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Preparation of Modified RNA Sequences for Biological Research

Stefan Pitsch*, Damian Ackermann, Cyrille Denarie, Frédéric Meylan, Muthulaniappan Meyyappan, Evelyne Muller, Andreas Peer, Sébastien Porcher, Luc Reymond, Alfred Stutz, Philipp Wenter, and Xiaolin Wu

Abstract: Our group is working on the development of reliable access to biologically relevant, long, and modified RNA sequences. Here, we briefly present the chemical synthesis of such sequences with 2′-O-triisopropylsilyloxymethyl (= 2′-O-tom) protected ribonucleoside phosphoramidites, their template-directed enzymatic ligation, and some examples of modified nucleotides, designed to promote structural and biological studies.

Keywords: Ligation · Nucleic Acids · Organic synthesis · Phosphoramidites · RNA

1. Introduction

During the last few years, several new, important and fascinating biological functions of RNAs have been uncovered. A wide-ranging family of 'non-coding' or 'functional' RNAs (ncRNAs, fRNAs) has been identified, which are of general importance for RNA processing, editing, targeting and regulation [1]. Among them, small nuclear RNAs (snRNAs) play a central role during the assembly of the mRNA-splicing complexes by associating with proteins to form ribonucleoprotein (RNP) complexes [2]. Small nucleolar RNAs (snoRNAs) interact sequence-specifically with rRNA to guide processing and modification by the corresponding RNA-editing enzymes [3]. Micro-RNAs (miRNAs) have shown to act as posttranscriptional downregulaters of protein expression by inducing the cleavage of specific mRNAs [4]. Very importantly, it has been discovered that the same intrinsic mechanism of gene-silencing can be triggered by artificial short interfering RNAs (siRNAs) [5]. The concepts of gene-expression have been enriched by the discovery of the phenomenon of 'alternative splicing' which leads to the generation of alternative proteins from one gene; this process is regulated by consensus sequences (*e.g.* polypyrimidine tracks) located in intron regions [6]. Among mRNAs, so-called 'riboswitch' motives have been found which regulate or control gene expression on the level of translation or transcription by changing their structure upon binding of metabolites, or 3′-end processing [7]. The understanding of translation has been dramatically improved by high-resolution X-ray structures of ribosomes and ribosome subunits [8].

The study and understanding of such RNA-related biological processes often requires (among many other factors) the preparation of appropriately functionalized RNA sequences. For crystallographic studies, it is important to include heavy elements at defined positions, for NMR work on RNA structures or on RNA-protein complexes, it is imperative to prepare uniformly or, preferentially, selectively isotope-labeled RNA sequences. Biological studies, carried out *in vitro* or in cultured cells, often depend on the preparation of fluorescent-labeled RNAs for detection or of EDTA-conjugates for structural probing. Interesting RNAs contain typically between 65 and 200 nucleotides and are often naturally modified nucleotides or cap-structures.

In our laboratory, we concentrate principally on the development of reliable synthetic and enzymatic methods for the efficient preparation of such RNAs, which includes the synthesis and automated assembly of phosphoramidite building blocks, the ligation of chemically or enzymatically prepared RNA fragments and their functionalization/modification. Working with relative long RNA sequences requires standardized procedures and disciplined work under sterile conditions, in order to avoid their degradation by ubiquitous ribonucleases. Importantly, we have worked out reliable HPLC methods for the purification of RNA sequences under denaturating conditions and their analysis by electrospray-ionization MS (ESI-MS).

2. Results

2.1. *Chemical Synthesis of RNA Sequences*

The chemical synthesis of RNA is intrinsically more demanding than the synthesis of DNA, because the additional 2′-OH group, present in RNA-nucleotides, must be protected during assembly. As a consequence, additional chemical steps are required, both for the introduction of the 2′-O-protecting groups into monomeric phosphoramidites, and for their removal from the assembled RNA sequences. Some years ago, we introduced a new method for the chemical synthesis of RNA sequences, based on the fluoride-labile 2′-O-[(triisopropylsilyl)oxy]methyl (= 2′-O-TOM) protecting group [9]. In the meantime, it has become one of three commercially exploited methods for the routine and high-throughput synthesis of oligoribonucleotides, and the corresponding phosphoramidite building blocks are commercially available [10]. Their as-

*Correspondence: Prof. Dr. S. Pitsch
Laboratory of Nucleic Acid Chemistry
Ecole Polytechnique Fédérale de Lausanne
EPFL-BCH
CH-1015 Lausanne
Tel.: + 41 21 693 9380
Fax: + 41 21 693 9355
E-Mail: stefan.pitsch@epfl.ch

sembly is adapted as closely as possible to established DNA chemistry and excellent coupling yields of >99% are achieved routinely under DNA-coupling conditions. The final deprotection under very mild conditions is fast, easy and efficient, and does not interfere with the integrity of the product RNA sequences. As an example of such a synthesis, the anion-exchange HPLC-trace and the ESI mass spectrum of a crude 55mer RNA sequence, which is part of the *Azoarcus* ribozyme [11], is shown in Fig. 1.

2.2. *Ligation of RNA Fragments*

Up to 100mer RNA sequences can be assembled with 2′-O-TOM protected building blocks, with the same quality as analogous DNA sequences. However, in our experience, sequences >55mers cannot be reliably purified anymore by HPLC or other methods, and therefore contain variable amounts of shorter sequences and other byproducts resulting from side-reactions during assembly and/or deprotection. In order to extend the length of homogeneous, chemically synthesized RNA-sequences beyond 55mers, or in order to prepare longer sequences by combining *in vitro* transcripts with chemically synthesized fragments, we have developed general chemical and enzymatic methods for the template-directed ligation of RNA-fragments. Usually, the template-directed enzymatic ligation of RNA-fragments is carried out with T4-DNA Ligase and DNA-templates (there are no template-depending RNA-ligases). We have discovered that short unnatural 2′-OMe-RNA templates or DNA/2′-OMe-RNA hybrid-templates are often superior, and we have meanwhile optimized this reaction for the predictable and large-scale synthesis of long and modified RNA-sequences. In this study, we found a correlation between the intrinsic structure of the RNA substrates and the efficiency of ligation and, as a consequence, were able to develop a set of structure-based conditions for optimal results [14]. In Fig. 2 the HPLC-trace of such a ligation between a 48mer and a 71mer RNA sequence is shown together with the ESI-MS spectrum of the isolated 119mer RNA product sequence. This sequence is part of the 189mer U2 snRNA involved in mRNA splicing and nuclear export [15].

2.3. *Modification and Functionalization of RNA Sequences*

Our 2′-O-TOM-chemistry is not only fully compatible with DNA synthesis and the traditional 2′-O-TBDMS-chemistry, but as a consequence, also with all known modified building blocks, such as the various fluorescent labels, sequence and terminus modifiers, backbone modifications, and unnatural nucleobases. Additionally, we have prepared a large set of 2′-O-TOM protected, fully compatible ribonucleoside phosphoramidites containing modified sugar moieties and nucleobases. Some recent examples are shown in Fig. 3.

Bulky and photolabile protecting groups were introduced into the O^6-position of guanosines, resulting in conformationally trapped bistable RNA sequences. Upon subsequent photolytic cleavage, the parent guanosine pairing was fully restored and refolding could occur. Such refolding processes could be monitored by real-time NMR spectroscopy, providing quantitative, time-resolved and structural information [16].

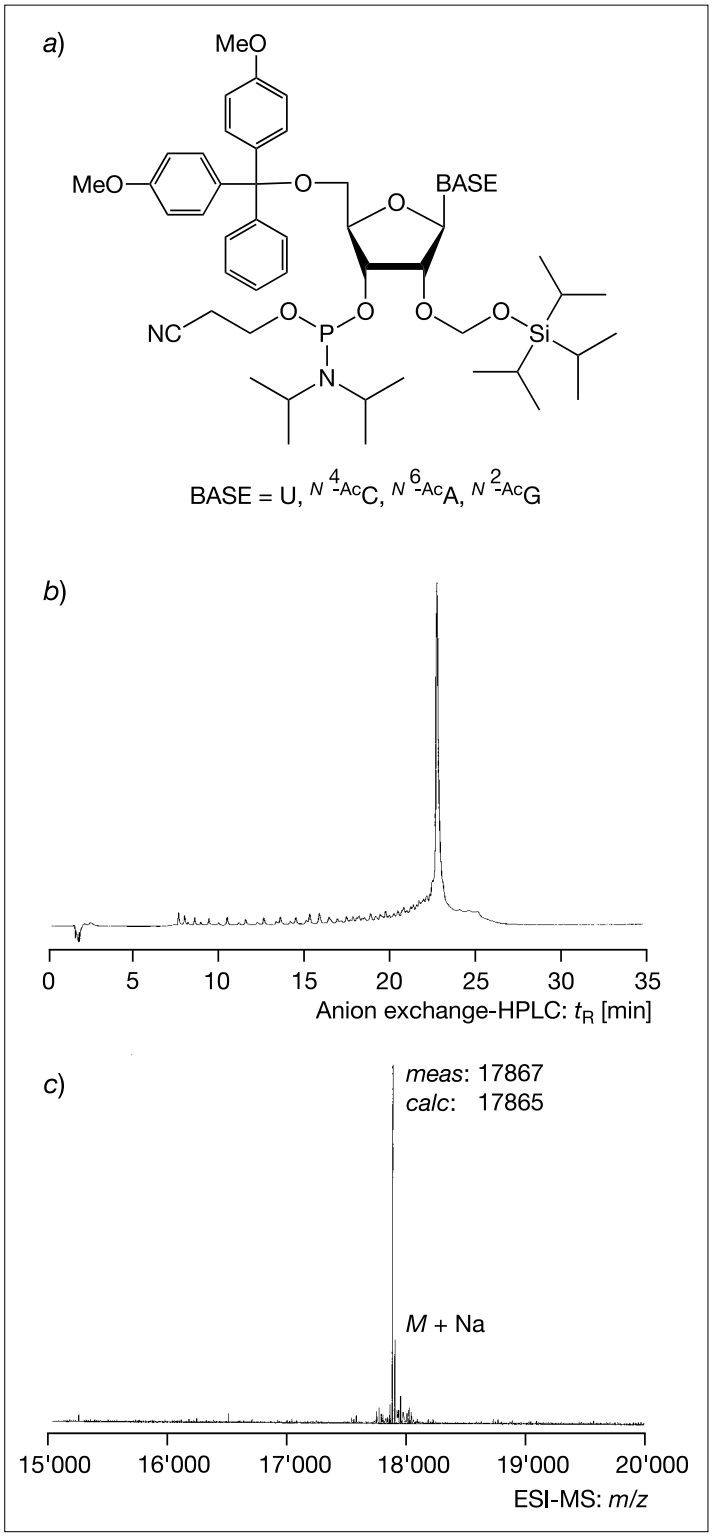

Fig. 1. Preparation of a 55mer RNA sequence by automated synthesis with 2′-O-TOM phosphoramidite building blocks. a) Structure of the four standard building blocks. b) Anion-exchange HPLC trace of the crude product, obtained under denaturating conditions according to [12]; detection at 260 nm. c) Deconvoluted electrospray ionization MS (negative mode) of the HPLC-purified product (yield 28%) obtained under our optimized conditions [12][13]. Abbreviation: Ac = acetyl.

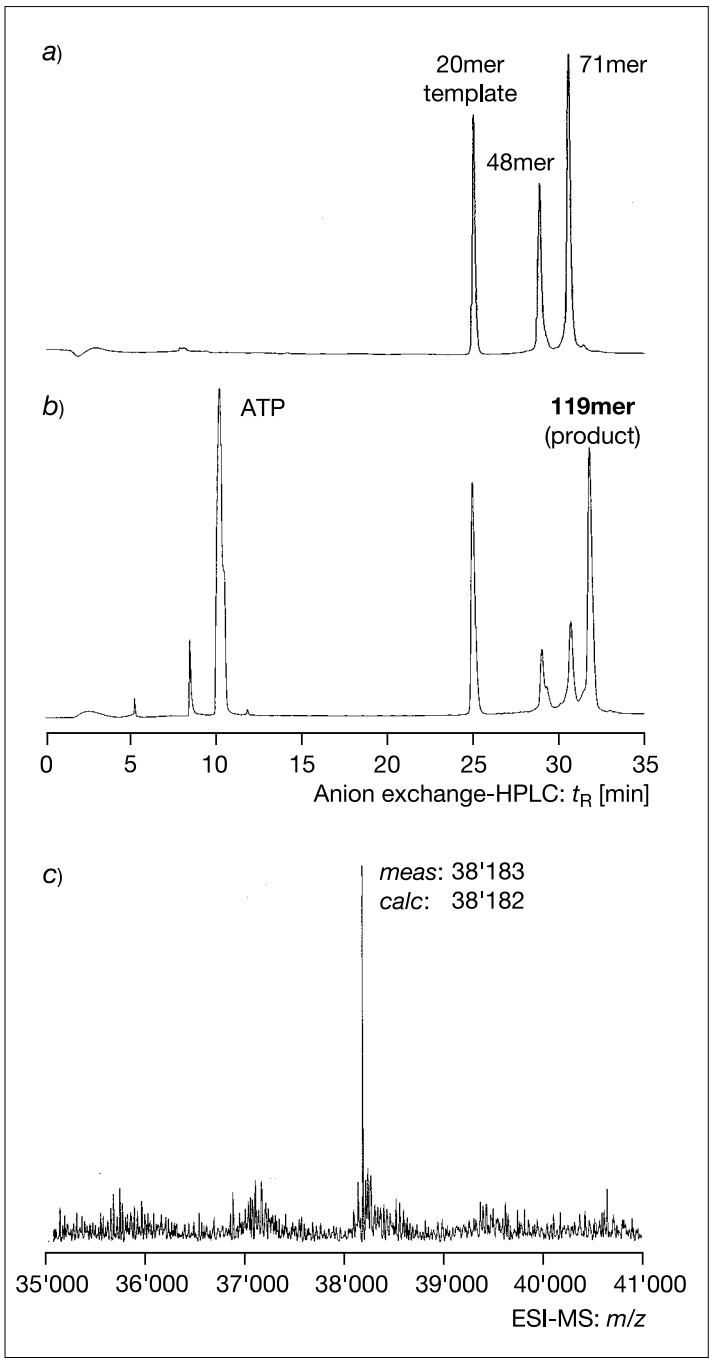

Fig. 2. Preparation of a 119mer RNA sequence by T4-DNA ligase mediated, template-dependant ligation under optimized conditions [14]. a) Anion exchange HPLC trace of the 20mer template and the starting materials (48mer and 71mer RNA sequence, the latter was prepared by an analogous ligation of a 27mer and a 44mer), detection at 260 nm. b) Analogous HPLC trace of the reaction mixture after 24h. c) Deconvoluted electrospray ionization MS (negative mode) of the HPLC-purified 119mer product sequence (yield 50%).

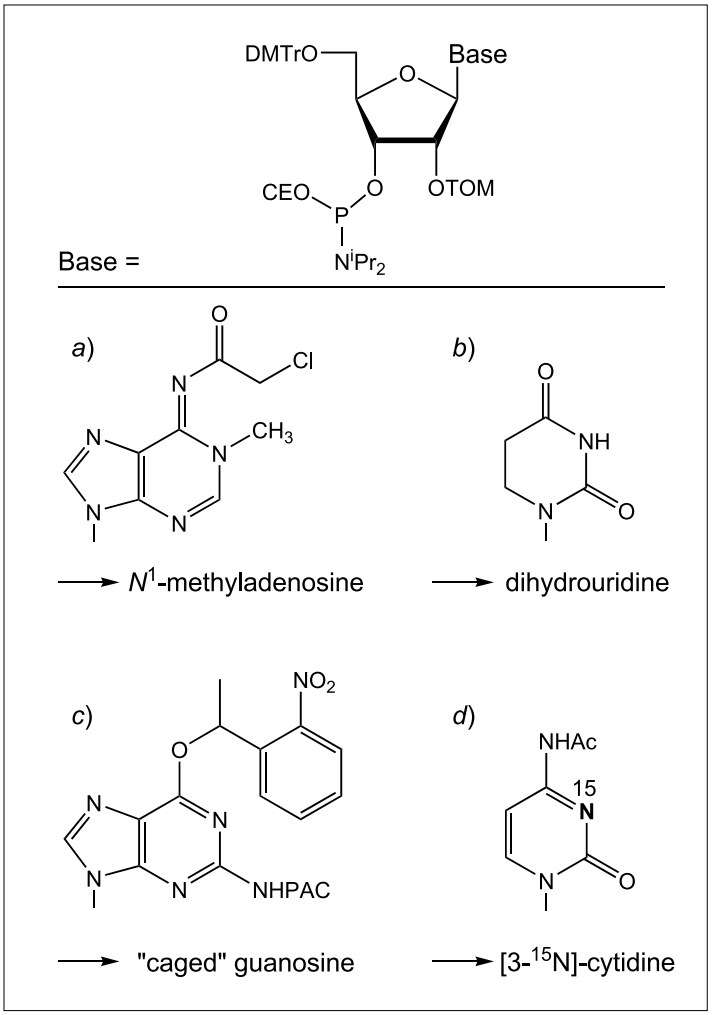

Fig. 3. Some representative examples of ribonucleoside phosphoramidites for the introduction of the indicated modified nucleotides into RNA sequences. a,b) An entire series of compatible building blocks for the introduction of naturally occurring, modified ribonucleotides such as N^1-methyladenosine and dihydrouridine have been prepared by organic synthesis [17]. c) Incorporation of this phosphoramidite building block leads to caged RNA sequences containing O^6-[(S)-(2-nitrophenyl)ethyl] guanosines, which are employed for time- and structure resolved studies of RNA folding [16]. d) The four phosphoramidite building blocks containing each a ^{15}N-label at the central Watson-Crick hydrogen bond position have been prepared. Their site-specific introduction into RNA-sequences provides a powerful tool for assignment and probing of putative secondary structure motives [12][16]. Abbreviations: DMTr = 4,4'-dimethoxytrityl; Ac = acetyl, CE = cyanoethyl, PAC = (4-isopropylphenoxy)acetyl.

Several naturally occurring alkylated and acylated nucleoside analogues have been prepared and introduced into tRNA sequences, in order to study their still debated influence on the function and structure of these important compounds [17]. Such posttranscriptional modifications are also present in most of the other RNA species and often required for correct folding or recognition and binding of proteins.

In order to overcome serious limitations in the detection of base-pair existence and dynamics by NMR, we are introducing ^{15}N- and ^{13}C-labels at representative base-pairs within defined secondary structure motives. In this context, we have prepared a variety of selectively isotope-labeled RNA-phosphoramidites, which are fully compatible with our standard phosphoramidites and can be incorporated anywhere in a RNA-sequence [12].

3. Outlook

This short article summarizes the most important methodological projects that we have carried out during the last ten years. Lately, more and more fascinating RNA-based or RNA-related biological processes have been found and many more will eventually be uncovered. The study of these processes requires modified RNA sequences as probes and in this context, we are planning to constantly extend our competences

in RNA synthesis, ligation and conjugation by developing new tools/building blocks, protecting groups, reaction conditions and analytical methods.

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