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# Conversion of adenine to 5-amino-4-pyrimidinylimidazole caused by acetyl capping during solid phase oligonucleotide synthesis

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ABSTRACT

The acetyl capping reaction used throughout solid phase oligonucleotide synthesis is meant to minimize *n−1* deletionmer impurities by terminating sequences that fail to couple to a phosphoramidite. However, the reaction is also responsible for the formation of a number of impurities. One capping-related impurity has an additional mass of 98 amu from the parent oligonucleotide. The *n+98 amu* impurity was found to result from modification of an adenine nucleobase. The structure of the impurity was determined by preparation of an oligonucleotide enriched in *n+98 amu*, enzymatic digestion to individual nucleosides, isolation of the pure nucleoside+98 amu species, crystallization, and X-ray crystallographic analysis. The *n+98 amu* impurity is an oligonucleotide in which one adenine residue has been converted to 5-amino-4-pyrimidinylimidazole. The mechanism of formation of the impurity was investigated, and a mechanism is proposed.



Antisense therapy is a powerful technology in which oligonucleotides regulate protein expression by binding mRNA and targeting it for degradation.[1] The pharmaceutical platform has experienced dramatic growth, with antisense oligonucleotides (ASOs) increasingly making their way into clinical trials and to the market.[2] It is important that drugs dosed in humans have a controlled impurity profile, both in terms of minimization of impurities and of understanding their structures and mechanisms of formation. As part of an ongoing investigation into ASO impurities,[3–6] we report here the characterization of a formerly unknown and frequently observed oligonucleotide impurity generated during the manufacturing process, the *n+98 amu* impurity. As its name implies, the impurity has a mass 98 amu larger than the parent oligonucleotide.

Our ASO manufacturing process utilizes a highly optimized version of the phosphoramidite approach,[7,8] in which the oligonucleotide is assembled one nucleotide at a time on a solid support with the aid of a computer-controlled automated synthesizer. Each nucleotide is attached using a four-step cycle (Scheme 1): (1) acidic removal of the DMTr protecting groups on the support, (2) phosphoramidite coupling, (3) oxidation or oxidative sulfurization, and (4) acetyl capping. Acetyl capping is accomplished by delivering equal volumes of two solutions through the column: Cap A composed of 2:3:5 *N*-methylimidazole (NMI):pyridine:solvent (v/v/v) and Cap B composed of 1:4 acetic anhydride ($Ac_2O$):solvent (v/v). Acetonitrile or toluene can be used as solvents. Capping is meant to acetylate alcohols that failed to couple during the coupling step and to minimize the formation of *n−1* deletionmer impurities.[9] Though capping has proven useful in cases of high coupling failure, there are drawbacks associated with the reaction. Among those drawbacks are "capping-related impurities" that form as byproducts during acetyl capping. After successfully characterizing one such impurity as an oligonucleotide with the $N^2$-acetyl-2,6-diaminopurine nucleobase in place of a guanine residue,[3] we turned our attention to another capping-related impurity, *n+98 amu*.

Capping-related impurities can be increased by conducting oligonucleotide synthesis with a large excess of acetyl capping. In a representative experiment, two samples of a 20-nucleotide sequence were synthesized, ASO **1** with no capping treatment and ASO **2** with delivery of 81 mL/mmol of Cap A and Cap B solution (corresponding to 86 molar equiv of $Ac_2O$ relative to ASO on support) over 15 min for cycles 1–19 (Table 1). The latter capping treatment is roughly 5 times our typical capping delivery volume and 10 times the contact time. Comparison of the average MS under the main product peak in each case shows a substantial increase in a number of capping-related impurities in ASO **2** relative to ASO **1** (Fig. 1), including abasic species resulting from 5-methylcytosine hydrolysis, $(P=O)_1$, *n+41 amu*,[3] *n+69 amu*, *n*

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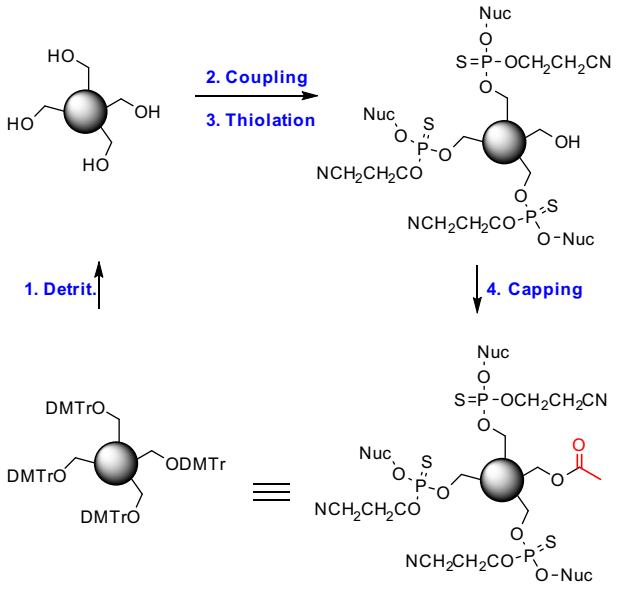


**Scheme 1.** Solid phase ASO synthesis with phosphoramidites.

*+83/84 amu*, and *n+98 amu*. ASO **2** was analyzed by high resolution MS (HRMS) with the results illustrated in Figure 2. Comparison of the monoisotopic mass of the *n+98 amu* impurity to that of the full length species indicated a mass difference of +98.0296 amu.

ASOs **1** and **2** were treated with formic acid at elevated temperature to hydrolyze purine nucleobases from the sequence. In addition to adenine and guanine, a species consistent with adenine +98 amu was clearly present in the ASO **2** hydrolysate. None of the species could be detected in the ASO **1** hydrolysate. No species consistent with guanine+98 amu was observed (See Supporting Information). This result suggests that the *n+98 amu* impurity is a result of modification of an adenine residue.

Indeed, polyadenosine sequences consistently generate a clear *n +98 amu* impurity when excessive capping is used. In the case of the $A_{20}$ DNA sequence, ASO **3** was synthesized with no capping treatment and ASO **4** with delivery of Cap A + B solutions containing 1581 equiv of acetic anhydride on the final cycle (Table 1). Comparison of MS data for ASO **3** to **4** shows a large amount of *n+98 amu* impurity (~8%) in the latter that is absent in the former. HRMS analysis of both samples (Fig. 3) reveals an exact mass difference between the impurity and full length species of +98.0264 amu.

To characterize the *n+98 amu* impurity, ASO **4** was enzymatically digested into individual nucleosides with a mixture of snake venom phosphodiesterase and alkaline phosphatase. The 2′-deoxyadenosine (dA) +98 amu component was identified in the mixture

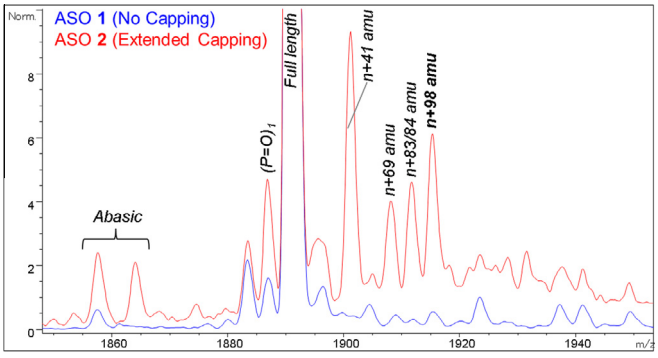


**Figure 1.** Average mass spectra (MS) under the main peaks for ASO **1** (blue) and ASO **2** (red). Capping-related impurities are labeled.

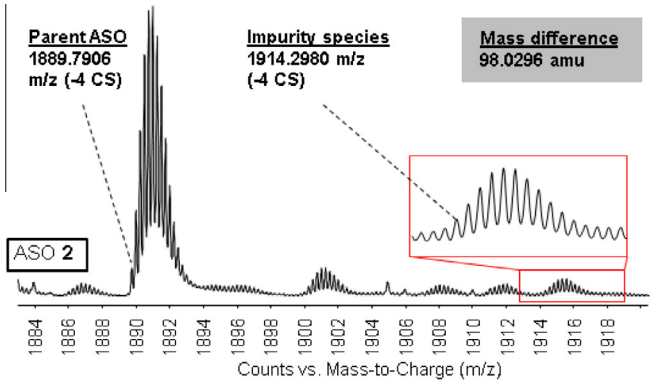


**Figure 2.** High resolution MS of ASO **2** (top) and ASO **1** (bottom) with a comparison of monoisotopic mass of the *n+98 amu* impurity and full length.

**Table 1**
Experimental details for ASO capping experiments

| ASO # | Sequence (5′–3′) | Equiv $Ac_2O$ delivered[a]/cycles |
|---|---|---|
| **1** | TGGTG CACAGTTTCT GGCAG[b] | No capping |
| **2** | TGGTG CACAGTTTCT GGCAG[b] | 86 equiv/cycles 1–19 |
| **3** | AAAAAAAAAAAAAAAAAAAA[c] | No capping |
| **4** | AAAAAAAAAAAAAAAAAAAA[c] | 1578 equiv/cycle 20 |

[a] Delivered as Cap A + B solutions. Equiv are relative to support loading.
[b] A = adenine, C = 5-methylcytosine, G = guanine, T = thymine, internucleotide linkages are phosphorothioates, underlined residues denote 2′-*O*-methoxyethyl (MOE) nucleotides, non-underlined residues denote 2′-deoxy nucleotides, and the 5′-alcohol of the ASO is protected as the 4,4′-dimethoxytrityl (DMTr) ether.
[c] A = 2′-deoxy adenosine, internucleotide linkages are phosphate diesters, and the 5′-alcohol of the ASO is unprotected.

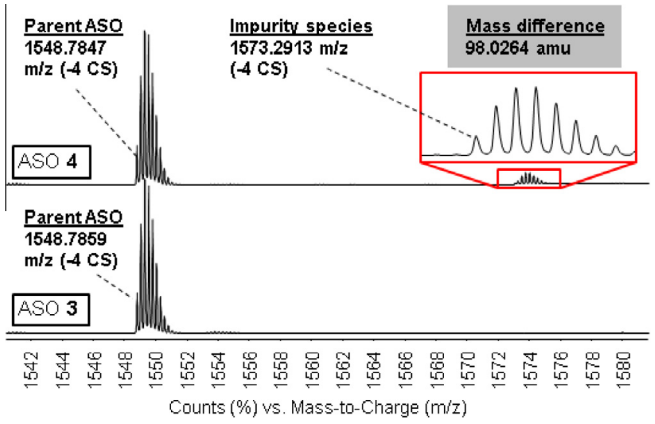


**Figure 3.** HRMS of ASOs **3** (bottom) and **4** (top) with a comparison of monoisotopic mass of the *n+98 amu* impurity and full length in the latter.

and purified by reversed-phase (RP) chromatography. Analysis of the purified dA+98 amu species by HRMS gave a mass consistent with the formula $C_{15}H_{19}N_4O_2$, corresponding to an additional $C_5H_6O_2$ (98.0368 amu) attached to the nucleoside. Analysis by NMR revealed some structural features of the modified nucleoside, such as the presence of two carbonyl groups and two methyl groups as well as the absence of one of the protons at the 2 and 8 positions of the former adenine ring, but it was not sufficient to solve the structure. The dA+98 amu structure was ultimately solved by X-ray crystallography (Fig. 4). The compound crystallized from ethanol/water as the trihydrate.

The mechanism of formation of the *n+98 amu* impurity was studied in detail. The protected dA compound **5** was treated with Cap A and B solutions for 6 days, quenched with MeOH, and then