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2-*C*-Methyluridine modified hammerhead ribozyme against the estrogen receptor

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ABSTRACT

A new synthesis of 2′-*C*-methyluridine phosphoramidite is presented. Special emphasis is dedicated to the improvement of the protection of the tertiary 2′-hydroxyl group. Comparison to previous protecting strategies and analysis of stability under 5′-DMTr removing conditions are discussed. The synthetic incorporation of this modified nucleoside into the catalytic core of a hammerhead ribozyme against the estrogen receptor α protein (ER-α), and transfection experiments in MCF-7 cell line are also presented.

The development of synthetic oligoribonucleotides with non conventional activities, like catalytic ribozymes,[1] aptamers[2] and more recently siRNA,[3] found applications in different fields such as therapeutics, biosensors and molecular biology. The susceptibility of RNA to the attack of nucleases limited its utilization, especially in medicine. A way to overcome this restriction is the use of chemical modifications, which must fulfill certain requisites.[4] Another important feature is related to RNA structure, in this sense sugar puckering of modified nucleosides can play an important role, like in the case of locked nucleic acids (LNA).[5]

(2′*R*)-2′-*C*-methylribonucleosides are attractive candidates to be used in modified RNA structures, because they showed increased nuclease resistance, when incorporated to RNA sequences;[6] and can adopt C-3′*endo* (RNA-like) conformation with *anti* orientation of the base.[7] An important attribute of 2′-*C*-methylribonucleosides is that they posses the 2′-hydroxyl. This functional group could be essential to mimic RNA polar interactions and hydrogen bonding of complex RNA tertiary structures. 2′-*C*-methyl nucleosides have been synthesized using two main strategies: (i) construction of the modified sugar moiety and posterior glycosidation reaction, and (ii) modification of the ribose portion of a natural ribonucleoside. Related to the first approach, glucose,[8] fructose[9] or ribose[10] have been employed as starting materials making use of regioselective deprotection at position 2[11] or of alkaline sugar degradation.[12]

The direct modification of the ribose moiety of a natural ribonucleoside can be also accomplished by two different routes, both involving as a common synthon, the 2′-ketonucleoside. In one case, the 2′-keto group is attacked by a methyl organometallic, yielding mixtures of the ribo and arabino epimeres. The second option consists in a Wittig reaction of the keto derivative followed by a stereoselective dihydroxylation.[7]

In order to introduce 2-*C*-methyl nucleosides in an oligonucleotide, proper phosphoramidite building blocks must be synthesized. This includes an adequate protection of the 2′-hydroxyl. In previous publications we used tetrahydropyranyl (THP) protection of the tertiary alcohol,[6] but unfortunately this group is not stable enough to the conditions used during 5′-dimethoxytrityl (DMT) deprotection throughout oligonucleotide solid phase synthesis.[13]

An interesting model for testing this modification is the hammerhead ribozyme.[14,15] This molecule catalyses the sequence specific hydrolysis of a phosphodiester linkage of RNA targets containing the UX motif (X = C, A or U). Taking into account these antecedents we decided to explore the preparation of 2′-*C*-methyl phosphoramidite by alkaline degradation of fructose, using an improved lactone reduction procedure and a more acid-stable protective group at the tertiary 2′-position. The modified phosphoramidite was introduced in the catalytic core of a hammerhead ribozyme directed against the ER-α[16] mRNA, which expression was measured by western blot in cancer MCF-7 cells.

In an attempt to improve 2′-*C*-methyl uridine preparation, by the construction of the 2-modified sugar moiety first, 2-*C*-methylribon-

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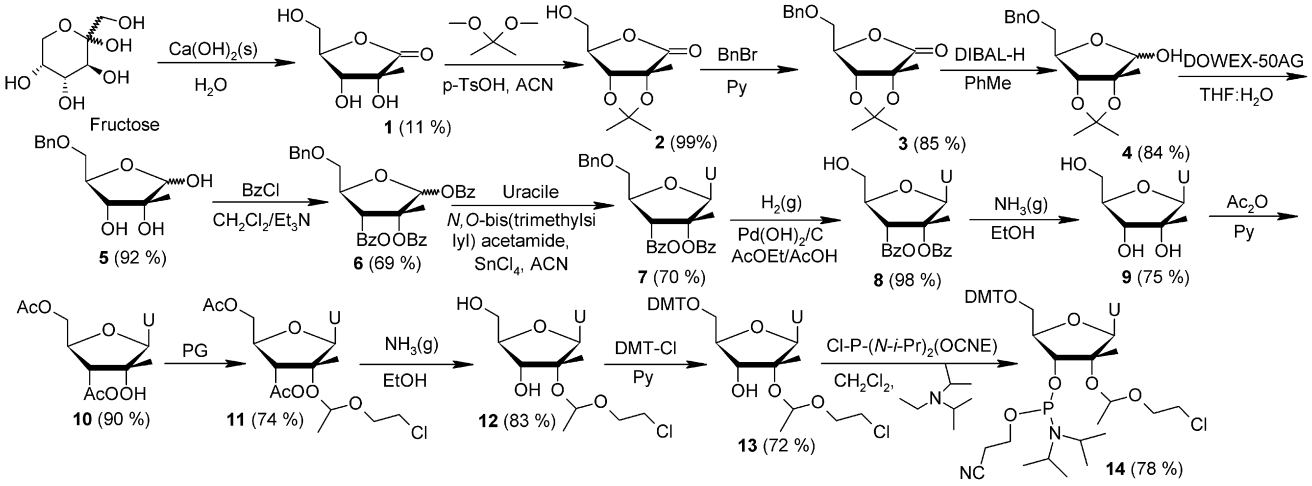

Figure 1. Synthesis of 2′-*C*-methylphosphoramidite (**14**) from fructose.

o-γ-lactone (**1**, Fig. 1) was prepared, starting from fructose and using an alkaline degradation with calcium hydroxide. In our experience the key step of this procedure is the exhaustive elimination of remaining calcium ions by ion exchange chromatography (see Supplementary data) and a percolation on silica gel, previous to crystallization. In spite the low yield of this step (11%), the low cost starting materials (food grade fructose, calcium hydroxide and water) justifies the procedure. Positions 2- and 3- were then protected using 2,2-dimethoxypropane to yield 2,3-di-*O*-isopropyliden-2-*C*-methylribo-γ-lactone (**2**, Fig. 1). This product was then benzylated in 85% yield, to give **3** (Fig. 1). This compound was reduced using 1.7 equivalents of DIBAL-H at −70 °C, obtaining 5-*O*-benzyl-2,3-*O*-isopropyliden-2-*C*-methyl-α,β-D-ribose (**4**, Fig. 1), practically as an equimolar anomeric mixture, in 84% yield. Subsequently, 2,3-protection was removed using an acid resin (DOWEX-50AG). Compound **5** was then perbenzoylated in 69% yield, to obtain 5-*O*-benzyl-1,2,3-tri-*O*-benzoyl-2-*C*-methyl-α,β-D-ribofuranose (**6**, Fig. 1). This intermediate was stereoselectively glycosylated under Vörbruggen conditions[10] with uracil to give 5′-*O*-benzyl-2′,3′-*O*-dibenzoyl-2′-*C*-methyluridine (**7**, Fig. 1) in 70% yield. The nucleoside was hydrogenated to remove the 5′-protection, using $Pd(OH)_2$/C as catalyst, practically in quantitative yield to afford **8** (Fig. 1), which was debenzoylated by NH_3(g) in ethanol, to give 2′-*C*-methyluridine (**9**, Fig. 1) in 75% yield.

The next step consisted in the preparation of the corresponding phosphoramidite. For this purpose, 3′ and 5′ positions were regioselectively acetylated. This alternative was preferred, because traditional Marckiewicz protection of positions 3′ and 5′, generates a steric hindrance that hampers the access to the 2′-tertiary alcohol.[13] In this way 3′,5′-di-*O*-acetyl-2′-*C*-methyluridine (**10**, Fig. 1) was prepared from **9** in 90% yield. Then, different protective groups, orthogonal to 5′-DMT protection, were tested. Classical *tert*-butyl-dimethylsilyl (TBDM) ether was attempted obtaining negative results using either the chloride or the triflate sililating agents. Then, (triisopropylsilyl)oxymethyl chloride (TOM-Cl),[17] was tested. TOM-Cl is known to have less steric hindrance. In order to introduce this protective group different bases (DBU, BDDDP, BuLi) and reaction conditions (different solvents and temperatures) were unsuccessfully assessed. Finally, 2-chloroethoxyethyl ether was tested, carrying out the reaction in the absence of solvent using an excess of 33 equiv of 2-chloroethylvinyl ether and pyridonium *p*-toluensulfonate as catalyst. In this way product **11** (Fig. 1) was obtained in 74% yield. The next step consisted in the removal of the acetyl groups under basic conditions to give 2′-*O*-(1-(2-choroethoxy)ethyl)-2′-*C*-methyluridine (**12**, Fig. 1) in 83% yield. Then, the 5′-position was protected using DMT-Cl to give **13** in 72% yield. Finally, the phosphoramidite **14** (Fig. 1) was prepared, by reaction of **13** with β-cyanoethoxy-*N*,*N*-aminochlorophosphine in a mixture of THF, diisopropylethylamine and CH_2Cl_2 under reflux, in 83% yield.

In order to test the enhanced acid stability of the 2′-protection, a comparative acid hydrolysis of 2′-*O*-tetrahydropyranyl-2′-*C*-methyluridine (**15**) and compound **12** was performed using 2.5% dichloroacetic acid in CH_2Cl_2.[7] The remaining protected nucleoside vs reaction time is depicted in Figure 2. As can be seen, (2-chloroethoxy)ethyl protection is more stable (half life ca. 50 min) than THP stable (half life ca. 7 min).

As a proof of concept and taking into account previous results[15,6,14], we decided to synthesize a modified hammerhead ribozyme carrying 2′-*C*-methyluridine in position 7- of the catalytic core. In order to test its applicability we selected the ER-α mRNA for the experiments in cell culture, motivated by its critical role in the proliferation of certain estrogen-dependent mammary breast tumors.[17]

For this purpose, a modified ribozyme designed to cleave the 956 nt of the mRNA of ER-α was prepared. In order to increase the ribozyme lifetime, the sequence was modified using 2′-*O*-methylnucleosides (blue capital letters captions, Fig. 3), two phosphorothiates at 3′ and 5′-ends (blue lower case, Fig. 3), the (2′*R*)-2′-*C*-methyluridine (red capital letters caption, Fig. 3) and keeping natural ribonucleotides (black captions, Fig. 3) at positions where

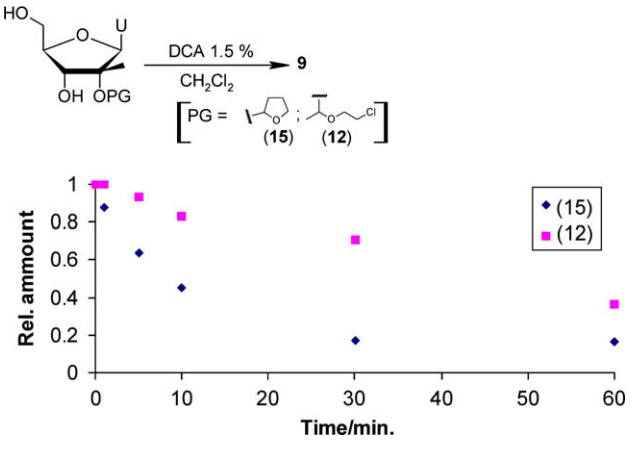

Figure 2. Relative acid stability of (**12**) and (**15**).

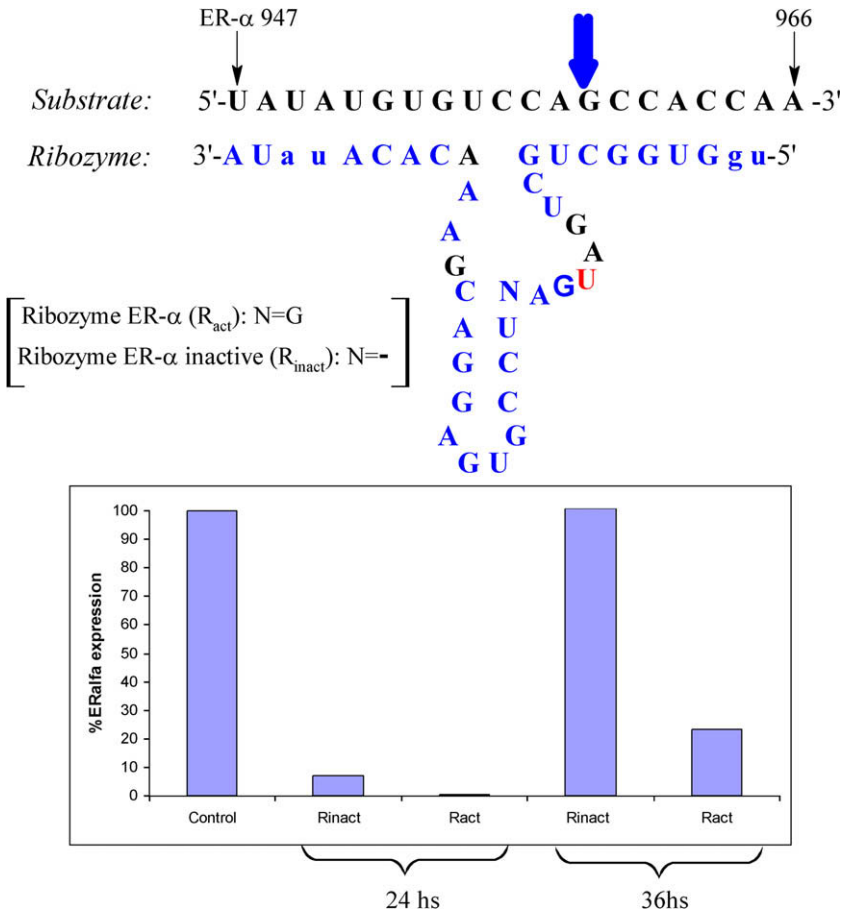

Figure 3. Hammerhead ribozyme sequence and western blot results.

2′-hydroxyl is important for activity (R_{act}, Fig. 3). An inactive ribozyme (R_{inact}, Fig. 3) was prepared by deletion of a G from the catalytic core (Fig. 3). This oligonucleotide has no catalytic activity but maintains its antisense function.

These sequences were used to transfect MCF-7 cells using cationic liposomes (Oligofectamine, Sigma), incubating the cells for 24 and 36 h. ER-α protein expression was verified at these times by western blot technique. A large protein inhibition was observed at 24 h by the active modified ribozyme (0.5%, R_{act}, 24 h, Fig. 3), showing also that the inactive ribozyme has inhibitory activity, presumably associated to its antisense performance (7.2%, R_{inact}, Fig. 3).

Down regulation of R_{inact} disappeared at 36 h of incubation (100%, R_{inact}, Fig. 3) while in the case of $R_{act}$the decreased in protein expression is still significative (23.4%, R_{act}, Fig. 3), probably as consequence of its catalytic effect.

In summary, the utility of a new modified 2′-*C*-methylphosphoramidite in the synthesis of RNA mimics was assessed and its successful application in an active ribozyme targeting the ER-α in MCF-7 breast cancer cells was shown.

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Supplementary data

Supplementary data (detailed description of the experimental procedures and NMR assignments) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.060.

References and notes

1. Strobel, S. A.; Cochrane, J. C. *Curr. Opin. Chem. Biol.* **2007**, *11*, 636.
2. Stoltenburg, R.; Reinemann, C.; Strehlitz, B. *Biomol. Eng.* **2007**, *24*, 381.
3. Corey, D. R. *J. Clin. Invest.* **2007**, *117*, 3615.
4. Kurreck, J. *Eur. J. Biochem.* **2003**, *270*, 1628.
5. Kaur, H.; Ravindra Babu, B.; Maiti, S. *Chem. Rev.* **2007**, *107*, 4672.
6. Gallo, M.; Kretschmer-Kazemi Far, R.; Scazakiel, G.; Iribarren, A. M. *Chem. Biodiv.* **2005**, *2*, 198.
7. Gallo, M.; Monteagudo, E.; Cicero, D.; Torres, H.; Iribarren, A. *Tetrahedron* **2001**, *57*, 5707.
8. Beigelman, L. N.; Eromolinsky, B. S.; Gurskaya, G. V.; Tsapkina, E. N.; Karpeisky, M. Y.; Mikhailov, S. N. *Carbohydr. Res.* **1987**, *166*, 219.
9. (a) Jenkins, S. R.; Arison, B.; Walton, E. *J. Org. Chem.* **1968**, *33*, 2490; (b) Walton, E.; Jenkins, S. R.; Nutt, R. F.; Holly, F. W. *J. Med. Chem.* **1969**, *12*, 306.
10. Harry-O'kuru, R. E.; Smith, J. M.; Wolfe, M. S. *J. Org. Chem.* **1997**, *62*, 1754.
11. Al-Husani, A. H.; Moore, H. W. *J. Org. Chem.* **1985**, *50*, 2597.
12. Yang, B. Y.; Montgomery, R. *Carbohydr. Res.* **1996**, *280*, 27.
13. Gallo, M. Doctoral Thesis, Universidad de Buenos Aires, 2002.
14. Beigelman, L.; Karpeisky, A. M.; Matulic-Adamic, J.; Haeberli, P.; Sweedler, D.; Usman, N. *Nucleic Acids Res.* **1995**, *23*, 4434.
15. Scher, S.; Kleba, C.; Häner, R.; Gauser, A.; Engels, J. W. *Bioorg. Med. Chem. Lett.* **1997**, *13*, 1791.
16. Lavrovsky, Y.; Tyagi, R. K.; Chen, S.; Song, C. S.; Chatterjee, B.; Roy, A. K. *Mol. Endocrinol.* **1999**, *13*, 925.
17. Pitsch, S.; Weiss, P. A.; Jenny, L.; Stutz, A.; Wu, X. *Helv. Chim. Acta* **2001**, *84*, 3773.