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Amides Are Excellent Mimics of Phosphate Linkages in RNA**

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After the discovery that RNA can catalyze chemical reactions, the number and variety of non-coding RNAs and the important roles they play in biology has been growing steadily. Backbone modified RNA may find broad applications in fundamental biology and biomedicine of non-coding 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 (RNAi) 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 non-ionic 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 work showed that 3'-CH₂-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 and co-workers^[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, 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

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duplexes. Despite the different geometry, amide AM1 appears to be an excellent mimic of the phosphate linkage in RNA. Complementing structural work on amide-modified DNA,^[4,6] our study provides the first detailed insight into how the AM1 amide is accommodated in an RNA duplex.

We started with designing of a new route for synthesis of the r(U_{AM1}A) dimer phosphoramidite, which was used to prepare the amide-modified RNA sequences. (Scheme 1). The TBS groups in the known 3'-allyl uridine **1**^[3] were replaced with 5'-O-methoxytrityl (MMT) and 2'-O-acetyl protections suitable for solid-phase RNA synthesis. Two-step oxidative degradation of the alkene gave the carboxylic acid (**6**) part of the r(U_{AM1}A) dimer.^[4a,b]

For synthesis of the amine part we designed a novel route involving selective protection of the 2'-OH of 5'-aminoadenosine with the triisopropylsilyloxymethyl (TOM) group. Treatment of 5'-azido-N-benzoyl adenosine **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 the 2'-protection of adenosine **7** was relatively low yielding, this strategy was advantageous because it eliminated difficult protecting group manipulations after the preparation of dimer.

Thus, dimer **10** was converted into **11** in one standard step of phosphoramidite synthesis. The route in Schemes 1 and 2 allowed, for the first time, synthesis of a phosphoramidite of amide-linked dimer, which was compatible with chemistry used on standard DNA/RNA synthesizers (our previous route^[3] gave an H-phosphonate dimer). Dimer **11** 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 thermal stability of RNA duplexes, detailed biophysical properties and structure of amide-modified RNA were not studied. Such information is important for fundamental understanding of how backbone modifications are accommodated in RNA and for design of amide-modified siRNAs.

Water is integral part of nucleic acid structure.^[7] However, the importance of hydration is frequently neglected when designing nucleic acid modifications. Hydrophobic modifications can be expected to have significant impact on nucleic acid structure and thermal stability by interfering with hydration. To gain insight into 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] Substitution of 10 out of 26 phosphates with amides slightly decreased the thermal stability of **OL2** (−0.5 °C per modification). The Δt_m per modification for the amide linkage is very similar to the destabilization observed for 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** compared to unmodified **OL1**. This result was surprising because **OL2** had more than 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 (Figure S3) and UV melting at different

oligonucleotide concentrations (Figure S4) confirmed that **OL1** and **OL2** were duplexes not hairpins) of similar structure. Similar results were obtained with GCGU_{AM1}ACGC **OL4**.

The solution conformations of **OL3** and **OL4**, determined from NMR 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 that all expected base pairs were formed, including the AU pairs flanking the amide modification (Figures S5–S7).

All ribose groups were in the C3'-endo conformation as indicated by strong H3'-H4' proton scalar coupling and undetectable H1'-H2' coupling. The backbone conformation was A-form for the three terminal base-pairs as indicated by H4'-H5'/H5'' and H3'-P scalar couplings and ³¹P chemical shifts (Table S1). 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, A5 γ in the *trans* conformation (~180°) instead of the canonical *g*⁺ conformation, and U4 ϵ in the *trans* conformation.

The amide linkage required a distance between adjacent sugars approximately 0.2 Å greater than in A-form RNA (measured from U4C3' carbon to the A5C4' carbon) and, more significantly, would result in a 2.6 Å displacement of the U4 sugar if modified and unmodified A5 sugars were co-localized (Figure 2a). Yet the distance between the adjacent AU base pairs was similar to the A-form distance and normal base pairs were formed (Figure 1 and 2b). The reorientation of the amide linkage was accommodated in large part by a ~12° rotation about the glycosidic bond of the adenine residue (cf. Figure 2a and 2b). This rotation was consistent with the relatively small NOE for A5H8-A5H3' (Figure S7). The slight displacements 3' and 5' 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 (~10° and ~8° relative to A-form values, respectively). Other dihedral angles in the model that were different from A-form values by more than 5° were C6 γ and U4 β . However, small rotations do not cause significant changes in scalar coupling constants or short-range NOEs and are not confirmed from 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 2'-OH via the TOM group, which should be applicable to other backbone-modified dimers as well. Our results reveal that the 3'-CH₂-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 and thermodynamic studies provide unique insights into how the AM1 amide, displaying different chemical structure and local conformation than 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 place to modify RNA. Taken together with recent results of Iwase and co-workers,^[5] our study suggests that amides may be promising modifications to try for optimization of siRNAs.

Experimental Section

See supporting information for details

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Figure 1.

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

Figure 2. Amide-modified linkage (color) compared to unmodified phosphate linkage (gray) showing only the linkage and the U4 and A5 sugar groups: (a) molecules are overlaid by alignment of the A5 sugars and (b) all atom alignment from the model in Figure 1.

Scheme 1.

Synthesis of Carboxylic Acid and Amine Monomers. Steps: (a) TBAF, THF, rt, 24 h, 95%; (b) p-methoxytrityl chloride, pyridine, rt, 12 h, 89%; (c) acetic anhydride, DMAP, pyridine, rt, 4 h, 91%; (d) OsO_4 , 4-methylmorpholine N-oxide, dioxane, rt, 10 h, then add NaIO_4 in water, rt, 10 h, 80%; (e) NaClO_4 , NaH_2PO_4 , DMSO, *t*-BuOH, water, rt, 30 h, 67%; (f) DIEA, Bu_2SnCl_2 , dichloroethane, rt, 1 h, then add TOM-Cl, 80 °C, 30 min, 30%; (g) H_2 , Pd/C, methanol, rt, 14 h 71%.

Scheme 2.

Synthesis of r(U_{AM1}A) Dimer Phosphoramidite. Steps: (a) HBTU, HOBT, DIEA, CH₂Cl₂, rt, 12 h, 54%; (b) DIEA, CIP(OCH₂CH₂CN)N(iPr)₂, CH₂Cl₂, rt, 4 h, 74 %.

Table 1
UV Thermal Melting, Calorimetry and Osmotic Stress Results of Amide-Modified RNA. ^[a]

Entry	Sequence	t_m °C	-ΔH kcal/mol			ΔS eu ^[d]	-ΔG ₃₁₀ kcal/mol ^[d]	Ethylene glycol Δn _w ^[e]	Glycerol Δn _w ^[e]	Acetamide Δn _w ^[e]
			DSC ^[b]	$\frac{d\Delta t_m}{dT_m}$ vs. T_m ^[c]	van't Hoff ^[d]					
OL1	CG(UA) ₃ CG	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 _{AmiA}) ₃ CG	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 _{AmiA} CGC	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] Oligonucleotide (2 μM for **OL1** and **OL2** and 4 μM for **OL3** and **OL4**) in 10 mM sodium cacodylate (pH 7.4), 0.1 mM EDTA, and 300 mM NaCl. Results ± standard deviations.

^[b] From differential scanning calorimetry.

^[c] From $\frac{d\Delta t_m}{dT_m}$ vs. T_m , ref. 8.

^[d] From van't Hoff analysis of melting curves.

^[e] Δn_w = number of water molecules released upon melting (for discussion of osmotic stress, see refs. 8 and 9). Results ± error estimates.