Backbone-Modified RNA

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Amides as Excellent Mimics of Phosphate Linkages in RNA**

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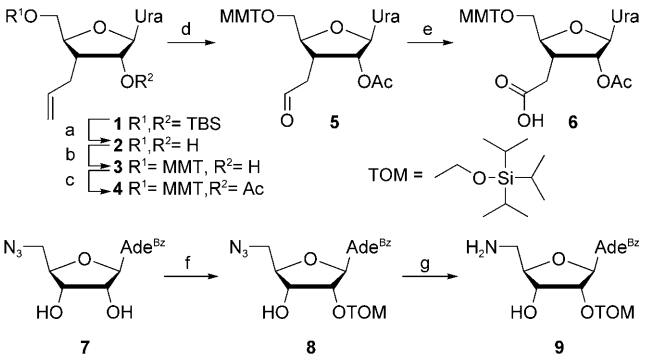
Since the discovery that RNA can catalyze chemical reactions, the number and variety of noncoding RNAs and the important roles they play in biology have been growing steadily. Backbone-modified RNA may find broad application in the fundamental biology and biomedicine of noncoding RNAs, providing that the modifications mimic the structure of the phosphodiester linkage and do not alter the conformation of RNA. In particular, the potential of RNA interference to become a new therapeutic strategy has revitalized interest in chemical modifications that may optimize the pharmacological properties of short interfering RNAs (siRNAs).[1] We are interested in hydrophobic nonionic mimics of the phosphate backbone, such as formacetals[2] and amides,[3] that may confer high nuclease resistance to siRNAs along with reduced charge and increased hydrophobicity. Earlier studies showed that 3′-CH_2-CO-NH-5′ internucleoside amide linkages (abbreviated here as AM1) were well-tolerated in the DNA strand of an A-type DNA–RNA heteroduplex.[4] Subsequently, we found that AM1 modifications did not change the thermal stability of RNA–RNA duplexes.[3] Most importantly, Iwase et al.[5] recently showed that AM1 amides were well-tolerated in the 3′ overhangs of siRNAs.

Taken together, these data suggest that amides may be good mimics of phosphate linkages in RNA; however, beyond simple melting-temperature measurements, the structural and thermodynamic properties of amide-modified RNA have not been established. Herein we present the first comprehensive structural and thermodynamic study that clearly shows that AM1 linkages do not disturb the A-type structure, thermal stability, and hydration of RNA duplexes. Despite the different geometry, amide AM1 appears to be an excellent mimic of the phosphate linkage in RNA. Our study complements structural studies on amide-modified DNA[4,6] and provides the first detailed insight into how the AM1 amide is accommodated in an RNA duplex.

We started by designing a new route for the synthesis of the r(U_{AM1}A) dimer phosphoramidite, which was used to prepare the amide-modified RNA sequences (Scheme 1). The



Scheme 1. Synthesis of the carboxylic acid and amine monomers **6** and **9**: a) TBAF, THF, room temperature, 24 h, 95%; b) *p*-methoxytrityl chloride, pyridine, room temperature, 12 h, 89%; c) acetic anhydride, DMAP, pyridine, room temperature, 4 h, 91%; d) OsO_4, 4-methylmorpholine *N*-oxide, dioxane, room temperature, 10 h; then $NaIO_4$ in water, room temperature, 10 h, 80%; e) $NaClO_2$, NaH_2PO_4, DMSO, *t*BuOH, water, room temperature, 30 h, 67%; f) DIEA, Bu_2SnCl_2, dichloroethane, room temperature, 1 h; then TOM-Cl, 80°C, 30 min, 30%; g) H_2, Pd/C, methanol, room temperature, 14 h, 71%. Bz = benzoyl, DIEA = *N*,*N*-diisopropylethylamine, DMAP = 4-dimethylaminopyridine, DMSO = dimethyl sulfoxide, TBAF = tetrabutylammonium fluoride.

tert-butyldimethylsilyl (TBS) groups in the known 3′-allyluridine **1**[3] were replaced with 5′-*O*-methoxytrityl (MMT) and 2′-*O*-acetyl protecting groups suitable for solid-phase RNA synthesis. Two-step oxidative degradation of the alkene gave the carboxylic acid part **6** of the r(U_{AM1}A) dimer.[4a,b]

For the synthesis of the amine part, we designed a novel route involving selective protection of the 2′-OH group of 5′-aminoadenosine with the triisopropylsilyloxymethyl (TOM) group. Treatment of 5′-azido-*N*-benzoyladenosine (**7**) with dibutyltin chloride followed by TOM chloride gave a mixture of 2′- and 3′-*O*-TOM nucleosides, from which the desired compound **8** was isolated in 30% yield. Reduction of the azide gave the amine **9**, which was coupled with the carboxylic acid **6** to give the dimer **10** (Scheme 2). Although protection of the 2′-OH group of adenosine **7** was relatively low-yielding, this strategy was advantageous because it eliminated difficult

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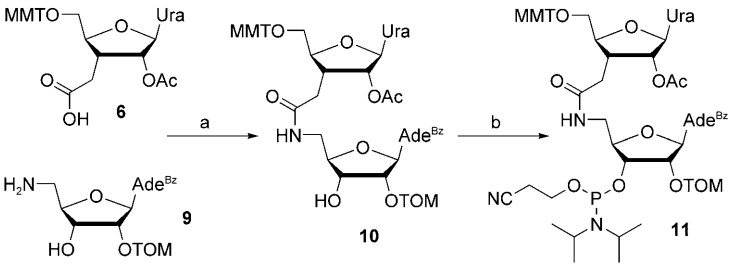

Scheme 2. Synthesis of the r(U_{AM1}A) dimer phosphoramidite **11**: a) HBTU, HOBt, DIEA, CH_2Cl_2, room temperature, 12 h, 54%; b) DIEA, $ClP(OCH_2CH_2CN)NiPr_2$, CH_2Cl_2, room temperature, 4 h, 74%. HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxy-1*H*-benzotriazole.

protecting-group manipulations after the preparation of the dimer.

Dimer **10** was converted into **11** in one standard step for phosphoramidite synthesis. Thus, the route in Schemes 1 and 2 enabled the synthesis of a phosphoramidite of an amide-linked dimer for the first time. Dimer **11** was compatible with chemistry used on standard DNA/RNA synthesizers (our previous route[3] gave an H-phosphonate dimer) and was used together with common 2′-*O*-TOM-protected ribonucleoside monomers (Glen Research) to synthesize a series of self-complementary amide-modified RNAs (Table 1) according to standard phosphoramidite chemistry on an Expedite 8909 instrument.

Although our preliminary study[3] established that the AM1 modification did not decrease the thermal stability of RNA duplexes, the detailed biophysical properties and structure of the amide-modified RNA were not studied. Such information is important for a fundamental understanding of how backbone modifications are accommodated in RNA and for the design of amide-modified siRNAs.

Water is an integral part of nucleic acid structure.[7] However, the importance of hydration is frequently neglected when nucleic acid modifications are designed. Hydrophobic modifications can be expected to have significant impact on the structure and thermal stability of nucleic acids by interfering with hydration. To gain insight into the hydration of the amide-modified RNA, we studied $CG(U_{AM1}A)_5CG$ (**OL2**), which contained 10 amide linkages per duplex and was similar to the modified DNA and RNA used in our previous osmotic-stress studies.[2,8] The substitution of 10 out of 26 phosphates with amides slightly decreased the thermal stability of **OL2** (−0.5 °C per modification). The ΔT_m value (change in melting temperature) per modification for the amide linkage is very similar to the destabilization observed for the phosphorothioate modification, which is considered to be the current state-of-the-art phosphate mimic. Remarkably, osmotic stress[8,9] showed that the relatively hydrophobic amides did not significantly change the hydration (Δn_W in Table 1) of **OL2** relative to that of unmodified **OL1**. This result was surprising, because **OL2** had more than a third of the polar phosphate linkages replaced by amides. Similar ΔH values, obtained by different methods, confirmed that the melting was a two-state transition. Circular dichroism (see Figure S3 in the Supporting Information) and UV melting at different oligonucleotide concentrations (see Figure S4) confirmed that **OL1** and **OL2** were duplexes (not hairpins) of similar structure. Similar results were obtained for $GCGU_{AM1}ACGC$ (**OL4**).

The solution conformations of **OL3** and **OL4**, determined from NMR spectroscopic experiments,[10] are compared in Figure 1 (see also Figure S8). Chemical shifts and 2D NOESY spectra indicated that the two structures were very similar and

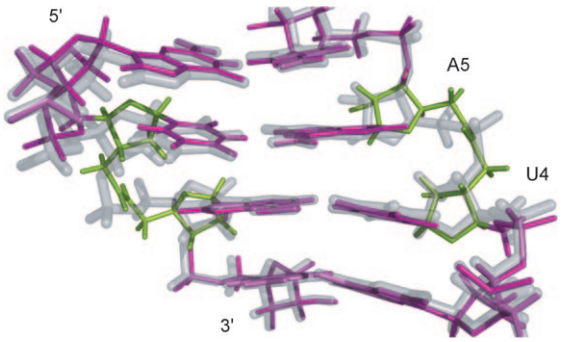

Figure 1. The central four base pairs of the solution structures of the amide-modified RNA **OL4** (purple, the amide linkage is highlighted in green) and the unmodified RNA **OL3** (gray), as determined by NMR spectroscopy.

Table 1: Results of UV thermal melting, calorimetry, and osmotic-stress studies for amide-modified RNA.[a]

RNA	Sequence	T_m [°C]	DSC[b]	$-\Delta H$ [kcal mol^{-1}] $d\alpha/d(1/T_m)$ vs. T_m[c]	van 't Hoff[d]	ΔS[d] [eu]	$-\Delta G_{310}$[d] [kcal mol^{-1}]	Δn_W[e] (cosolute: ethylene glycol)	Δn_W[e] (cosolute: glycerol)	Δn_W[e] (cosolute: acetamide)
OL1	$CG(UA)_5CG$	54.0 ± 0.3	83.1 ± 0.8	77.1 ± 3.9	80.7 ± 6.6	221 ± 20	12.4 ± 0.4	39 ± 9	45 ± 8	77 ± 5
OL2	$CG(U_{AM1}A)_5CG$	49.3 ± 0.2	71.9 ± 1.5	83.1 ± 3.6	73.7 ± 6.1	203 ± 19	10.9 ± 0.3	38 ± 3	38 ± 3	76 ± 7
OL3	GCGUACGC	58.3 ± 0.4	68.2 ± 1.8	69.2 ± 7.0	68.1 ± 8.8	179 ± 26	12.5 ± 0.6	30 ± 3	41 ± 3	65 ± 7
OL4	$GCGU_{AM1}ACGC$	56.5 ± 0.2	57.1 ± 0.3	61.6 ± 6.1	70.3 ± 2.3	187 ± 7	12.3 ± 0.2	36 ± 3	33 ± 3	44 ± 3

[a] The oligonucleotides (2 μM for **OL1** and **OL2**; 4 μM for **OL3** and **OL4**) were studied in a buffer solution containing 10 mM sodium cacodylate (pH 7.4), 0.1 mM ethylenediaminetetraacetic acid, and 300 mM NaCl. Results are given with the standard deviation (±). [b] $-\Delta H$ value determined by differential scanning calorimetry. [c] $-\Delta H$ value determined from a plot of $d\alpha/d(1/T_m)$ against T_m.[8] [d] $-\Delta H$ value determined by van't Hoff analysis of melting curves. [e] Δn_W = number of water molecules released upon melting (for a discussion of osmotic stress, see references [8] and [9]). Results are given with error estimates (±).

that all expected base pairs were formed, including the AU pairs flanking the amide modification (see Figures S5–S7). All ribose groups were in the C3′-*endo* conformation, as indicated by strong H3′–H4′ scalar coupling and undetectable H1′–H2′ coupling. The backbone conformation was the A form for the three terminal base pairs, as indicated by H4′–H5′/H5″ and H3′–P scalar couplings and ^{31}P chemical shifts (see Table S1 in the Supporting Information). The conformation of the amide linkage was determined from strong A5H4′–H5′ scalar coupling, strong U4H3′–U4H6′ scalar coupling, strong coupling of the amide proton to the U4H6′ proton, a weak NOE between the amide proton and U4H2′, and a medium NOE between the amide proton and A5H4′. The restraints indicated a *trans* amide bond, a *trans* conformation of the γ dihedral angle of A5 instead of the canonical g^+ conformation, and a *trans* conformation of the ε dihedral angle of U4.

The amide linkage required a distance between adjacent sugars approximately 0.2 Å greater than in A-form RNA (measured from the U4C3′ carbon atom to the A5C4′ carbon atom) and, more significantly, would result in a 2.6 Å displacement of the U4 sugar if modified and unmodified A5 sugars were colocalized (Figure 2a). However, the

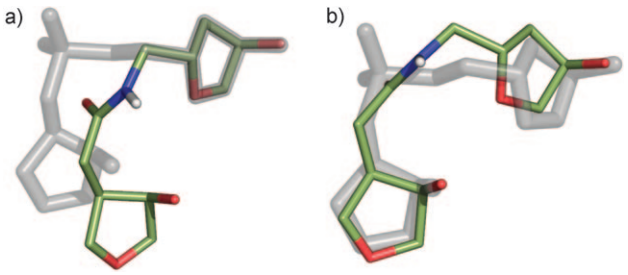

Figure 2. Comparison of the amide-modified linkage (in color) with the unmodified phosphate linkage (gray; only the linkage and the U4 and A5 sugar groups are shown): a) molecules overlaid by alignment of the A5 sugars; b) all-atom alignment from the model in Figure 1.

distance between the adjacent AU base pairs was similar to the A-form distance, and normal base pairs were formed (Figures 1 and 2b). The reorientation of the amide linkage was accommodated in large part by an approximately 12° rotation about the glycosidic bond of the adenine residue (Figure 2a,b). This rotation was consistent with the relatively small NOE for A5H8–A5H3′ (see Figure S7). The slight displacements on the 3′ and 5′ side of A5 and U4, respectively (see Figure 1), were accommodated in the model on the 3′ side by rotation of the A5 ξ dihedral angle and on the 5′ side by rotation of the U4 γ angle (ca. 10 and 8° relative to the A-form values, respectively). Other dihedral angles in the model that were different from A-form values by more than 5° were the C6 γ and U4 β angles. However, small rotations do not cause significant changes in scalar coupling constants or short-range NOEs and were not confirmed by the data.

In summary, we have developed a new route to amide-modified RNA that is compatible with standard phosphoramidite chemistry. The hallmark of the route is the selective protection of the 2′-OH group with the TOM group, which should be applicable to other backbone-modified dimers as well. Our results reveal that the 3′-CH_2-CO-NH-5′ amide linkages have surprisingly little effect on the global A-type structure, thermal stability, and hydration of RNA and appear to be excellent mimics of phosphodiester linkages. NMR spectroscopic studies and thermodynamic studies provided unique insight into how the AM1 amide, which has a different chemical structure and local conformation to those of phosphodiesters, is accommodated in an RNA duplex. The fact that the relatively hydrophobic amide does not disturb RNA hydration is surprising and suggests that the phosphate linkage may be in general a good position for the modification of RNA. Taken together with recent results of Iwase et al.,[5] our study suggests that amides may be promising modifications for the optimization of siRNAs.

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