

Troubleshooting the Synthesis of Modified Oligonucleotides

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Over the past decade, the level of sophistication regarding oligonucleotide synthesis has grown tremendously. Because the applicability of unmodified oligonucleotides is fairly limited, a great deal of time and money is spent on developing modified oligonucleotides. Most researchers find that some degree of oligonucleotide "tweaking" is necessary to get the desired properties for diagnostic and therapeutic applications.

Although the diagnostic and therapeutic industries have different needs, there is considerable overlap in the chemistries being developed. The diagnostic industry is confronted with two primary challenges: stringency and sensitivity (and consequently background noise). Unfortunately, these two requirements are almost mutually exclusive. In order to have high sensitivity, one must have a robust system with probes that will bind available targets with high affinity. However, in driving the equilibrium towards duplex formation, there is an increased binding to non-targets, leading to false positives. In order to solve these problems, researchers have turned to a number of oligonucleotide modifications, including base, sugar and backbone modifications to control hybridization properties. Conjugating reporter groups, such as fluorophores, chemiluminescent reagents and microchips can enhance sensitivity.

The therapeutic industry has a slightly broader range of problems to solve. Although there is disagreement about the severity of the various issues, and the solutions, most would agree that the following are the primary considerations:

1. *In vivo* stability
2. Strong hybridization properties to target while maintaining high stringency - *in vivo*
3. Circulatory lifetime
4. Tissue and cellular targeting
5. Metabolic properties (toxicity)

It was obvious from the outset that unmodified oligonucleotides would not become a viable drug candidate. A series of modifications enhancing the ability of the oligonucleotide to overcome these obstacles was introduced; phosphorothioates being the most popular of the second generation drugs. These modifications led to the discovery of new modes of action, such as the aptamers of Gilead and ISIS.

As our understanding of biological processes becomes more sophisticated, the demands on chemists increase to produce more radical departures from nature in order to control them. Now we are entering the third generation of drugs. Most of the major players have introduced oligonucleotides with more sophisticated chemistries: Gilead's propynyls, Lynx's phosphoramidates, ISIS's MMIs and MOEs, and Genta's chiral methylphosphonates.

With each new twist, the problems multiply for the synthetic chemists responsible for the preparation of these compounds. Compatibility issues between the new generations of oligonucleotides and the classic methods of synthesis abound. In some cases, such as with peptide nucleic acids, the entire synthesis scheme had to be redeveloped.

A brief review of several case histories demonstrating how to solve some synthesis problems are presented.

Case History 1: Synthesis

Problem

In developing the use of chirally pure methylphosphonate dimer synthons to prepare oligonucleotides, it was found that the phosphoramidite synthons rapidly lost their ability to couple, even while stored dry under anhydrous conditions.

Observations

1. Amidite was pure by ¹H and ³¹P NMR, HPLC, MS.

2. Synthons coupled well immediately after purification (>95%), only to rapidly lose efficiency over the next couple of days, in some cases to 20% or less.
3. Extensive co-evaporations and dry down times were not successful in recovering efficiency.
4. Using analytical techniques described above, no change in synthon was observed, despite loss of coupling efficiencies.

Experiments

1. Repurify on a silica dimer that coupled at 25% efficiency (95% originally).

Purpose: Try to remove undetectable impurity (salts, etc.)

Result: No improvement.

Conclusion: Contaminant co-migrates with product. Not from original synthesis of synthon, or would have been present from beginning.

2. Activate synthon with tetrazole in NMR tube. Compare to DMT-T amidite standard.

Purpose: Determine if synthon converts cleanly to tetrazolide intermediate.

Result: Very little active material observed with dimer synthon (>10%). Most of the amidite converted to hydrolyzed side-product.

Conclusion: It's water.

3. Treat dimer synthon with high quality molecular sieves.

Test by NMR and coupling.

Purpose: Try to remove water, and thereby increase coupling efficiency.

Result: After 2 days over sieves, NMR showed over 50% active reagent. Coupling efficiency >95%.

Conclusion: Molecular sieves are a viable solution.

Follow Up

All the dimer synthons are now treated with 3 Å molecular sieves for two days prior to use. In fact, this is done with any new or modified reagent. It is better to be safe, than to lose the results of many hard weeks in the lab. We now prepare our own molecular sieves.

Case History 2: Deprotection

Problem

2'-O-silyl RNA monomers have become the most popular reagents for the synthesis of RNA, mainly because of the "first in" principle and cost savings. However, there are a number of complaints about biologically inactive products being produced with these synthons. At the time of this work, tetrabutylammonium fluoride (TBAF) was the reagent of choice for removal of the silyl group.

Observations

1. Variable quality of RNA.
2. Quality of pyrimidine rich sequences were not as high as purine rich.
3. Biological quality of longer strands (40mer +) often poor.
4. Oligos that appeared pure on 20 cm gels showed extra banding when run on 40cm gels. (Prof. Paul Miller, Johns Hopkins University, originally made this observation.)

Experiments

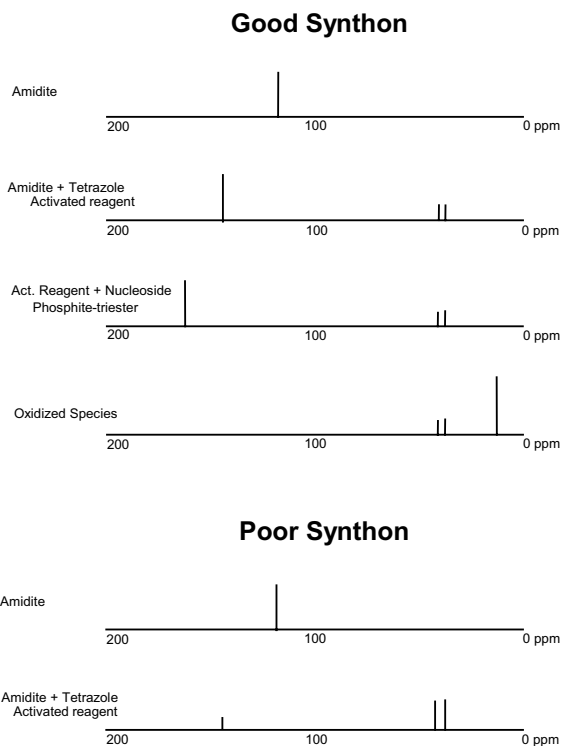
1. Synthesize alternating AG and CU RNA oligos, deprotect as usual, analyze on gel.

Purpose: Repeat Miller's observation, and to begin exploring purine vs. pyrimidine issue.

Result: Significant banding of CU oligo with no major band, AG oligo was a clean single band

Conclusion: Definitely a difference between AG and CU oligos.

³¹P NMR Experiment to Test for Water in Amidite



2. Retreat CU oligo with fresh TBAF.

Purpose: Determine if incomplete deprotection was problem.
Result: Multiple bands collapsed into single band.

Conclusion: Incomplete removal of silyl. Water content of both TBAF reagents were determined by Karl Fisher titration. The first contained over 10% water, the fresh bottle contained ~ 6%.

3. Prepare dimers CT, UT, AT and GT and isolate with silyl groups intact. Treat with TBAF containing variable amounts of water. Follow removal of silyl group by HPLC.

Purpose: Quantify affect of water in TBAF on rate of deprotection of individual bases in a model system in a realistic environment with a neighboring phosphate.

Result: Pyrimidines very sensitive to water content of TBAF, with the rate of desilylation rapidly declining with more than 5% water in the TBAF. Purines appear completely impervious, handling up to 20% water with no observable reduction in rate of desilylation.

Conclusion: It's water again.

4. Treat TBAF with molecular sieves to reduce water. Treat aliquot of reagent containing 20% water with sieves, compare ability to deprotect.

Purpose: Determine if we can not only protect TBAF against water, but actually recover wet reagent. Is water affecting the reagent itself, or the reaction?

Result: The TBAF treated with molecular sieves dried to only 2% water after 3 days. It completely deprotected the CT and UT dimers within 6 hours. The original untreated TBAF with 20% water deprotected about ½ of the dimer by that time.

Conclusion: Molecular sieves come to the rescue again.

Follow Up

We treat the TBAF upon arrival with sieves to ensure proper dryness, and remove the need to test each bottle by Karl Fisher prior to use. (We found that most brand new bottles contain more than 5% water, some contain as much as 8%, which will not deprotect pyrimidines under normal conditions.) We use the small TBAF (5 mL) bottles from Aldrich so that they are not in use for a long period of time. The combination of

fresh reagent well dried with sieves has led to a track record of no failed deprotections over several hundred syntheses.

Needless to say, we now treat every water sensitive reagent with high quality molecular sieves prior to use. This work was published by Hogrefe et al. in the paper: *The Effect of Excess Water on the Rate of Desilylation of Tetrabutylammonium Fluoride*.

Case History 3: Deprotection Problem

The synthesis of methylphosphonate oligonucleotides is no trivial matter. One problem is that the backbone is very sensitive to base and will completely degrade under normal deprotection conditions using ammonium hydroxide. The use of ethylenediamine (EDA) as an alternative was established by Paul Miller. The problem was that EDA transaminated N⁴-benzoyl cytidine, leading to undesired EDA adducts. We could see these compound as later eluting species on gel, but not on RP-HPLC. This problem was exacerbated during scale up. Miller used a primary treatment with hydrazine to remove the benzoyl group prior to treatment with EDA. This method was not amenable to scale up. Use of a brief treatment with ammonium hydroxide was not very effective at scale and led to loss of product due to insolubility of the oligo in aq. ammonium hydroxide. Somehow the NH₄OH permanently bound the oligo to the support. Not only that, but degradation of MP oligos to single nucleosides showed other modifications as well.

We had two problems here. One was to fix a known problem, EDA adduct formation with benzoyl-C, the second was to first determine what caused the unknown modification, then fix it. To top it off, we also had a solubility problem that limited our options.

Observations

1. During scale up transamination of benzoyl-C with EDA was found to occur with a rate of up to 15% per C.
2. Other modifications were > 10%, but still significant.
3. EDA is the best solvent for MP oligos.
4. Other pre-treatments, such as NH₃ saturated isopropanol, etc., for the removal of bz-C prior to EDA failed.

Experiments

1. Synthesize 9mers consisting of dA, dC, dG or T surrounded by T's using C-bz, C-ibu, G-ibu, and A-bz. Deprotect using:
 - a. first hydrazine, then EDA/ethanol (Miller)
 - b. first NH₄OH, then EDA/ethanol (Sarin)
 - c. EDA/ethanol alone

Purpose: Determine extent of problem, compare methods.
Result: dA, dC-ibu and T oligomers deprotect cleanly in all systems. dC-Bz yielded EDA adduct in every case. dG yielded unidentified modification in methods a and c, but not with b. Unfortunately, b yielded ½ the product the other methods did.
Conclusion: The first problem was neatly solved with a simple change, ibu instead of bz protection on dC. However, dG is also modified, something not reported in the literature. We tried O₆ protected dG-ibu (diphenylcarbonyl, DPC), but that exacerbated the problem.

2. Treat dG-ibu and dG-ibu-DPC with methods a, b and c.
Purpose: Look at modification of dG in isolated environment.
Result: No modification.

Conclusion: dG-ibu must be modified during synthesis, making it susceptible to transamination during deprotection. We suspected dimethylamino pyridine (DMAP), which we were required to use because MP oligomers are base labile enough to be cleaved by N-methylimidazole (NMI).

3. Cycle 20 artificial couplings over support bound dG-ibu (the other bases were done as well). By deblocking, then capping the support prior to running coupling cycles, the monomer is not elongated into an oligo. Each of the reagents, dichloroacetic acid, oxidizer, caps, amidites, etc., were replaced with acetonitrile (ACN) one at a time and the pro-

cess repeated. The process was then repeated with the variation of replacing all but one of the reagents with ACN. The supports were deprotected by methods a, b, and c above.

Purpose: To study the effect of each reagent on ibu-dG under simulated oligo synthesis conditions, both independently and in conjunction with other reagents.

Result: DMAP formed adducts with ibu-dG and DPC-ibu-dG in methods a and c, but not in b.

Conclusion: It is not surprising that ibu-dG formed EDA adducts. This was reported by Eadie. What was surprising was that the putative solution, O₆ DPC protected dG, was significantly more modified. The NH₄OH pre-treatment served to revert the modification back to desired product.

Follow Up

Now that we knew the culprit, and how to fix it, we merely had to apply our knowledge. We couldn't use method b, which called for a pre-treatment of NH₄OH before EDA, because it yielded very low yields on scale-up. EDA is the best solvent for that step, but to use it as the principle deprotecting reagent a new capping routine would have to be discovered to avoid NMI or DMAP. Fortunately, one last experiment gave us the needed clue. It was found that a very short treatment of 10 min with 30% NH₄OH, or a 30 min treatment with 2% NH₄OH in ACN/EtOH was sufficient to revert the DMAP-dG adduct. Furthermore, it was found that the 2% NH₄OH solution degraded the MP backbone at a very acceptable low rate of 1%/hour.

We solved the problem by first treating the support bound oligo with the mild NH₄OH solution to revert dG adducts, followed with one volume of EDA after 30 min to complete the deprotection. Our deprotections went cleanly and our yields were acceptable. And we had a one pot method that was faster, cleaner and more simple than before. Also, the use of dC^{bu} instead of dC^{oz} and this deprotection method eliminated the transamination side product. This work was also published by Hogrefe et al. in the paper: *Deprotection of Methylphosphonate Oligonucleotides Using a Novel One-Pot Procedure*.

Case History 4: Purification

Problem

In the search for an uptake enhancer, a certain peptide with biological activity of its own was deemed a viable candidate for conjugation to the terminus of an oligo. It was synthesized and purified using RP-HPLC with 0.1% Trifluoro acetic acid (TFA)/ACN as the eluent. The oligo was found to be too active. It had the same activity profile as the free peptide.

Observations

1. Conjugated oligo was clean on HPLC. Free peptide and oligonucleotide separated very well independently.
2. Conjugated oligo clean on mass spec, until we requested closer examination of lower mass region. Sure enough, there was the peptide. To quantitate the peptide, two different solvents had to be used, one to determine the oligo, the other to determine the peptide. The intensity of the peptide peak was compared to an internal standard, gramicidin. This took several months to complete.

Experiments

1. Mix peptide and oligo together (10/1 peptide/oligo). Analyze by Reverse Phase-HPLC and quantitate area of peptide and oligo.

Purpose: Try to determine if peptide sticks to oligo, and in what ratio.

Result: 3 molecules of peptide bound to every oligo.

Conclusion: The peptide sticks to the oligo.

2. Attempt same experiment with normal phase and AX columns.

Purpose: Try other HPLC approaches.

Result: Same results as with RP.

Conclusion: This complex is really tight!

3. Try every molecular weight cut off filter, solid phase extraction, and separation tool known to man, short of a Waring blender.

Purpose: Try to rip the complex apart.

Result: Nothing worked.

Conclusion: This complex is really, really tight!

4. Try electrophoresis.

Purpose: Try to rip the complex apart using fact that peptide is positively charged.

Result: Removed peptide.

Conclusion: Electrophoresis is the route of choice.

Follow Up

After successfully removing the free peptide, the compound was found to be completely inactive. Although not happy about the result, we were not entirely displeased. Have you ever contemplated pilot plant scale electrophoresis gels? Not a thought for the timid.

Case History 5: Purification

Problem

The lesson from the last case was that analytical methods are needed along with purification methods. The lesson here is to believe what you see. Prior to the development of mass spectroscopy (MS) for the analysis of Methylphosphonate (MP) oligonucleotides, you based purity on the integrated area of the putative product peak as determined by HPLC (MP oligos, having no charge, do not run on gel or CE well). What more can you do? First off, you keep looking for something new, and secondly, you believe what you see.

After much trial and tribulation, Genta developed a new purification scheme using normal phase chromatography. It worked very, very well for a number of compounds early on and was used exclusive of other techniques. However, one particular compound was going to be conjugated and therefore subjected to RP-HPLC analysis.

Observations

1. A new peak corresponding to 30% of the total area was seen in front of the main product peak. This information was passed on, but discounted as an artifact of the system, even though it repeated.
2. At this time, ESI-MS of MP oligos became available to us. Low and behold, the compound contained 30% n-1 product.

Experiments

1. Full length oligo and n-1, n-2, n-3 oligos were prepared and subjected to analysis.

Purpose: Determine if failure products resolve from product.

Result: n-1 did not resolve. n-2, etc. did resolve.

Conclusion: There was no separation of n and n-1.

2. Prepare a number of compounds containing different deletions.

Purpose: Explore dependence of chromatographic properties on sequence.

Result: T's do not effect retention time of oligos using normal phase HPLC.

Conclusion: AMP oligo containing a 5' terminal T will not resolve from an n-1 product, if it terminates in A, C, or G; n-2 if the n-1 terminates in T, etc.

Follow Up

The chromatographic properties of MP oligos on normal phase HPLC was so well defined by base composition that our consultant, LCResources, was able to develop a formula predicting the retention times of various oligomers. However, that did not solve our problem. The simple solution to this problem was to design our oligomers such that they don't end with a T.