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SULFURIZING REAGENT II - STABLE IN SOLUTION AND OPTIMIZED FOR RNA SULFURIZATION

Sulfurizing Reagent II

3H-1,2-benzodithiol-3-one 1,1-dioxide, popularly known as Beaucage Reagent (1),¹ has found common use for the sulfurization of oligonucleotides because of its high efficiency, fast reaction time, and widespread availability. The one mild flaw in Beaucage Reagent is that, although it is quite stable in acetonitrile solution in a silanized amber bottle, it has relatively poor stability in solution once installed on the DNA synthesizer. Consequently, we have not been able to supply a sulfurizing solution, preferring to supply the powdered reagent along with an appropriate silanized bottle. The customer then weighs an appropriate amount of reagent into the silanized bottle and adds acetonitrile at a concentration of 1g/100mL.

Sulfurizing Reagent II

A new sulfurizing reagent must exhibit all the good properties of Beaucage Reagent while adding good stability in solution on the synthesizer AND offering strong ability to sulfurize RNA linkages. We are happy to offer Sulfurizing Reagent II, 3-((Dimethylamino-methylidene)amino)-3H-1,2,4-dithiazole-3-thione, DDTT (2).

Use of Sulfurizing Reagent II in DNA Synthesis

Sulfurizing Reagent II in a 0.05M solution was compared to Beaucage Reagent in the synthesis of DNA phosphorothioates. The quality of the products was identical with both reagents. In DNA synthesis, the cycle efficiency seemed to be optimal for Sulfurizing Reagent II when using a 60 second sulfurizing time.

Use of Sulfurizing Reagent II in RNA Synthesis

Our experiments demonstrate that a 0.05 M solution of Sulfurizing Reagent II is recommended for the synthesis of RNA phosphorothioates. A sulfurizing time of 6 minutes generated oligophosphorothioates of high quality. This was true for both TOM-RNA and TBDMS-RNA monomers. As shown in Figure 2 using a model sequence, 5'-UUUUUUUUTT-3', Beaucage Reagent was significantly more sluggish than Sulfurizing Reagent II. Individual RNA sequences, especially those containing stretches of purine nucleoside residues are more difficult to sulfurize irrespective of the reagent used. To obtain a high degree of sulfurization with those oligonucleotides,

FIGURE 1: STRUCTURES OF SULFURIZING REAGENTS



Sulfurizing Reagent (Beaucage) (1) Sulfurizing Reagent II (DDTT) (2)

RNA SULFURIZATION

The most common usage for oligonucleotide phosphorothioates has been in the production of antisense oligodeoxynucleotides destined for use in identifying or modifying gene expression. Now, phosphorothioate linkages are popping up in the RNA world and sulfurizing RNA linkages with reagents like Beaucage Reagent has proved to be much more difficult than DNA linkages.

The phosphorothioate (PS) linkage is a not-so-expensive way of increasing the stability of nucleic acids and increasing nuclease resistance of RNA. Now, it has been shown² that fully PS oligos can promote the delivery of siRNA in cell culture. This siRNA uptake is sequence-independent and the length seems to vary between 30 and 70 nucleotides depending on the cell line. Even though this method is not yet as efficient as the cationic lipids, it opens the way to possible new methods. Reasons that may explain this are not understood at this time.

Another paper³ describes a method for the inactivation of micro RNA (miRNA) that may help to elucidate their functions. It uses 2'-OMe-RNA oligonucleotides (23-mers, complementary to a target miRNA) with a cholesteryl group at the 3' terminus and phosphorothioates at positions 1 and 2 at the 5' end and at the last four positions at the 3' end.

These oligos are called antagomirs. These molecules promote the cleavage of complementary miRNAs and thus should allow analysis of their function. The role of the PS linkages presumably is the stabilization against degradation in the mouse experiments as it is standard in the antisense field in such in vivo situations.

And finally, a recent paper⁴ shows that PS does not systematically abolish siRNA activity, opening the way for some potentially less expensive stabilization of such molecules. Incorporation of 2'-OMe (in the sense strand) in combination with PS linkages should confer to siRNA increased resistance to degradation by nucleases, as well as prolonged serum retention. And it is also possible that such easy modification of siRNA may increase the specificity by eliminating sense strand recruitment in the RISC complex and thus reducing a source of off-target effect.

FIGURE 2: CYCLE EFFICIENCY USING SULFURIZING REAGENTS AND U-TOM-RNA MONOMER

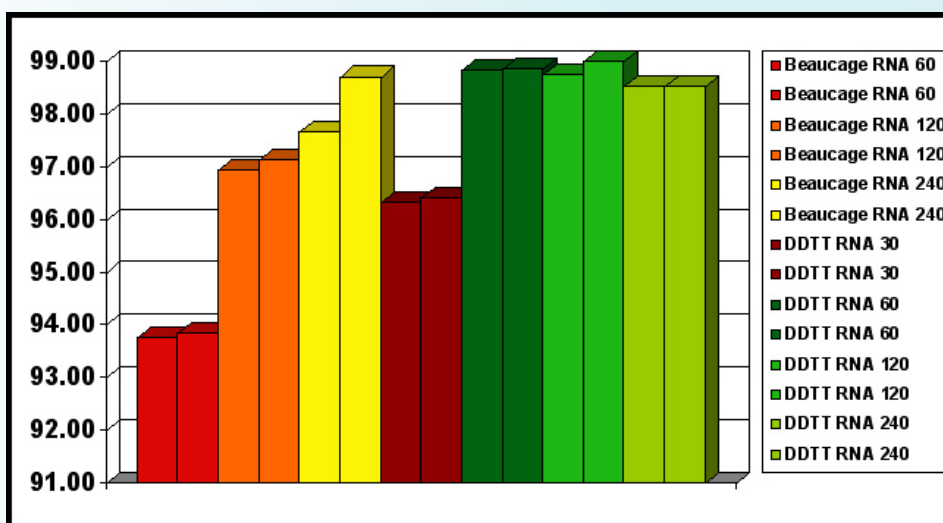
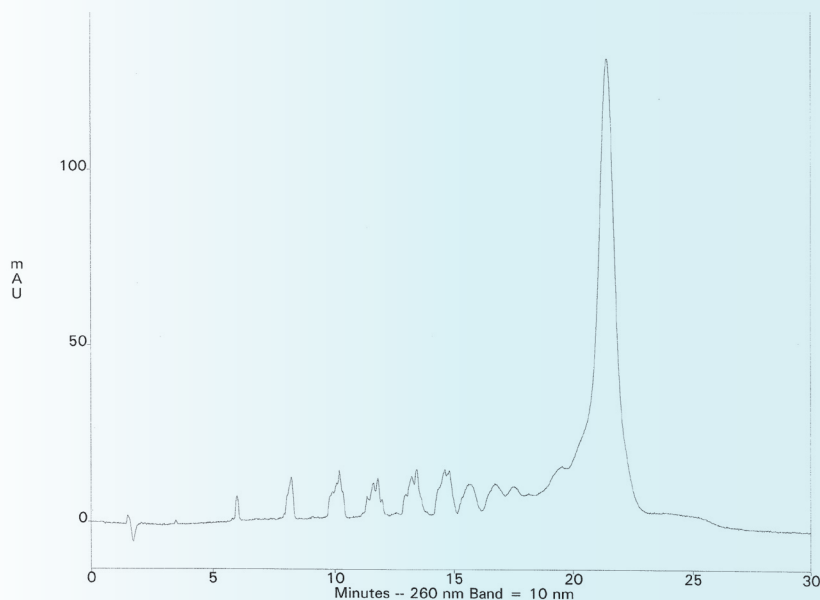


FIGURE 3: IEX HPLC OF RNA OLIGO SULFURIZED WITH SULFURIZING REAGENT II



Oligonucleotide: CUG GAC UUC CAG AAG AAC ATT

Column: Dionex B100, 4.6mm x 250mm at 60 °C

Buffers: A- 10mM NaClO₄, 25mM TRIS-HCl, 20% Acetonitrile, pH 7.4 ; B- 600mM NaClO₄, 25mM TRIS-HCl, 20% Acetonitrile, pH 7.4

Flow rate: 1mL/Min

TABLE 1: SULFURIZATION TIMES

Target Oligonucleotide	Time (seconds)
DNA	60
RNA	360

Note: Oligos containing many G residues may require more aggressive sulfurizing conditions. A 0.1M solution of Sulfurizing Reagent II in pyridine with a sulfurizing time of 600 seconds may be required.

TABLE 2: SOLUBILITY OF SULFURIZING REAGENT II

Concentration of Sulfurizing Reagent II	Solvents and their ratio (v/v)	
	Pyridine:ACN	Pyridine:THF
0.1M	100:0	40:60
0.06M	50:50	-
0.05M	40:60	20:80
0.03M	30:70	-
0.02M	20:80	0:100

Notes: Dissolve Sulfurizing Reagent II in pyridine first and then add acetonitrile (ACN) or tetrahydrofuran (THF). Molecular Weight is 205.31

a 0.1 M solution of Sulfurizing Reagent II and/or extended contact time may be required (Table 1). A representative HPLC analysis⁵ of an siRNA 20 mer oligo is shown in Figure 3.

Solubility and Stability of DDTT

The solubility of Sulfurizing Reagent II in mixtures of anhydrous pyridine and acetonitrile or anhydrous pyridine and THF is relatively limited and increases with the increasing concentration of pyridine. Some useful compositions are listed in Table 2. Under these conditions, Sulfurizing Reagent II forms stable solutions that do not display any loss of functional activity or precipitation of the reagent for a period of over 6 months. To prepare solutions of the desired concentration, we recommend first dissolving Sulfurizing Reagent II

in the calculated amount of pyridine, which may require mild heat, followed by diluting the obtained solution with the required volume of acetonitrile or THF.

We are happy to offer Sulfurizing Reagent II as a powder and as a 0.05M solution in pyridine/acetonitrile.

References:

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5. B.J. Bergot and W. Egan, *Journal of Chromatography*, 1992, **599**, 35-42.