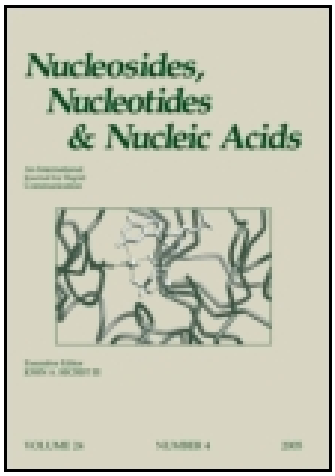



Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lncn20

# EFFICIENT ASSESSMENT OF MODIFIED NUCLEOSIDE STABILITY UNDER CONDITIONS OF AUTOMATED OLIGONUCLEOTIDE SYNTHESIS: CHARACTERIZATION OF THE OXIDATION AND OXIDATIVE DESULFURIZATION OF 2-THIOURIDINE

E. Sochacka [a]
[a] Institute of Organic Chemistry, Technical University of Łódź , Żeromskiego 116, Łódź, 90-924, Poland
Published online: 19 Aug 2006.

To cite this article: E. Sochacka (2001) EFFICIENT ASSESSMENT OF MODIFIED NUCLEOSIDE STABILITY UNDER CONDITIONS OF AUTOMATED OLIGONUCLEOTIDE SYNTHESIS: CHARACTERIZATION OF THE OXIDATION AND OXIDATIVE DESULFURIZATION OF 2-THIOURIDINE, Nucleosides, Nucleotides and Nucleic Acids, 20:10-11, 1871-1879, DOI: 10.1081/NCN-100107198

To link to this article: http://dx.doi.org/10.1081/NCN-100107198

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(10&11), 1871–1879 (2001)

# EFFICIENT ASSESSMENT OF MODIFIED NUCLEOSIDE STABILITY UNDER CONDITIONS OF AUTOMATED OLIGONUCLEOTIDE SYNTHESIS: CHARACTERIZATION OF THE OXIDATION AND OXIDATIVE DESULFURIZATION OF 2-THIOURIDINE

**E. Sochacka**

Institute of Organic Chemistry, Technical University of Łódź,
Żeromskiego 116, 90-924 Łódź, Poland
Department of Biochemistry, NCSU, Raleigh,
NC 27695-7622, USA



## ABSTRACT

In order to efficiently assess the chemical stability of modified nucleosides to the reagents and conditions of automated oligonucleotide synthesis, we designed, developed and tested a scheme in which the modified nucleoside, directly attached to a solid support, is exposed to the cyclic chemistry of the instrument. Stability of 2-thiouridine against different oxidizers was investigated. *Tert*butyl hydroperoxide (1 M) in anhydrous acetonitrile was a more effective oxidizer for the incorporation of 2-thiouridine into oligonucleotide chains than the same oxidizer in methylene chloride. Carbon tetrachloride/water in the presence of a basic catalyst was superior in maintaining the thiocarbonyl function, but its utility for RNA synthesis has yet to be fully tested, whereas 2-phenylsulfonyloxaziridine was a very efficient reagent for oxidative desulfurization of 2-thiouridine.

## INTRODUCTION

Recently an increasing interest has been generated for the chemical synthesis of oligonucleotides containing 2-thiouridine and its 5-substituted derivatives as model compounds for structure and function studies (antisense strategy, ribozyme activity, priming HIV-1 reverse transcription, ribosome binding)[1–5]. The most effective methods of oligonucleotide synthesis are phosphoramidite and H-phosphonate approaches. Both of these synthesis methods require a P (III) to P (V) oxidation step[6], which can result in the undesired loss of the 2-thio function.

It is important to consider the thioketo group at the 2 position of the pyrimidine ring not only as a domain of simple oxidation resulting in the corresponding uridine, but also as a site for an oxidative desulfurization leading to the 4-pyrimidinone nucleoside[7–11] (Fig. 1). More detailed studies showed, that the ratio of products **2** to **3** and the amount of unreacted 2-thiouridine strongly depend on the conditions used for oxidation (oxidizing agent, the presence of water, reaction solvent, pH, molar excess of the reagents, substituent at the 5-position of 2-thiouracil)[10,11].

The chemical reactivity of 2-thiouridines under oxidative conditions is incompatible with the standard protocols used for the automated syntheses of oligonucleotide chains[6,9]. Oxidative desulfurization of $s^2U$ leading to the 4-pyrimidinone nucleoside ($H^2U$) is a major reaction when aqueous iodine, the standard oxidizing reagent, is used in the repeated oxidation steps required for the phosphoramidite approach to RNA synthesis[9]. However, no problems were reported with the incorporation of 2-thiothymidine by using iodine oxidation with H-phosphonate chemistry[12]. The difficulty of introducing 2-thiopyrimidine nucleosides into DNA with the standard iodine oxidation protocol for phosphoramidite synthesis was overcome by using toluoyl as a protecting group for the base moiety[13]. In RNA, Kumar and Davis have reported that 2-thiouridine could be incorporated successfully

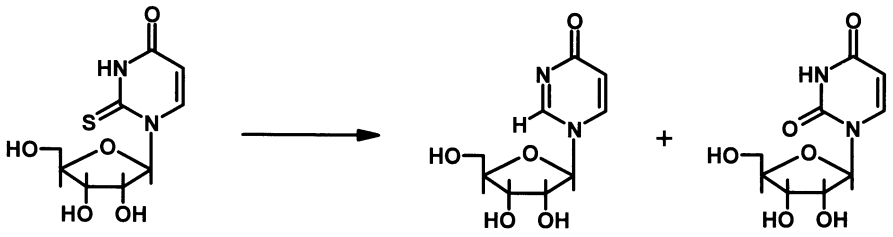
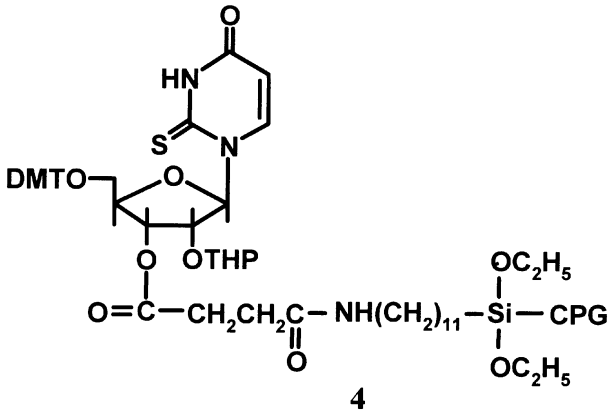



*Figure 1.* Products of 2-thiouridine oxidative desulfurization and oxidation.




4

*Figure 2.* 2-Thiouridine derivative attached to lcaa CPG.

into oligonucleotides without base protection by replacing iodine with 10% *tert*butyl hydroperoxide in acetonitrile as the oxidizing agent[14].

Thiolation of the C-2 atom of uridine is only one example of the many modifications that are chemically and structurally interesting, biologically important, and also susceptible to alteration under the commonly used conditions of synthesizer protocols. Therefore, it is important to thoroughly investigate the conditions of automated chemical synthesis that could irreversibly effect modified nucleoside chemistry in oligonucleotides and the yields of the correct oligomer. Although reactivity of protected and unprotected modified nucleosides can be assessed by treatment with reagents used on the synthesizer, conditions identical to those on the instrument are difficult to duplicate during solution synthesis in the test tube.

Here, we present an efficient and effective method of using the instrument to analyze the effects of synthesizer protocols on modified nucleoside stability. In this method, the modified nucleoside is attached to a solid support. We have compared the stability of 2-thiouridine derivative **4** (Fig. 2) against different oxidizers commonly used in phosphoramidite and H-phosphonate oligonucleotide solid phase synthesis.

## RESULTS AND DISCUSSION

Oligomers with the sequences corresponding to tRNA's anticodon stem and loop domain with a 2-thiolated nucleosides were chemically synthesized in our studies on the structure-function relation of wobble position 2-thiouridines[4]. Using automated solid phase synthesis, irreproducible yields of 2-thiouridine-containing oligoribonucleotides were observed when utilizing 1 M *tert*butyl hydroperoxide in methylene chloride as the oxidizer. To improve the yield of the desired 2-thiouridine-containing oligonucleotide, we

undertook a more detailed and systematic study of 2-thiouridine stability under treatment with the various oxidizers used in solid phase synthesis. The specificity of the automated solid phase oligonucleotide synthesis (a very small scale, a large molar excess of oxidizing agent) produces results on the instrument that often differ from those observed in solution when testing of new oxidizers. Our model compound 5′-*O*-dimethoxytrityl-2′-*O*-tetrahydropyranyl-2-thiouridine derivative **4** attached by succinyl linker to a polymer support (Fig. 2) provided a means to overcome this problem and compare the performance of the different oxidizers under the conditions of solid phase synthesis methodology.

Synthesis of the 2-thiouridine derivative **4** was achieved from 2-thiouridine by standard protection of 2′ and 5′ hydroxyl functions with tetrahydropyranyl (THP) and dimethoxytrityl (DMT) groups, respectively[15] and then by the introduction of 3′-*O*-succinate[16]. The 3′-*O*-succinate was converted to its 4-nitrophenyl ester, which was reacted with long chain alkylamine controlled pore glass (lcaa-CPG)[16] to give a support with a loading of 54 μmol of 2-thiouridine per gram. A 2-thiouridine functionalized support was used for the preparation of several 1 μmol columns for an Applied Biosystems 394 oligonucleotide synthesizer.

In our studies we tested the following oxidizing agents used for phosphoramidite synthesis: 1 M solution of *tert*butyl hydroperoxide (*t*BuOOH) in acetonitrile[14] or methylene chloride[17] and carbon tetrachloride/N-methylmorpholine (NMM)/pyridine/water/acetonitrile 2.5/1.0/6.0/0.2/9.3 (v/v)[18]. We also examined oxidizers used in the H-phosphonate approach: 2% iodine in pyridine/water 98/2[19], 10% water in carbon tetrachloride/triethylamine/N-methylimidazole (NMI) (90/0.5/0.5)[20] and 0.2 M 2-phenylsulfonyloxaziridine (PSO) in methylene chloride[21].

The experiments were conducted following a modified ABI protocol of phosphoramidite synthesis in which coupling and detritylation steps were omitted. The time of oxidation was varied for each oxidizer as listed in Table 1 (entry 2, 3, 4). The protocol corresponds to 10 coupling cycles and therefore the model compound was exposed to 10 oxidation steps. To mimic H-phosphonate chemistry only one oxidation step was performed (entry 5, 6, 7). As a control, the recovery of 2-thiouridine from CPG support in the absence of any oxidizer was determined (entry 1).

The acid labile THP protecting group was used for the 2′OH function of **4**. This allowed the removal of THP and DMT groups simultaneously under the prolonged treatment (30 min) with 3% trichloroacetic acid as the last step of machine aided experiments. To check that the same amount of ribose residue was attached to CPG for each oxidant tested, DMT cation release was measured with almost identical readings obtained for each trial. Finally the beads were incubated overnight with anhydrous ethanolic ammonia to release the nucleoside material. After filtration of the polymer support and evaporation of the solvent, the residue was dissolved in water.

***Table 1.*** Summarized Results of 2-Thiouridine Transformation to 4-Pyrimidinone Nucleoside and Uridine Under Various Oxidizing Conditions Used in Oligonucleotide Solid Phase Synthesis.

| No. | Oxidizer | Time for Oxidation | $H^2U$ (%) | U (%) | $s^2U$ (%) |
|---|---|---|---|---|---|
| 1 | None | | | | 100 |
| 2 | 1 M *t*BuOOH in acetonitrile | 12 min* | 2 | 8 | 66 |
| 3 | 1 M *t*BuOOH in methylene chloride | 4 min* | 6 | 13 | 54 |
| 4 | $CCl_4$/NMM/pyridine/$H_2O$/acetonitrile 2.5/1.0/6.0/0.2/9.3 (v/v) | 5 min* | | | 92 |
| 5 | 2% $I_2$ in pyridine/$H_2O$ 98/2 (v/v) | 10 min** | 27 | 15 | 13 |
| 6 | 10% $H_2O$ in $CCl_4$/NMI/$Et_3N$ 90/0.5/0.5 (v/v) | 10 min** | | | 84 |
| 7 | 0.2 M PSO in methylene chloride | 5 min** | 96 | | |

*time for each of ten oxidation steps; **time for the total oxidation process.

Small aliquots were analyzed by reversed-phase HPLC using isocratic elution with 0.01 M phosphate buffer/methanol (97.5/2.5 v/v, pH 5.3) (Fig. 3). A solution of equimolar concentration of 2-thiouridine ($s^2U$), uridine and the 4-pyrimidinone ($H^2U$) nucleoside was used as the standard for the determination of the composition of the reaction mixture for each oxidation experiment.

1 M *t*BuOOH in acetonitrile or methylene chloride resulted in 2-thiouridine recovery in 66% and 54% yield, respectively. Although the oxidation time was three times longer with *t*BuOOH in acetonitrile (Table 1, entry 2) than in methylene chloride (entry 3), the first condition was found to retain more 2-thiopyrimidine nucleoside under synthesis conditions. Treatment with carbon tetrachloride/water mixture in the presence of basic catalysts had little effect upon 2-thiouridine (entry 4 and 6). The best recovery of 2-thiouridine (92%) was obtained with carbon tetrachloride/N-methylmorpholine/pyridine/water/acetonitrile (2.5/1.0/6.0/0.2/9.3) (entry 4).

Aqueous iodine oxidation (entry 5) with the time suitable for an H-phosphonate approach produced mainly the product of oxidative desulfurization ($H^2U$) with 2-thiouridine recovered in only 13% yield. Testing of 2-phenylsulfonyloxaziridine resulted in complete loss of sulfur in 2-thiouridine. In our experiment this reagent appeared as a very efficient desulfurization agent, thus only the 4-pyrimidinone nucleoside was detected in the reaction mixture at the condition for entry 7 (Table 1). A large scale experiment with 2′,3′,5′-tri-*O*-acetyl-2-thiouridine confirmed that the 2-phenylsulfonyloxaziridine treatment could be used as a convenient and very efficient desulfurization method for 2-thionucleoside transformation to 4-pyrimidinone, both at the nucleoside level in solution and for post-synthetic modification at the level of oligomer.

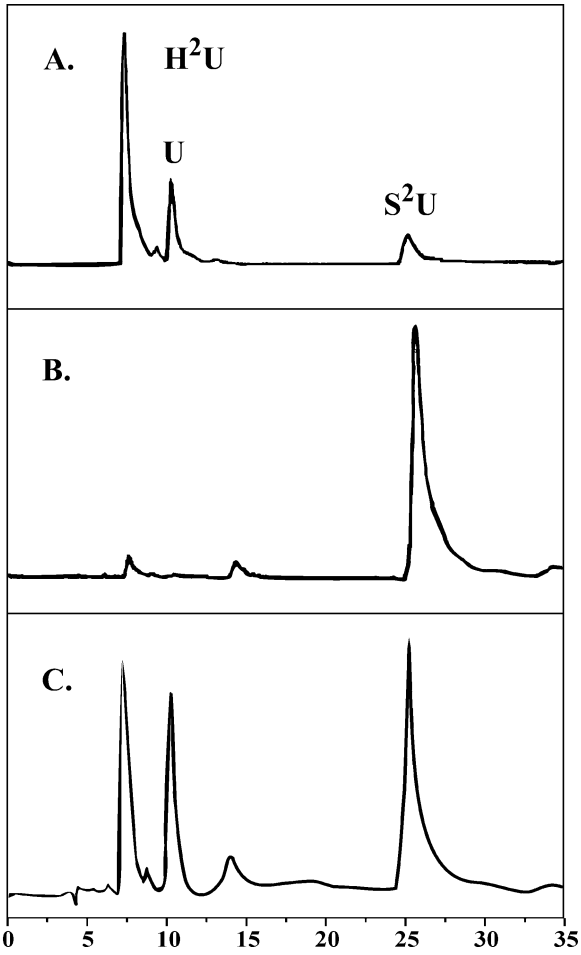



***Figure 3.*** HPLC traces of: (A) equimolar mixture of $H^2U$, U and $s^2U$; (B) the elution profile for the test that utilized $CCl_4$/NMM oxidizer (entry 4, Table 1); (C) the elution profile for the test that utilized 1 M *t*BuOOH in methylene chloride as the oxidizer.

## CONCLUSIONS

With 2-thiouridine conjugated to a solid support, we analyzed its stability under various oxidation conditions of the automated oligonucleotide synthesizer. *t*BuOOH (1 M) under anhydrous conditions is presently the preferred oxidizer for the chemical introduction of 2-thiouridine by the phosphoramidite approach in automated oligonucleotide synthesis. Anhydrous acetonitrile is a better solvent for *t*BuOOH oxidation than methylene chloride. The best yield of unchanged 2-thiouridine was obtained when a carbon tetrachloride/water mixture in the presence of basic catalyst was used as the oxidizer in both phosphoramidite and H-phosphonate methods. It is

necessary to underline that the efficiency of these conditions for P (III) moiety oxidation in RNA synthesis need to be thoroughly checked. Aqueous iodine oxidation cannot be used for the introduction of 2-thiouridine by phosphoramidite or H-phosphonate chemistries. The 2-phenylsulfonyloxaziridine was found as a very efficient desulfurization agent for 2-thiouridine at the nucleoside level as well as for the post-synthetic 2-thiouridine modification in an oligomer. We conclude that analysis of modified nucleoside stability, or that of novel protecting groups, to the conditions of automated oligonucleotide synthesizers is efficiently accomplished on the instrument with the nucleoside in question coupled directly to a solid support.



## EXPERIMENTAL

### *Lcaa-CPG Derivatization with 2-Thiouridine*

5′-*O*-DMT-2′-*O*-THP-3′-*O*-succinate p-nitrophenyl ester of 2-thiouridine was obtained according to published procedures[15,16]. The structure of this compound was confirmed by FAB MS and $^1$H NMR.

$^1$H NMR ($CDCl_3$, δ ppm): 1.5–2.0 (m, 6H), 2.6–2.9 (m, 4H), 3.4–3.6 (m, 4H), 3.8 (s, 6H), 4.3–4.4 (m, 1H), 4.6–4.8 (m, 2H), 5.3–5.6 (m, 2H), 7.1 (s, 1H), 6.8–7.3 (m, 17H), 7.9 (d, 1H) FAB MS: $m/z$ 745 $(M\text{-}H)^-$ for 5′-*O*-DMT-2′-*O*-THP-3′-*O*-succinate.

Lcaa-CPG derivatization was performed according to a standard protocol[16] and the support with a loading of 54 μmol of 2-thiouridine per gram was obtained. Using this support, several 1 μM columns were prepared.

### *Machine Aided 2-Thiouridine Oxidation Experiments*

The experiments mimicking solid phase synthesis with $s^2U$ attached to lcaa-CPG were performed using a Perkin-Elmer Applied Biosystems Model 394 synthesizer. The standard 1 μM RNA synthesis protocol was changed as follow: i) for experiments mimicking phosphoramidite synthesis, the detritylation and coupling steps were omitted; ten oxidation steps were performed and the oxidation times were set individually for each oxidizer as listed in Table 1, entry 2, 3, 4; ii) for H-phosphonate approach one oxidation step was mimicked (entry 5, 6, 7, Table 1).

The final 3% trichloroacetic acid treatment was prolonged to 30 min to remove simultaneously DMT and THP protecting groups. DMT readings obtained from each column (entry 1 to 7) were in the same range. Nucleoside material was cleaved from the beads by incubating the sample for 18 hours at room temperature in ethanolic ammonia. Then the samples were dried using a Savant Speed Vac and finally each sample was dissolved in 5 mL of water.

*HPLC Analysis of Nucleoside Composition*

100 μL aliquot of each sample was analyzed by reversed phase HPLC using a Nucleosil C-18 column (3.2 mm i.d. × 250 mm, Supelco) and isocratic elution with 0.01 M potassium dihydrogen phosphate/methanol 97.5/2.5, pH 5.3 at a flow 0.75 mL/min. Detection was accomplished with a Waters 990 photo-diode array detector monitoring at 260 nm.

Equimolar mixture of 2-thiouridine, uridine and 4-pyrimidinone nucleoside was used as a standard for the composition analysis of samples. The retention time for 4-pyrimidinone nucleoside (**2**), uridine (**3**) and 2-thiouridine (**1**) were 7.7 min, 10.7 min, and 26.2 min, respectively. To calculate the yield of 4-pyrimidinone nucleoside, uridine and 2-thiouridine obtained after each oxidation experiment the following correction factors were applied to the integrated areas of nucleoside peaks at 260 nm: $H^2U = 3.2$; $U = 1.8$; $s^2U = 1.0$.

## ACKNOWLEDGMENTS

I thank Paul F. Agris and Richard Guenther (NCSU) and Andrzej Małkiewicz (Technical University of Łódź) for helpful discussions and comments on manuscript.

I also thank Winnell Newman of the NCSU Nucleic Acids Facility for the help in the experiments on oligonucleotide synthesizer.

This work was supported by the Polish Commitee for Scientific Research, Grant no. 0877/T09/96/11, and a National Science Foundation Grant, MCB9986011.

## REFERENCES

1. Murlidharan Nair, T.; Myszka, D.G.; Davis, D.R. Nucleic Acids Res. **2000**, *28*, 1935–1940.
2. Testa, S.M.; Disney, M.D.; Turner, D.H.; Kierzek, R. Biochemistry **1999**, *38*, 16655–16662.
3. Komatsu, Y.; Kumagai, I.; Ohtsuka, E. Nucleic Acids Res. **1999**, *27*, 4314–4323.
4. Ashraf, S.S.; Sochacka, E.; Cain, R.; Guenther, R.; Malkiewicz, A.; Agris, P.F. RNA **1999**, *5*, 188–194.
5. Sundaram, M.; Durant, P.C.; Davis, D.R. Biochemistry **2000**, *41*, 12575–12584.
6. *Protocols for Oligonucleotides Synthesis and Analogs*, ed. Agrawal, S. *Methods Mol. Biol.* **1993**, *20*.
7. Watanabe, K. Biochemistry **1980**, *19*, 5542–5549.
8. Ogihara, T.; Mitsunobu, O. Chem. Lett. **1982**, 1621–1624.
9. Kuimelis, R.G.; Nambiar, K.P. Tetrahedron Lett. **1993**, *34*, 3813–3817.
10. Crestini, C.; Mincione, E.; Saladino, R.; Nicoletti, R. Tetrahedron **1994**, *50*, 3259–3272.

11. Saladino, R.; Mincione, E.; Crestini, C.; Mezzetti, M. Tetrahedron **1996**, *52*, 6759–6780.
12. Ishikawa, T.; Yoneda, F.; Tanaka, K.; Fuji, K. Bioorg. Med. Chem. Lett. **1991**, *1*, 523–526.
13. Kuimelis, R.G.; Nambiar, K.P. Nucleic Acids Res. **1994**, *22*, 1429–1436.
14. Kumar, R.K.; Davis, D.R. J. Org. Chem. **1995**, *60*, 7726–7727.
15. Sochacka, E. Nucleosides Nucleotides **1998**, *17*, 327–338.
16. Sproat, B.S.; Lamond, A.I. In *Oligonucleotides and Analogues; A Practical Approach*; Ekstein, F., Ed.; Oxford University Press: NY, 1991; 71–72.
17. Sproat, B.S.; Colonna, F.; Mullah, B.; Tsou, D.; Andrus, A.; Hampel, A.; Vinyak, R. Nucleosides Nucleotides **1995**, *14*, 255–273.
18. Padiya, K.J.; Salunkhe, M.M. J. Chem. Res. (S) **1998**, 804–805.
19. Stawinski, J. In *Handbook of Organophosphorus Chemistry*; Engel, R., Ed.; Marcel Dekker, Inc: NY, 1992; 377–435.
20. Sinha, N.D.; Striepeke, S. In *Oligonucleotides and Analogues; A Practical Approach*; Ekstein, F., Ed.; Oxford University Press: NY, **1991**; 185–210.
21. Wada, T.; Sato, Y.; Honda; F.; Kawahara, S.; Sekine, M. J. Am. Chem. Soc. **1997**, *119*, 12710–12721.

Received February 7, 2001
Accepted July 2, 2001