Fast and Reliable Automated Synthesis of RNA and Partially 2′-*O*-Protected Precursors ('Caged RNA') Based on Two Novel, Orthogonal 2′-*O*-Protecting Groups

Preliminary Communication

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Two sets of RNA phosphoramidites, carrying the (fluoride-labile) 2′-*O*-[(triisopropylsilyl)oxy]methyl (=tom) group and the (photolabile) [(*R*)-1-(2-nitrophenyl)ethoxy]methyl (=(*R*)-npeom) group, were prepared (see **1**–**4** and **5**–**8**, resp.). The two protecting groups were completely orthogonal to each other. Three ribozyme-substrate constructs, protected each by a (*R*)-npeom group, were synthesized; on photolysis, efficient cleavage of this remaining protecting group occurred (*Scheme 3*). It could be demonstrated that the presence of one (*R*)-npeom group within a RNA strand has only a minor influence on the pairing properties of corresponding duplexes.

1. Introduction. – In contrast to the automated assembly of DNA, the routine synthesis of RNA is restricted to relatively short sequences (< 40 nucleotides). Structurally, this difference is related to the additional 2′-*O*-protecting groups which sterically interfere with the coupling process and require an additional deprotection step. From the large number of protecting groups investigated so far [1], the (*tert*-butyl)dimethylsilyl (=tbdms) group has found the widest application [2]. We have developed a synthetic method for the introduction of the known [3] (photolabile) [(2-nitrobenzyl)oxy]methyl (=nbom) group into ribonucleosides [4]. With this 2′-*O*-protecting group, better coupling yields and shorter coupling times could be realized (as compared to the tbdms-group) [3][4]. This result can probably be attributed to its lower steric demand [1][3][4]. Meanwhile, we have successfully applied our synthetic method to the introduction of related, formaldehyde acetal derived 2′-*O*-protecting groups [5]. This work describes the automated synthesis of RNA and caged [6][7] RNA with phoshoramidites protected by the [(triisopropylsilyl)oxy]methyl (=tom) group (→**1**–**4**)[1]) and the [(*R*)-1-(2-nitrophenyl)ethoxy]methyl (=(*R*)-npeom) group (→**5**–**8**)[2]), respectively (*Fig. 1*). The sequences prepared in the context of this work represent three different caged constructs of the hammerhead-ribozyme motif [9–11][3]).

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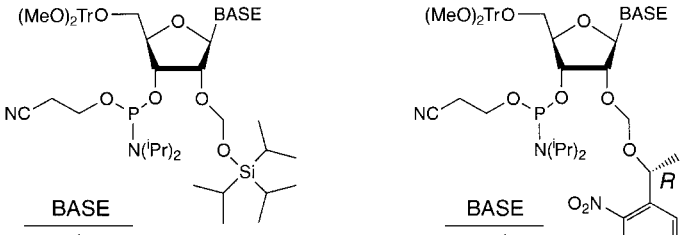
[1]) The full paper on the preparation of the phosphoramidites **1**–**4** and the assembly and deprotection of sequences derived therefrom is in preparation [8].

[2]) The (*R*)-configuration of the protecting group was chosen arbitrarily.

[3]) One example of such a caged ribozyme substrate has been described already by *Chaulk* and *MacMillan* [7], who protected one adenosine residue by the 2′-*O*-(2-nitrobenzyl) group. They observed partial cleavage of the protecting group by F^- ions (required for deprotection of 2′-*O*-silyl groups) and, therefore, have chosen the orthogonal (acid-labile) 2′-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl] (Fpmp)-protecting group. The nbom group [3][4] is also cleaved efficiently by F^- ions [12].

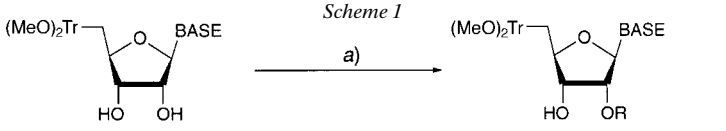


BASE		BASE	
1	ac^4**C**	**5**	ac^4**C**
2	ac^6**A**	**6**	ac^6**A**
3	ac^2**G**	**7**	ac^2**G**
4	**U**	**8**	**U**

Fig. 1. *Phosphoramidites prepared from the precursors* **15**–**22** (*Scheme 1*) *according to* [4][5] (ac = acetyl)

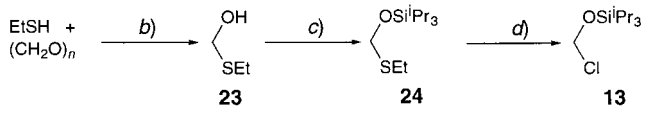
2. Results and Discussion. – From the *N*-acetylated, 5′-*O*-dimethoxytritylated nucleosides **9**–**12** and the alkylating agents tom-Cl (**13**) and (*R*)-npeom-Cl (**14**), respectively, the protected nucleosides **15**–**22** were obtained according to our published procedure [4][5] (*Scheme 1*)[4]) and transformed into phosphoramidites **1**–**8** or immobilized on solid support according to [4][5]. The alkylating agents **13** and **14** were prepared in analogy to known procedures from **23** and **25** *via* **24** and **26**, respectively (*Scheme 1*).

Scheme 1

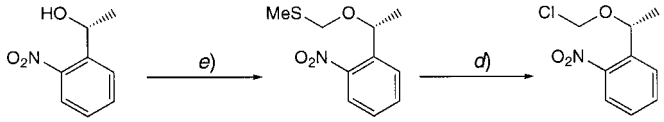


BASE		BASE	R = tom	R = (*R*)-npeom
9	ac^4**C**	ac^4**C**	**15** (45%)	**19** (65%)
10	ac^6**A**	ac^6**A**	**16** (40%)	**20** (55%)
11	ac^2**G**	ac^2**G**	**17** (60%)	**21** (85%)
12	**U**	**U**	**18** (40%)	**22** (55%)

$(MeO)_2Tr = (MeO\text{-}C_6H_4)_2PhC$, ac = MeCO, tom = $^iPr_3SiOCH_2$, npeom = $NO_2C_6H_4CH(Me)OCH_2$



23 **24** **13**



25 **26** **14**

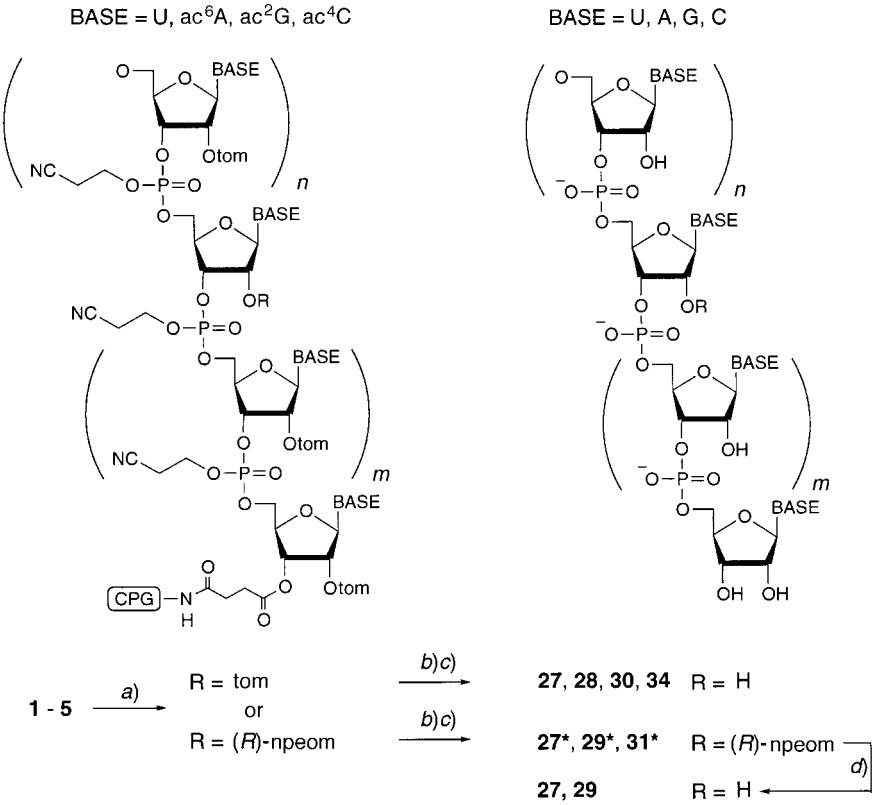
a) Bu_2SnCl_2, $^iPr_2NEt$ in $(CH_2Cl)_2$, 25°, 1 h; then **13** (→ **15**–**18**) or **14** (→ **19**–**22**), 70°, 30 min (according to [4][5]). *b*) NaOH, H_2O (cat.), 25 → 40°, 1 h; quant. [13]. *c*) $^iPr_3SiCl$, 1*H*-imidazole, CH_2Cl_2, 25°, 14 h, 85%. *d*) SO_2Cl_2, CH_2Cl_2, 25°, 1 h; 85% [13]. *e*) DMSO, Ac_2O, AcOH, 25°, 6 d (according to [3][14]).

[4]) The 3′-*O*-protected regioisomers were isolated as minor products. All described compounds were fully characterized. The R_f values and NMR spectra of nucleosides **19**–**22** and phosphoramidites **5**–**8** were nearly identical with those of the nbom-protected analogues [4].

With the phosphoramidites **1**–**5**, individual coupling yields >99% were obtained under standard DNA-coupling conditions[5]). The combination of acetyl (ac) and tom as base- and sugar-protecting group, respectively, allowed a short, mild and complete deprotection (*Scheme 2*). Under these conditions, the (*R*)-npeom group was completely stable[3]). The crude sequences **27**, **27***, **28**, **29***, **30**, **31***, and **34** were desalted on *Sephadex G-10* [5] and purified by HPLC (*Table*).

Fig. 2 shows the capillary electrophoresis (=CE) chromatograms of the crude and purified sequences **28** and **31***. They demonstrate that phosphoramidites **1**–**5** can be assembled to relatively long RNA sequences and with an efficiency equivalent to DNA

Scheme 2



tom = $^{i}Pr_3SiOCH_2$, npeom = $NO_2C_6H_4CH(Me)OCH_2$, CPG = controlled pore glass

a) Automated 1.5- or 15-μmol synthesis (50 or 500 mg of CPG, loading: 30 μmol g^{-1}) on a *Pharmacia Gene Assembler*; detritylation with 4% $CHCl_2COOH/(CH_2Cl)_2$; 15 μmol: 7 min coupling (400 μl of 0.1M **1**–**5**/600 μl of 0.35M 1-(benzylthio)-1*H*-tetrazol (= BnSTet) in MeCN); 1.5 μmol: 2.5 min coupling (120 μl of 0.1M **1**–**5**/360 μl of 0.30M BnSTet in MeCN); capping/oxidation: standard conditions [15]. *b*) 10M $MeNH_2$, $EtOH/H_2O$ 1:1, 25°, 3 h. *c*) 1M $Bu_4NF \cdot 3\,H_2O$, THF, 25°, 12 h. *d*) 5 μM **27*** or **29***, 10 mM $MgCl_2$, 50 mM Tris · HCl (pH 8), H_2O, 25°, photolysis.

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[5]) The commonly employed 2′-*O*-tbdms-protected phosphoramidites require longer coupling time and usually give lower coupling yields [1].

Table. *Preparation and Characterization of Sequences* (*Scheme 2*)

	r(5′-sequence-3′)[a]	Scale [μmol]	Coupling yield[b]) [%]	Isolated yield[c])		MS[e]) [*m*/*z*]	
				a.u. (260 nm)[d])	mg [%]	calc.[f])	found
27	ACGGUCGGUCGCC	1.5	99.4	45	2(25)	4131	4133
27*	ACGGUC*GGUCGCC	15	99.2	350	11(20)	4310	4314
28	GGCGACCCUGAUGAGGCCGAAAGGCCGAAACCGU	15	99.3	380	12(10)	11051	11053
29*	GGCCGAAACUCGUAAGAGUC*ACCAC	15	99.1	350	11(10)	8181	8180
30	GUGGUCUGAUGAGGCC	15	99.1	280	9(15)	5153	5152
31*	ACGGUC*GGUCGCCGUUUGGCGACCCUGAU-GAGGCCGAAAGGCCGAAACCGU	1.0	99.5	50	2(10)	–[g])	
34	GGCGACCGACCGU	1.5	99.3	50	2(25)	4154	4152

[a]) C* = 2′-*O*-[(*R*)-npeom]-protected cytidine nucleotide. [b]) Average coupling yield, determined by the trityl assay. [c]) Yield after purification by ion-exchange HPLC (*Nucleosil-SAX* or *Dionex-GenPak*); by CE, the purity was estimated >98% (**31***: *ca.* 90%). [d]) a.u. = absorption unit. [e]) MALDI-TOF MS; matrix: 2,4-dihydroxyacetophenone (ammonium citrate). [f]) For fragment $[M-H]^-$ (**28**: $[M-2H+K]^-$). [g]) Not measured.

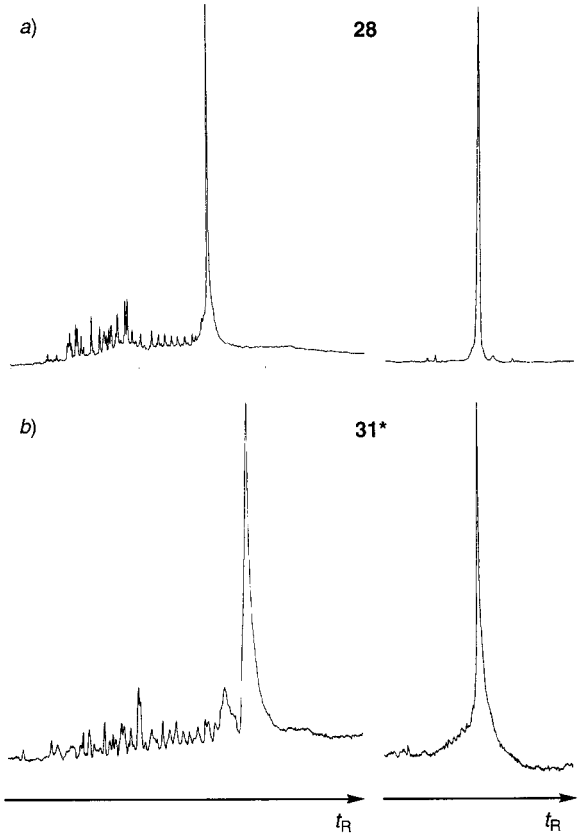


Fig. 2. *CE Traces from crude (left) and purified (right) RNA sequences* (measured at 260 nm)

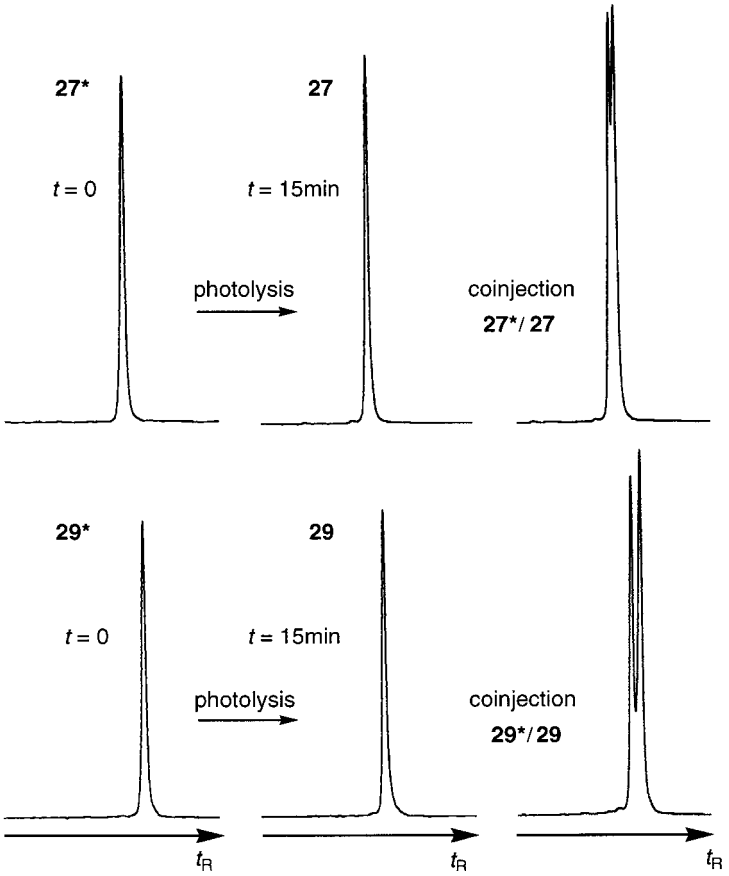


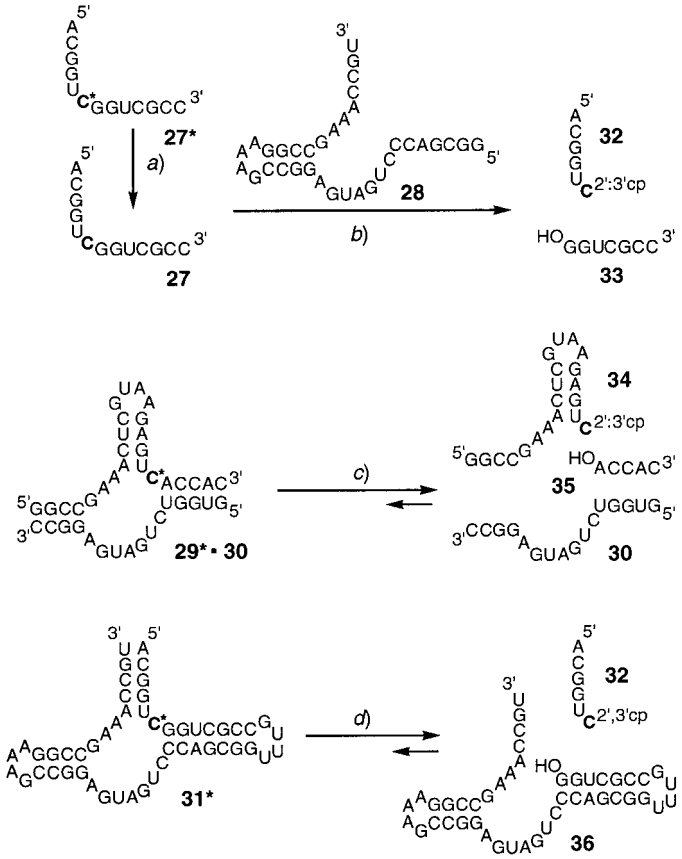
Fig. 3. *HPLC Traces of photolysis experiments* (measured at 260 nm).

synthesis. In an exploratory experiment, the photolysis of the substrates **27*** and **29*** was carried out under ribozyme-cleaving conditions (10 mM $MgCl_2$, pH 8) [9–11]. The corresponding HPLC traces in *Fig. 3* show a fast and complete removal of the remaining photolabile (*R*)-npeom protecting groups[6]).

In *Scheme 3* and *Fig. 4*, the cleavage experiments with the three ribozyme constructs **27**·**28**, **29**·**30** and **31** (obtained from their protected precursors) are shown. All reactions were carried out in the presence of Mg^{2+} ions at pH 8 and were induced by an irradiation period of 15 min. In the first experiment, a catalytic amount of ribozyme **28** was added after irradiation of the substrate-precursor **27***. After 3 h, the released sequence **27** had been cleaved completely (→ **32**/**33**), whereas without prior irradiation, no trace of cleavage was observed. In the second reaction, an equimolar mixture of the protected substrate **29*** and the ribozyme **30** was irradiated for 15 min. After 1 h, the equilibrium between scission and ligation had been reached (→ **34**/**35**/**29**). No reaction was observed in the control experiment without irradiation. A fast

[6]) The photolysis product of the sequence **27*** was identical with the authentic sequence **27** (MS, HPLC, CE).

Scheme 3. *Ribozyme-Catalyzed Cleavage Reactions* (*Fig. 4*)



C* = C[(*R*)-npeom]2′, C2′:3′p = cytidine 2′,3′-cyclophosphate

a) Photolysis. *b*) 5 μM **27** (from **27***), 0.5 μM **28**, 10 mM $MgCl_2$, 50 mM Tris·HCl (pH 8), H_2O, 40°, 3 h. *c*) 5 μM **29***, 5 μM **30**, 10 mM $MgCl_2$, 50 mM Tris·HCl (pH 8), H_2O, 25°, photolysis. *d*) 1 μM **31***, 2 mM $MgCl_2$, 20 mM Tris·HCl (pH 8), H_2O, 25°, photolysis.

cleavage reaction was also observed after irradiation of the ribozyme-substrate conjugate **31***; again equilibrium had been reached after 1 h (→ **32**/**36**/**31**), and the control experiment showed no cleavage at all.

These experiments demonstrate that the 2′-*O*-[(*R*)-npeom]-protected sequences are inactive precursors that are transformed into active substrates by photolysis.

The X-ray structures of the ribozymes **28** and **30** have been determined with other substrate analogues [10][11]. Using molecular-modeling experiments (MacroModel), we concluded that the (*R*)-npeom-protected sequences **27*** and **29*** should not strongly interfere with the conformation of the substrate-ribozyme pairing complexes. The thermodynamic parameters of duplex formation of **27*·37** differs only slightly from the corresponding parameters of the RNA duplex **27·37** (*Fig. 5,a*). The transition

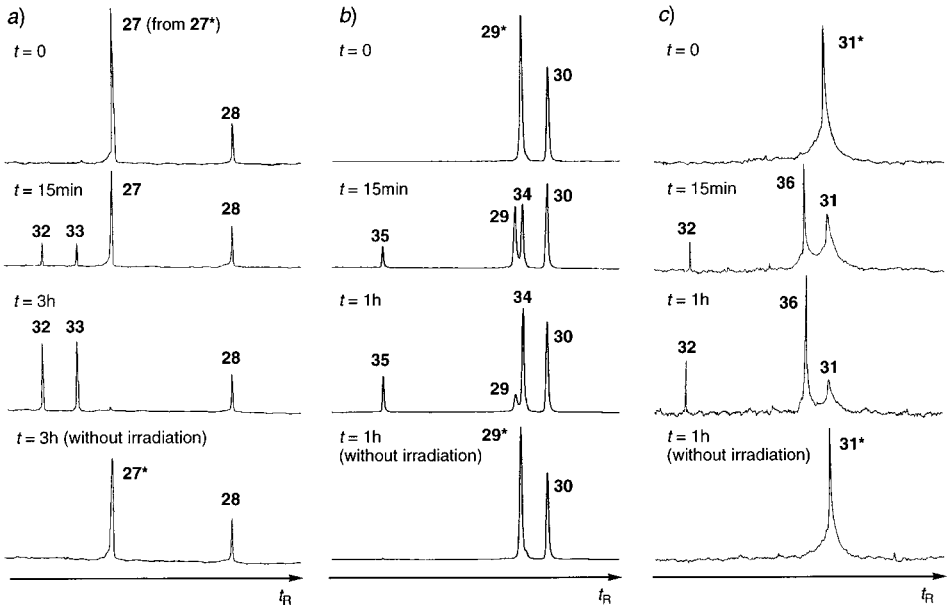


Fig. 4. *Ribozyme-catalyzed cleavage reactions* (*Scheme 3*): a) c) *CE traces* (measured at 260 nm); *b*) *HPLC traces* (measured at 260 nm). Aliquots from the reaction mixture were quenched by addition of EDTA.

curves of the ribozyme **28** with the substrate **27** and the precursor **27**** are nearly identical (*Fig. 5,b*). The cleavage of **27** by **28** was competitively inhibited by the substrate analogue **27*** (*Fig. 5,c*). These results are compatible with a minor influence of the additional protecting group on the strength and conformation of corresponding pairing complexes.

The two presented orthogonal 2′-*O*-protecting groups allowed an efficient, large-scale preparation of ribozymes and caged substrates. We are now trying to obtain single crystals in order to investigate by X-ray spectroscopy the fascinating, not yet fully understood [9] ribozyme cleavage process.

This work was supported by the ETH Zürich and the *Alfred-Werner-Stipendium*.

Experimental Part

General. Photolysis: 250-W Hg Lamp, 1-cm *Pyrex* filter, 15 min, 25°. CE: *BioFocus 3000* (*BioRad*) with a coated *BioCap-XL* column (75 μm × 40 cm, no. 1483082), run buffer (no. 1845026) + 6M urea, elution with 15 kV at 40°, detection at 260 nm. HPLC: *Nucleogel SAX 1000-8/46* (*Macherey & Nagel*, No. 719469), 0.5 ml/min; $A \rightarrow B$ in 45 min (*A:* 10 mM sodium phosphate in H_2O, pH 11.5; *B:* 10 mM sodium phosphate/1M NaCl in H_2O, pH 11.5), detection at 260 nm, elution at 25°.

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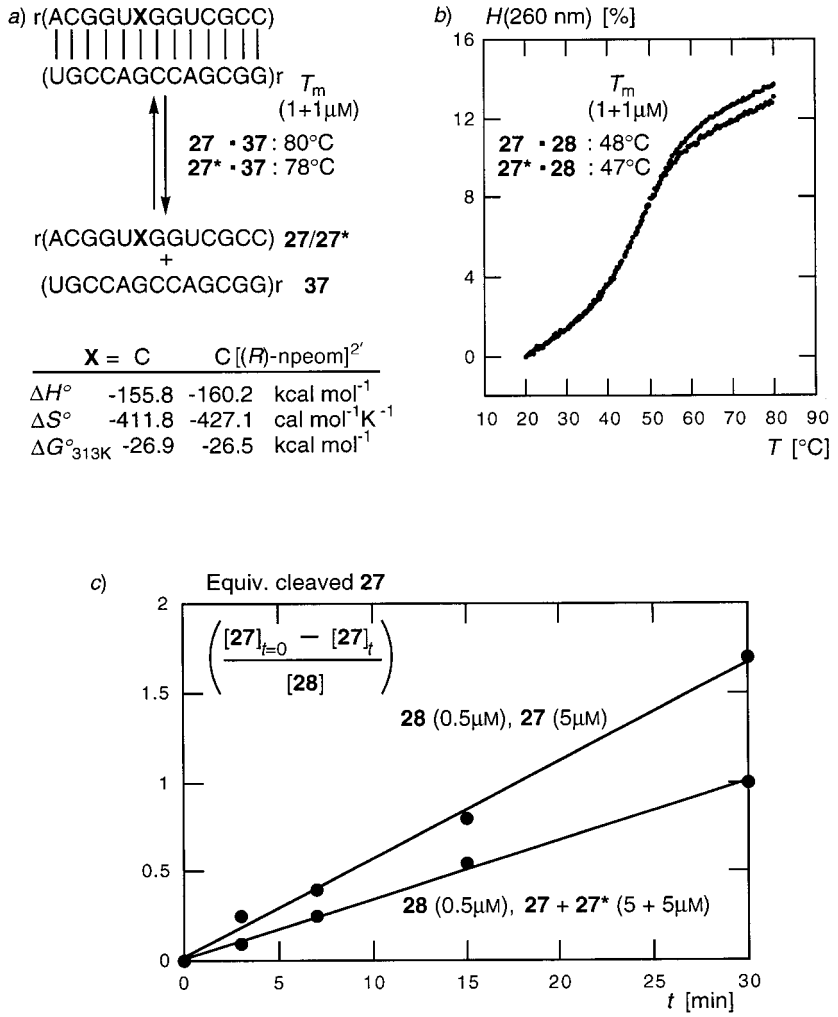

Fig. 5. *Pairing behavior of partially 2′-O-[(*R*)-npeom]-protected sequences.* Conditions: *a*) 150 mM NaCl, 10 mM Tris·HCl (pH 7.4) (thermodynamic parameters and transition temperatures determined according to [5]; estimated experimental error ±5%); *b*) as in *a*); *c*) 5 μM **27** (with and without 5 μM **27***), 0.5 μM **28**, 10 mM $MgCl_2$, 50 mM Tris·HCl (pH 8), H_2O, 40° (values determined from CE traces).

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Received June 24, 1999