

$$[P_1]/[R]_0 = \frac{k_1}{k}(1 - e^{-kt}) \quad (1)$$

$$[P_2]/[R]_0 = \frac{k_2}{k}(1 - e^{-kt}) \quad (2)$$

with $k = k_1 + k_2$ and $[P_1]/[P_2] = k_1/k_2$). The central and right-hand panels in Figure 2 show that, whereas cleavage of **7** occurs in the CpG, CpU, and UpG bonds, **8** is cleaved selectively in the UpA bond. Rate constants for such reactions, as for other slow reactions, were calculated from initial rates.

The rate constant *k* for the cleavage of all scissile bonds under the reaction conditions are collected in Table 1. We first note that the copper(II)−calixarene complexes behave as effective and selective artificial ribonucleases. The CpA sequence turns out to be the most reactive, independent of catalyst identity. More in general, the cleavage patterns appear to be independent of catalyst identity, and so does the reactivity order **6** > **7** ≈ **8**, with the notable exception of **3**−Cu_2 which is totally inactive toward **7**.

There is an undeniable tendency for the catalytic efficiency to increase with the number of metal units in a way suggesting that the subunits in the trimetallic and bimetallic complexes do not act as independent units but show variable degrees of cooperation among metal ions. Interestingly, the 1,3-distal regioisomer **4**−Cu_2 is more effective than its 1,2-vicinal regioisomer **3**−Cu_2 in the cleavage of **7**, but the reverse holds in the cleavage of **6** and **8**. The *N*-benzylated mononuclear complex **1**−Cu (see footnote *d* to Table 1) cleaves the CpA bond of **6** one order of magnitude more slowly than the mononuclear calixarene complex **2**−Cu and is inactive toward the less reactive CpC bond. This finding suggests that the calixarene moiety plays an active role in the catalysis. A possible reason for this is the hydrophobicity of the calixarene moiety which can enhance the binding to the aromatic nucleobases.

Table 1. Cleavage of Oligoribonucleotides **6**−**8** Promoted by Copper(II)−Calixarene Complexes; Pseudo First-Order Rate Constants (10^6 *k*, s^{-1})[a] in Water, pH 7.4, 50 °C[b,c]

Catalyst	*Substrate*		
	6	**7**	**8**
2-Cu [d]	5′-ACCAUC-3′	5′-CGCUGA-3′	5′-AGGUUAA-3′
	C|C 0.3 C|A 5.8	*e*	*e*
3-Cu_2 (1,2-vicinal)	5′-ACCAUC-3′	5′-CGCUGA-3′	5′-AGGUUAA-3′
	C|C 4.6 C|A 35	*e*	U|A 13
4-Cu_2 (1,3-distal)	5′-ACCAUC-3′	5′-CGCUGA-3′	5′-AGGUUAA-3′
	C|C 2.8 C|A 13	C|G 3.7 C|U 1.3 U|G 0.4	U|A 5.2
5-Cu_3	5′-ACCAUC-3′	5′-CGCUGA-3′	5′-AGGUUAA-3′
	C|C 11 C|A 110	C|G 6.4 C|U 1.1 U|G 1.1	U|A 9.5

[a] Rate constants for the cleavage of **6** by **3**−Cu_2 and **5**−Cu_3 from time-course kinetics, error ±10%. All other *k* values from initial rates, error ±15%. [b] Runs carried out on <0.2 nM substrate solutions, in the presence of 50 μM catalyst and 50 μM SDS. [c] The width of the arrows pointing to the scissile bonds gives an indication of the relative reactivity. [d] In the presence of 50 μM monometallic catalyst **1**−Cu, $k_{CA} = 4.6 \times 10^{-7}$ s^{-1}. No cleavage of the CpC bond was observed in 5 h. [e] No reaction in 5 h.

Cleavage of 9−11. Since the reactivity of a given phosphate bond depends on the position in the oligomer, as well as on the base sequence of the oligomer itself, it was felt that investigation of substrates with different structural features was appropriate. Accordingly, the catalytic cleavage of oligoribonucleotides **9**−**11** was investigated under the same conditions used for the cleavage of **6**−**8**. The CpA phosphodiester bond, which is the most

5′-*pCAGGCC-3′	5′-*pCCGGCA-3′	5′-*pACUAUC-3′
9	**10**	**11**

reactive bond in the first set of oligonucleotides, is in the 5′-terminal position in **9**, and in the 3′-terminal position in **10**. The reverse holds for CpC. Hexanucleotide **11**, which differs from **6** for the presence of UpA in place of CpA was chosen to compare the reactivity of different pairs in otherwise identical oligoribonucleotide structures.

(16) From the log *K* value of 12.63 for binding of copper(II) to [12]aneN$_3$ (see: Zompa, L. J. *Inorg. Chem.* **1978**, *17*, 2531−2536) and the value of 12.6 for the pK_a of the ligand (see: Kimura, E.; Shiota, T.; Koike, T.; Shiro, M.; Kodama, M. *J. Am. Chem. Soc.* **1990**, *112*, 5805−5811) we calculate that at pH 7.4 and 50 μM ligand and copper(II) ion, the fraction of ligated metal ion is 0.97 and is 0.94 at a concentration of 10 μM.

(17) The trimetallic complex **5**−Cu_3 is still catalytically active at 5 μM concentration. The rate of cleavage of the CpA bond in **6** at 5 μM is 1 order of magnitude lower than that at 50 μM, which provides an indication that the conditions of the catalytic experiments are well below saturation. This implies that the affinity of the catalyst for the oligoribonucleotide does not exceed 10^4 M^{-1}.

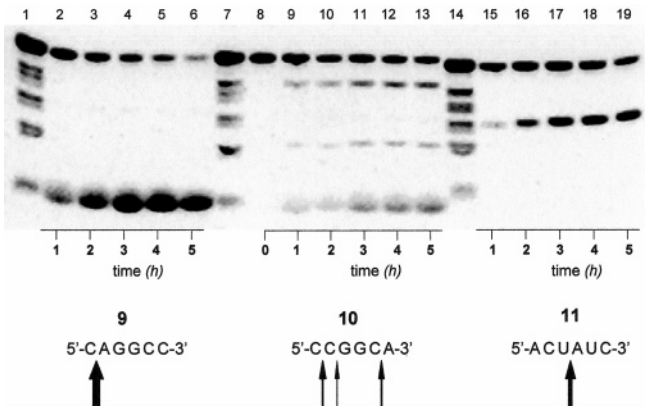


Figure 4. Gel electrophoretic autoradiograms corresponding to the cleavage of oligoribonucleotides **9**−**11** promoted by 50 μM **4**−Cu_2. Lanes 1, 7, and 14 correspond to the aspecific hydrolysis in 0.1 M NaOH.

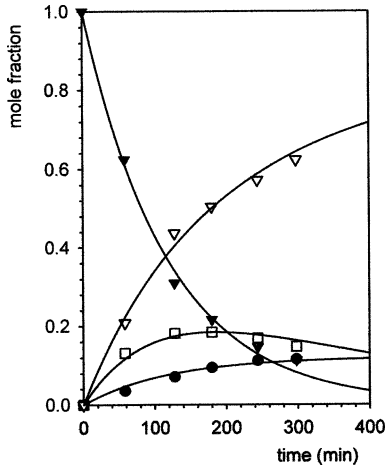


Figure 5. Reaction of 5′-p*CCGGCA (**10**) promoted by 50 μM 1,2-vicinal bimetallic complex **3**−Cu_2. Mole fractions of reactant (▼), and of fragments 5′-p*C (▽; CpC cleavage), 5′-p*CC (●; CpG cleavage), and 5′-p*CCGGC (□; CpA cleavage) vs time. The points are experimental, and the curves are calculated from integrated rate equations derived from Scheme 2 and best fit values of rate constants reported in Table 2.

The autoradiograms related to the cleavage of **9**−**11** in the presence of the 1,3-distal bimetallic complex **4**−Cu_2 are shown in Figure 4. The CpA bond in **9** is cleaved very effectively and with high selectivity. The cleavage of the same bond in **10** is much slower, and is accompanied by rupture of the CpC and CpG bonds. In the case of **11**, the only scissile bond is UpA, which is cleaved with good efficiency.

The 1,2-vicinal bimetallic complex **3**−Cu_2 is more effective than its 1,3-distal regioisomer **4**−Cu_2 by 5-fold in the cleavage of all the scissile bonds in **10**. Time−concentration data for the reaction of **10** in the presence of **3**−Cu_2 are plotted in Figure 5. It is apparent that data points deviate from the behavior expected on the basis of simple parallel first-order reactions. The concentration of the fragment CCGGC increases in the early stages of the reaction, then reaches a maximum, and eventually decreases, thus indicating that CCGGC undergoes further cleavage. Considering that cleavage of the CpC bond in **10** is the fastest, we assume that CCGGC undergoes further cleavage at the CpC site, as shown in Scheme 2. The set of differential equations derived from Scheme 2 are amenable to analytical integration (see Supporting Information). Best fit of data points to the integrated rate equations gave the following values of

Scheme 2. Cleavage of **10** Promoted by **3**−Cu_2

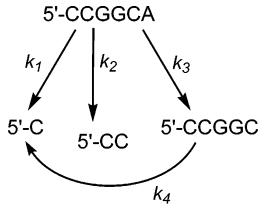


Table 2. Cleavage of Oligoribonucleotides **9**−**11** Promoted by Copper(II)−Calixarene Complexes; Pseudo-First-Order Rate Constants (10^6 k, s^{-1})[a] in Water, pH 7.4, 50 °C[b,c]

Catalyst	*Substrate*		
	9	**10**	**11**
2-Cu	5'-CAGGCC-3'	5'-CCGGCA-3'	5'-ACUAUC-3'
	C|A 7.1	[d]	U|A 2.1
3-Cu_2 (1,2-vicinal)	5'-CAGGCC-3'	5'-CCGGCA-3'	5'-ACUAUC-3'
	C|A 130	C|C 76 C|G 17 C|A 47	U|A 25
4-Cu_2 (1,3-distal)	5'-CAGGCC-3'	5'-CCGGCA-3'	5'-ACUAUC-3'
	C|A 110	C|C 15 C|G 3.2 C|A 10	U|A 50
5-Cu_3	5'-CAGGCC-3'	5'-CCGGCA-3'	5'-ACUAUC-3'
	C|A 300	C|C 17 C|G 2.1 C|A 11	U|A 51

[a] Rate constants for the cleavage of **9** by **3**−Cu_2, **4**−Cu_2, and **5**−Cu_3, of **10** by **3**−Cu_2, and of **11** by **4**−Cu_2 and **5**−Cu_3 from time-course kinetics, error ±10%. All other k values from initial rates, error ±15%. [b] Runs carried out on <0.2 nM substrate solutions, in the presence of 50 μM catalyst and 50 μM SDS. [c] The width of the arrows pointing to the scissile bonds gives an indication of the relative reactivity. [d] No reaction in 5 h.

rate constants: $k_1 = 7.6 \times 10^{-5}\ s^{-1}$; $k_2 = 1.7 \times 10^{-5}\ s^{-1}$; $k_3 = 4.7 \times 10^{-5}\ s^{-1}$; $k_4 = 5.4 \times 10^{-5}\ s^{-1}$. The curves calculated with the optimized values of the parameters show a close adherence to data points (Figure 5).

Rate constants for the cleavage of all the scissile bonds in **9**−**11** are collected in Table 2. Many of the reactivity and selectivity features emerged from the reactions of oligoribonucleotides **6**−**8** are also observed in the reactions of **9**−**11**. The much lower efficiency of the monometallic catalyst **2**−Cu compared with **3**−Cu_2 and **4**−Cu_2 clearly indicates that the two metal ions in the bimetallic complex act in a synergic fashion. There are cases in which the efficiency of bimetallic complexes is either superior (cleavage of **10** by **3**−Cu_2) or virtually identical (cleavage of **11** by **4**−Cu_2 and cleavage of the CpU bond in **7** by **4**−Cu_2) to that of the trimetallic complex **5**−Cu_3. There are also cases in which **5**−Cu_3 is the best cleaving agent in the lot, but the advantage due to the third metal ion hardly exceeds a factor of 2, showing that a convincing evidence of the operation of trimetallic catalysis is lacking.

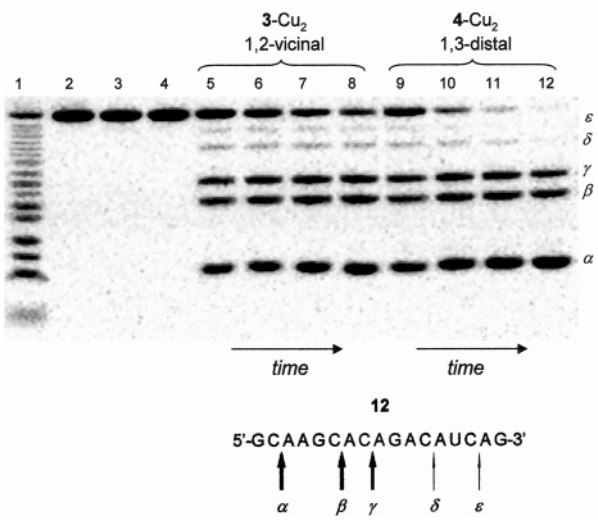


Figure 6. Gel electrophoretic autoradiograms corresponding to the cleavage of **12** in the presence of 10 μM **3**−Cu_2 (lanes 5−8) or 10 μM **4**−Cu_2 (lanes 9−12). Quenching times were 110, 195, 270, and 375 min in both experiments. Lane 1 corresponds to the aspecific hydrolysis in 0.1 M NaOH. Lanes 2−4 are blank experiments carried out in the absence of metal complex.

Cleavage of 12. Substrate **12** is a heptadecamer featuring six pyrimidine-p*N* sequences. Five of them are CpA, the most

5'-*pGCAAGCACAGACAUCAG-3'
12

reactive unit among the previously examined oligonucleotides (Tables 1 and 2), and one is UpC, which in **6** was not cleaved by any metal complex. Catalytic experiments on **12** could not be carried out under the conditions used for oligonucleotides **6**−**11**, due to a poor and irreproducible recovery of the radioactivity on the electrophoresis gels. Good results were obtained by increasing the SDS concentration to 150 μM and decreasing the metal complex concentration to 10 μM. The autoradiograms corresponding to the **3**−Cu_2 and **4**−Cu_2 promoted cleavage of **12** under the given conditions are shown in Figure 6. All the CpA bonds are cleaved whereas other bonds do not undergo appreciable scission in more than 6 h. CpA bonds labeled as α, β, and γ are cleaved much more rapidly than the bonds labeled as δ and ϵ, which would suggest that far-away CpA bonds are cleaved more slowly than bonds closer to the 5′-terminal position.

The time−concentration profiles in Figure 7 are related to the cleavage of the three most reactive CpA bonds in **12** promoted by **3**−Cu_2. The shapes of the profiles suggest that fragments resulting from β and γ cleavage undergo further cleavage of the CpA bond closest to the 5′-terminus, as shown in Scheme 3. With the reasonable assumption that $k_4=k_5=k_1$, the set of differential equations consistent with Scheme 3 is amenable to analytical integration (see Supporting Information). Numerical values of the rate constants (Table 3), obtained by best fit of data points to the rate equations, were used to calculate the curves in Figure 7. A similar treatment was applied to the cleavage of **12** in the presence of **4**−Cu_2. As shown in Table 3, the two isomeric bimetallic complexes exhibit quite similar catalytic activities, with a modest superiority of **3**−Cu_2 over **4**−Cu_2.

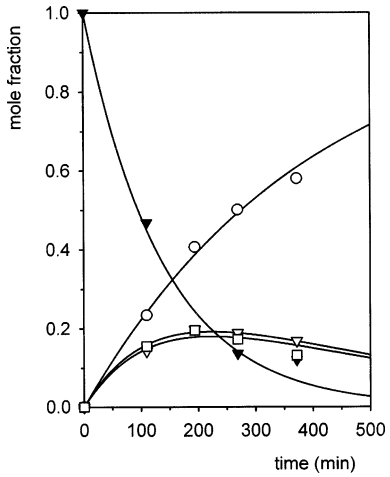
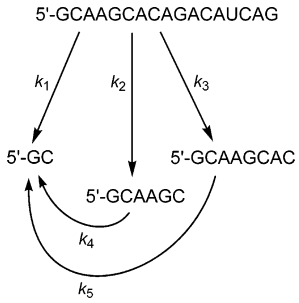


Figure 7. Reaction of 5′-p*GCAAGCACAGACAUCAG (**12**) promoted by 10 μM 1,2-vicinal bimetallic complex **3**−Cu_2. Mole fractions of reactant (▼) and fragments 5′-p*GC (○; α-CpA cleavage), 5′-p*GCAAGC (▽; β-CpA cleavage), and 5′-p*GCAAGCAC (□; γ-CpA cleavage) vs time. The points are experimental, and the curves are calculated from integrated rate equations derived from Scheme 3 and best fit values of rate constants reported in Table 3.

Scheme 3. Cleavage of **12** Promoted by **3**−Cu_2 or **4**−Cu_2



Discussion

In the preceding section we have shown that di- and trinuclear metal complexes **3**−Cu_2, **4**−Cu_2, and **5**−Cu_3 exhibit pronounced ribonuclease activity − at concentrations as low as 10−50 μM and under conditions close to physiological − toward oligoribonucleotides composed of different monomeric units. Whenever multiple cleavages were observed, time−concentration data showed a good adherence to kinetic schemes involving either competitive or competitive-consecutive first-order reactions.

The results listed in Tables 1−3 offer a varied phenomenological picture. A prominent feature of the data is the remarkable selectivity of the cleavage reactions. The most reactive phosphodiester bond is that in CpA, followed by UpA and CpC. Other scissile bonds, namely, CpG, CpU, and UpG are cleaved less effectively. The reactive bonds always have a pyrimidine base in the 5′-position, with a marked preference for cytosine over uracyl. However, not every *N*p*N*′ bond in which *N* is a pyrimidine base is reactive enough to be cleaved under the conditions of the catalytic runs, as no reaction of UpU in **8** and UpC in **6** was observed. The base in 3′-position can be either a pyrimidine or a purine base, but the decided superiority of adenine is clearly indicated by the high reactivity of CpA and UpA phosphodiester bonds.

The catalytic rate enhancement (k/k_{bg}) shown by the trinuclear complex **5**−Cu_3 in the cleavage of CpA bond in **9** with respect to the estimated value of the background reaction of CpA

Table 3. Cleavage of **12** Promoted by **3**−Cu_2 and **4**−Cu_2; Pseudo-First-Order Rate Constants (10^6 k, s^{-1})[a] in Water, pH 7.4, 50 °C[b,c]

catalyst	*substrate*		
	12		
3-Cu_2 (1,2-vicinal)	5'-GCAAGCACAGACAUCAG-3'	C|A (α)	42
	α β γ δ ε	C|A (β)	41
		C|A (γ)	38
4-Cu_2 (1,3-distal)	5'-GCAAGCACAGACAUCAG-3'	C|A (α)	25
	α β γ δ ε	C|A (β)	32
		C|A (γ)	33

[a] From time-course kinetics, error ±10%. [b] Runs carried out on 0.1 nM substrate solutions, in the presence of 10 μM catalyst and 150 μM SDS.[c] The width of the arrows pointing to the scissile bonds gives an indication of the relative reactivity.

dinucleoside monophosphate ($k_{bg} = 6.1 \times 10^{-10}$ s^{-1}, pH 7, T = 50 °C)[6a] amounts to ca. 5×10^5-fold. This is one of the highest values recorded for a synthetic metallonuclease in the cleavage of a phosphodiester bond in ribonucleotide dimers or higher oligomers.[5a,j,l] The corresponding figures for the **3**−Cu_2 catalyzed cleavage of CpC and CpG in **10** are 2×10^5 and 5×10^4, respectively.

In our previous work on the cleavage of diribonucleoside monophosphates CpA, GpA, CpG, CpC, ApG, GpG, GpU, UpG, and UpU we found that rate enhancements relative to background brought about by **3**−Cu_2 and **5**−Cu_3 (1 mM catalyst, water solution, pH 7.0, 50 °C) were on the order of 10^4-fold, largely independent of the base identity, with the exception of UpU and UpG, for which rate enhancements were 1 order of magnitude higher. Selectivity in the reactions of dinucleoside monophosphates containing uracyl as the 5′-nucleobase was explained in terms of a deprotonated uracyl moiety acting as an anchor site for the metal catalyst.[18] Comparison with the data from the present work reveals completely different selectivity patterns. The highly reactive CpA and CpC pairs in the oligoribonucleotides display no enhanced reactivity as dinucleoside monophosphates. Moreover, the UpG bond in **7** is cleaved quite slowly, whereas the UpU bond in **8** is not cleaved at all. These observations clearly indicate that the modes of interaction of the copper(II) complexes with a 5′-labeled oligoribonucleotide and with a diribonucleoside monophosphate are markedly different, which is not surprising, inter alia, in view of the presence of a doubly charged binding site in the former.

(18) Enhanced cleavage rates of uracyl-containing dinucleoside monophopshates by dinuclear zinc(II) complexes of [12]aneN_3-based ditopic ligands were explained along the same lines. See ref 6b.

The paucity of quantitative data for the cleavage of single-stranded oligoribonucleotides by di- and trimetallic complexes[13] prevents a comparison of the results from the present work with strictly analogous data. Still, we cannot help noting striking similarities with literature reports on nonmetallic cleaving agents. In a thorough investigation of the nonenzymatic cleavage of short, single-stranded oligoribonucleotides by polyamines such as spermidine, putresceine, and spermine, Kierzek[19] reported that the most reactive phosphodiester bonds are *N*pA and *N*pC, where *N* is a pyrimidine base. Furthermore, ribonuclease A mimicks composed of a bisquaternary DABCO moiety as RNA binding domain and a histamine or hystidine residue as cleaving unit, were reported to cleave oligoribonucleotides primarily in the CpA and UpA sites, with a moderate preference for the former bond.[20,21] However, it is difficult to decide whether such similarities are fortuitous, or may be taken as an indication of similar mechanisms of action, in spite of the widely differing nature of the cleaving agents.

Many artificial ribonucleases are composed of a catalytic group, such as a ligated metal ion, and a highly structured probe for substrate binding and sequence recognition.[5a] In simple synthetic metalloribonucleases, such as those described in this work, the tasks of substrate binding (recognition) and activation of the bound substrate are assigned to ligated metal ions. Most likely, the primary binding site for our metallocatalysts is the doubly charged, labeled phosphate group present in the 5′-terminal position of all investigated oligoribonucleotides.

We have already touched upon the apparently important role of the distance of a scissile bond from the 5′-terminus. Reactivity ratios of 27:10:1 are calculated from the data in Tables 1 and 2 for the **5**−Cu_3 catalyzed cleavage of the CpA bond in **9**, **6**, and **10**, respectively. Similarly the CpC bond in **10** is cleaved by **3**−Cu_2 16 times more rapidly than in **6**, whereas its rate of cleavage in **9** is not fast enough to compete with the cleavage of the CpA bond. Although reactivity ratios vary from catalyst to catalyst, the reactivity orders **9** > **6** ≥ **10** for the CpA bond and **10** > **6** > **9** for the CpC bond are independent of catalyst identity. However, this is not always the case. The UpA bond in **11** is cleaved more rapidly by **5**−Cu_3 and **4**−Cu_2 than in **8**, but the rates of cleavage in the presence of **3**−Cu_2 are almost the same. Still odder is the cleavage of the CpG bond, whose reactivity order is **7** > **10** in the presence of **5**−Cu_3, **7** ≈ **10** in the presence of **4**−Cu_2, and **7** ≪ **10** in the presence of **3**−Cu_2. Furthermore, the three most reactive bonds in **12**—labeled as α, β, and γ—are cleaved by the dinuclear catalysts at very similar rates (Table 3) without displaying any reactivity decrease on increasing the distance from the 5′-terminus. A reasonable conclusion is that the distance of a scissile bond from the 5′-terminus is indeed an important factor, but other poorly understood factors are also important.

On the basis of the simple view that binding of a metal cation to a doubly charged phosphate is expected to be stronger than to a singly charged phosphate, we speculate that the primary binding interaction between catalyst and oligoribonucleotide involves one of the metal centers and the ^{32}P-radiolabeled 5′-

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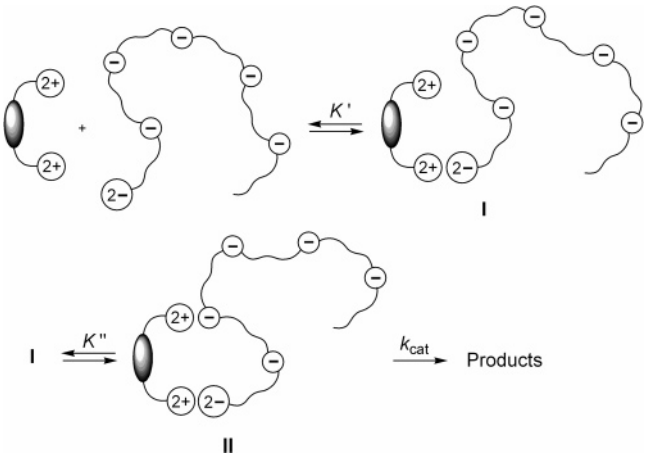
(21) Koval'ov, N.; Kuznetsova, I.; Burakova, E.; Sil'nikov, V.; Zenkova, M.; Vlassov, V. *Nucleosides, Nucleotides Nucleic Acids* **2004**, *23*, 977−981.

Scheme 4. Catalysis by a Dinuclear Copper(II)−Calixarene Complex in the Cleavage of a Hexaribonucleotide Labeled at the 5′-Terminus with a ^{32}P Phosphate Dianion



terminal phosphate dianion, as schematically shown in **I** (Scheme 4). One of the possible productive complexes (e.g., **II**) is depicted as a dinuclear metallomacrocycle arising from intramolecular (intracomplex) binding of one of the singly charged phosphates of the nucleotide backbone with the other metal center. A similar mechanism was proposed by Kuusela et al.[22] for the zinc(II)-promoted hydrolysis of oligonucleotides bearing a phosphate dianion at the 3′-terminus, where the divalent metal ion was suggested to bridge two phosphate groups that are several nucleotidic units apart.

Since our reactions have been carried out under conditions approaching subsaturation,[17] the rate of cleavage of a given bond is a function of both the stability of the productive complex ($K' \times K''$) and of its reactivity (k_{cat}). Other things being equal, the quantity K'' is expected to decrease on increasing the ring size[23] of the metallomacrocycle intermediate **II**,[24] which is well in keeping with the finding that scissile bonds close to the 5′-terminal are (usually) cleaved more easily.

To summarize, di- and trinuclear azacrown-copper(II) complexes implanted on the upper rim of the calix[4]arene scaffold cleave single-stranded RNA oligonucleotides with high efficiency and selectivity under pH and temperature conditions close to physiological. An extensive kinetic investigation of the cleavage reactions afforded pseudo-first-order rate constants for the cleavage of the most labile phosphodiester bonds. With few exceptions, substantial levels of cooperation between metal centers were observed in the reactions promoted by dinuclear complexes, irrespective of whether the substitution pattern was 1,2-vicinal or 1,3-distal. However, in no case was the contribution from a third ligated metal ion at the upper rim such to unambiguously support the operation of trimetallic catalysis.

Interestingly, the observed CpA selectivity, which resembles that shown by natural RNases A, was obtained without the use of antisense oligonucleotides, generally employed to develop site-directed RNA cleaving agents. Surprisingly, this selectivity is completely different from that previously observed in the cleavage of diribonucleoside monophosphates by the same complexes, where a high preference for the cleavage of UpU and UpG sequences was observed.

The reported results may also have a general significance for the entire class of synthetic metallonucleases because the base selectivity shown by some of them in the cleavage of dinucleoside monophosphates, usually ascribed to a preferred binding of the metal ion to a specific nucleobase, may change in favor of the intrinsically most reactive *NpN′* bonds when using oligoribonucleotide or RNA substrates having a terminal doubly charged phosphate group. In these cases, the catalyst−phosphate interactions and local structural changes may become the dominant factors.

Experimental Section

Materials and Methods. Azacrown ligands **1**−**5** were available from previous investigations.[6a] Oligoribonucleotides **6**−**12** were purchased from Dharmacon Research (Lafayette, CO) and ^{32}P-5′-end-labeled with T4 polynucleotide kinase according to standard procedures.[25] Concentrations of radioactive oligonucleotides were determined from specific activities, while concentrations of solutions of nonradioactive samples were determined from absorbance readings at 260 nm and extinction coefficients were estimated by the nearest-neighbor method.[26] Cleavage experiments were carried out in water (50 μM SDS and 15 mM PIPES ± NaOH, adjusted to pH 7.4, 50 °C), unless otherwise stated. The ionic strength was adjusted to 0.07 M with NaCl. Solutions of $CuCl_2$ were used immediately after preparation to minimize formation of insoluble hydroxides. The aqueous solutions were prepared using deionized (Millipore) and sterilized distilled water.

Kinetic Measurements. Cleavage experiments were carried out in sterilized polypropylene micro test tubes stored inside metal boxes that were immersed in a thermostated water bath. Copper(II) complexes of ligands **1**−**5** were formed in situ by addition to the buffer (15 mM PIPES in water, pH 7.4) of a concentrated ethanol solution of the ligand and of the calculated stoichiometric amount of the $CuCl_2$ aqueous solution. The resulting solution was left at room temperature overnight to allow quantitative formation of the metal complex.[27] Reactions were started by addition of the aqueous solution of the substrate, previously heated at 90 °C for 2 min (to disrupt potential aggregates) and equilibrated for 10 min at the reaction temperature. The total volume of the reaction mixture was 200 μL. At proper time intervals, aliquots (25 μL) of the reaction mixtures were withdrawn and added to 30 μL of CH_2Cl_2. After the addition of 2.5 μL of a 0.5 M EDTA solution (final concentration 50 mM), the solutions were briefly shaken with a Vortex mixer and centrifuged. The aqueous phase was separated and quenched by addition of formamide and excess EDTA. Radiolabeled substrates and products were separated on 7 M urea/20% polyacrylamide gels. The dried gels were exposed to a Kodak phosphor screen that was analyzed by a Personal Molecular Imager. Quantitative analysis was carried out using the software Quantity-One (Bio-Rad). Nonlinear

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(24) Such a decrease is predicted to be substantial, but not dramatic. The number of rotatable single bonds, including copper(II)−phosphate coordinative bonds, which are involved in the closure of the smallest dinuclear metallomacrocycle is 11, and each additional nucleotide unit contributes 5 additional rotatable bonds. On a purely entropic basis, the *EM* for closure of a strainless macrocycle decreases from the value of 0.37 for the 11-rotor case to one of 0.037 M for the 31-rotor case. A further drop to 0.021 M is calculated for the 46-rotor case, which corresponds to the cleavage of the γ-CpA bond in the 17-mer **12**. See ref 23.

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least-squares calculation of kinetic data was carried out using the program SigmaPlot 2002 for Windows, version 8.0 (SPSS Inc.).

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Supporting Information Available: Kinetic treatment of rate data according to integrated rate equations derived from Schemes 2 and 3. This material is available free of charge via the Internet at http://pubs.ac.org.

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