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Towards a Total Synthesis of Aminoacylated t-RNAs

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Abstract: In the ribosomal biosynthesis of proteins, the aminoacylated t-RNAs serve as carriers of the amino acids. A total synthesis of these biological key-compounds would allow the site-specific incorporation of modifications within the t-RNA and attachment of any desired amino acid. Such analogues are useful tools for structural and functional studies, and for the incorporation of unnatural amino acids into proteins.
Our retrosynthetic scheme for the preparation of aminoacylated t-RNAs includes the synthesis of truncated t-RNAs, the synthesis of aminoacylated RNA-fragments, and their subsequent ligation. In this article, we present our approaches toward the realization of these three tasks.

Keywords: Ligation · Oligonucleotides · Protecting groups · RNA synthesis · t-RNA

Stefan Pitsch was born on April 28, 1964 in Basel. From 1984–1988 he studied chemistry at the Department of Biological Sciences at the ETH Zürich. His doctoral studies with Prof. Albert Eschenmoser were related to prebiotic sugar phosphate chemistry. After his PhD in 1993, he continued working for Prof. Eschenmoser as a postdoctoral researcher, carrying out research in the field of nucleic acid chemistry. During his subsequent stay with Prof. G. Arrhenius in La Jolla, USA, he worked with mineral catalysts for organic reactions. Five years ago, he returned to the ETH Zürich to work in the group of Prof. A. Vasella on his habilitation thesis which was submitted last spring. Presently he is lecturing in biological and organic chemistry and leading a small research group at the ETH Zürich. His main research interest is the development of reliable methods for the synthesis of oligoribonucleotides and modified analogues.

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1. Introduction

We are interested in a general, non-enzymatic synthesis of aminoacylated t-RNAs for studies related to the process of transcription and for the ribosome-mediated incorporation of unnatural amino acids into proteins. Currently, artificial aminoacylated t-RNAs are obtained by enzymatic ligation of a truncated t-RNA (produced by T7-polymerase-mediated transcription of an appropriate gene construct) with an aminoacylated r(CA) dimer [1]. This approach results in small quantities of t-RNA analogues, containing only the four canonical ribonucleosides. A total synthesis, in contrast, would allow the synthesis on a larger scale and the site-directed incorporation of any desired modification.
The ester bond of aminoacylated (oligo)ribonucleotides is an activated, energy-rich bond with a free energy of hydrolysis comparable to the one of ATP hydrolysis [2]. At 25 °C and pH 7.4, for instance, we determined a half-live of only 0.8 h for a L-phenylalanyl-nucleotide (Fig. 1). Therefore, in our planned synthesis of aminoacylated RNA-sequences we had to choose reaction conditions and protecting groups carefully, in order to avoid cleavage of the critical ester bond. Specifically, we had to avoid the commonly used acyl-type nucleobase protecting groups which are removed with strong nucleophiles such as NH_3 or $MeNH_2$. In a first approach to our target compounds, we developed a RNA synthesis which was based on novel phosphoramidite building blocks containing photolabile sugar and nucleobase protecting groups [3]. With these building blocks the first aminoacylated RNA-sequences could be prepared, but we soon realized that the purification and handling of such amino acid/RNA conjugates was still too difficult. Therefore, we decided to modify the original concept by preparing stabilized precursors that could be transformed into the target structures by a final photolytic step. It was well known that both N-acylation and the absence of a neighboring 2'-OH group stabilize the ester bond of aminoacylated nucleoside derivatives towards hydrolysis [4]. By model studies we were able to confirm these observations and to determine the individual stabilization of the protecting groups at the nucleoside and at the amino acid portion, respectively. As compared to the unprotected aminoacylated nucleotide, simultaneous N- and 2'-O-protection resulted in a 70-fold increase of stabilization at 25 °C and pH 7.4 (Fig. 1).

Our retrosynthetic scheme for the preparation of stabilized precursors of aminoacylated t-RNAs is shown in Scheme 1. We are planning to attach N- and 2'-O-protected aminoacylated RNA-fragments to the 3'-end of truncated, chemically synthesized t-RNAs by

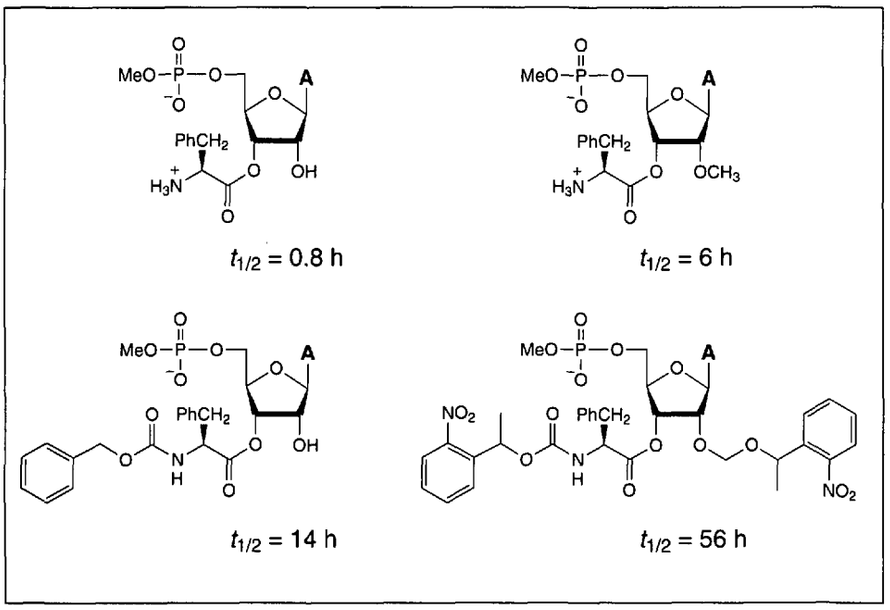
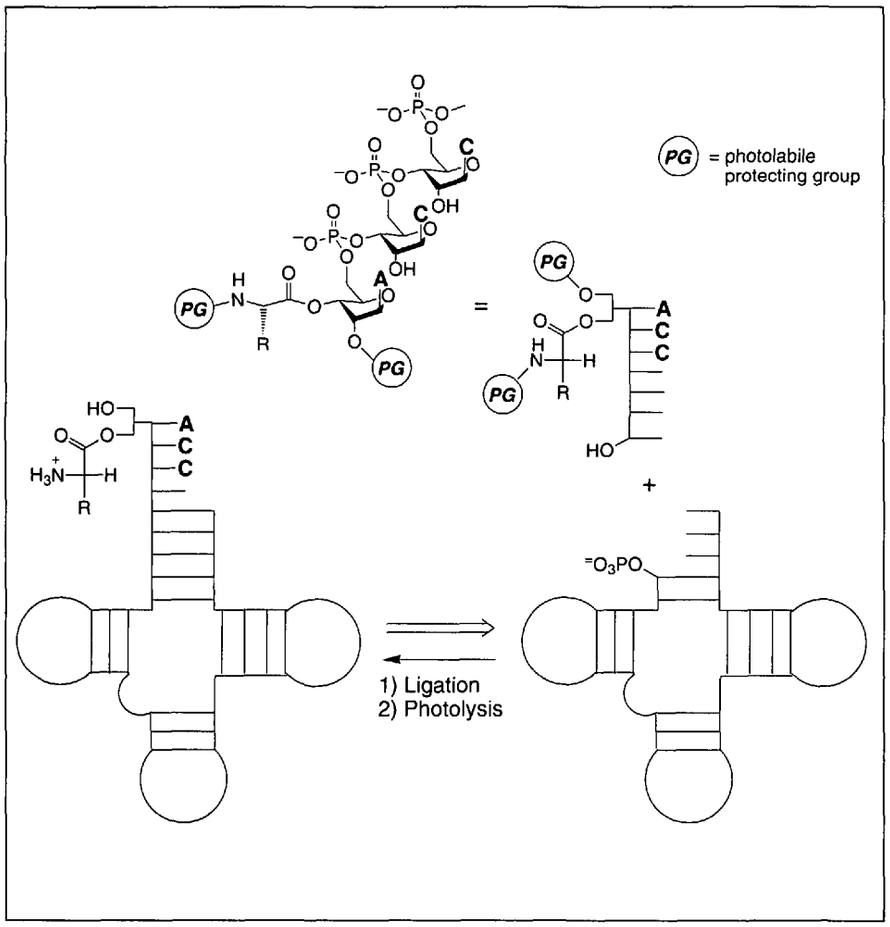


Fig. 1. The stability towards hydrolysis of the ester linkage formed between an amino acid and a nucleotide is increased by N- and 2'-O-protection. Conditions: 0.1M Tris-HCl (pH 7.4), H_2O, 25 °C.



Scheme 1. Retrosynthetic analysis: Ligation of stabilized N- and 2'-O-protected aminoacylated RNA fragments to truncated t-RNAs, followed by the liberation of the labile aminoacylated t-RNAs by photolysis.

chemical ligation. This non-enzymatic ligation requires a template which is provided by the 5'-region of the truncated t-RNA; its 3'-end serves thereby as primer.

According to the presented retrosynthetic analysis, three different problems have to be solved: 1) the chemical synthesis of relatively long RNA-sequences, 2) the synthesis of aminoacylated RNA-fragments, 3) the ligation of RNA-sequences. Here, we present our approaches towards the realization of these three tasks.

2. Results

2.1. *RNA Synthesis*

Compared to DNA, each nucleotide unit of RNA contains a 2'-OH-group, which has to be protected during the assembly. Unfortunately, these supplementary protecting groups sterically interfere with the coupling process and require an additional deprotection step. From the large number of 2'-O-protecting groups investigated so far, the tBDMS group [5] has found the widest application. However, several factors, including the relatively low coupling yields, obtained with such building blocks, were not optimal and limited the length of the chemically synthesized RNAs to about 40 nucleotide units.

In this context, we recently developed the novel 2'-O-[(triisopropylsilyl)oxy] methyl (= tom) protected RNA-building blocks [6] (Fig. 2). By combining the advantages of the tBDMS-protecting group with a sterically non demanding linker, our tom-group allows the synthesis of RNA-sequences (consisting of up to 100 nucleotides) with excellent coupling yields (> 99.3% under DNA-coupling conditions) and a short and reliable deprotection. As an example, Fig. 3 shows the capillary electrophoresis chromatogram of a crude 69mer sequence (containing one deoxyribonucleoside) which was prepared from 2'-O-tom protected phosphoramidites under DNA-coupling conditions.

Typically, t-RNAs consist of about 80 units, including the four canonical ribonucleotides and a great variety of modified nucleotides. Our 2'-O-tom protected phosphoramidites will allow the straightforward preparation of truncated t-RNAs (consisting of about 75 nucleotides). We are about to prepare building blocks which additionally will permit the introduction of modified nucleotides, such as *pseudo*-uridine.

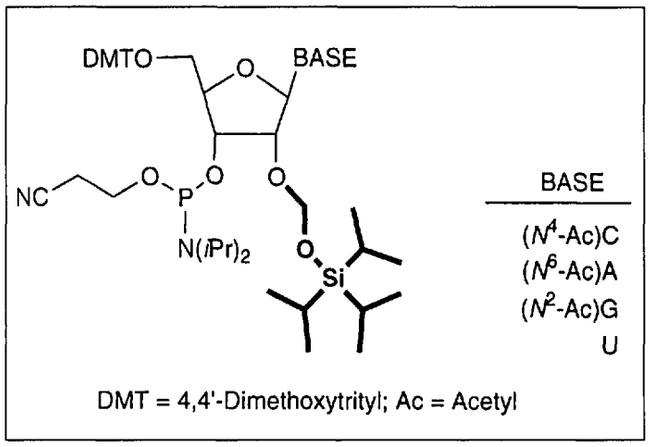


Fig. 2. Structure of our 2'-O-tom protected phosphoramidite building blocks employed in the routine synthesis of RNA-sequences.

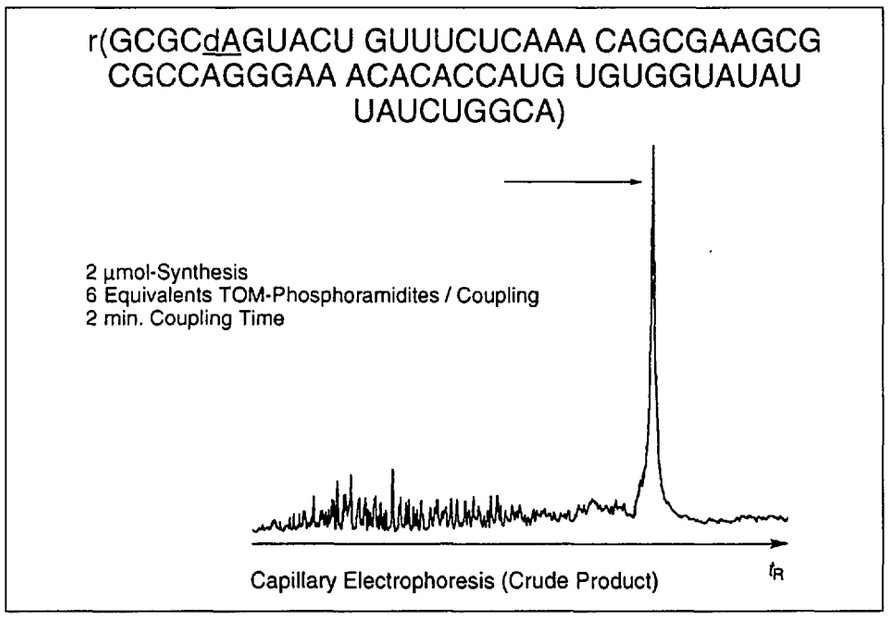


Fig. 3. Example of a crude 69mer RNA-sequence prepared with 2'-O-tom-protected phosphoramidites under DNA-coupling conditions. The arrow indicates the full-length product.

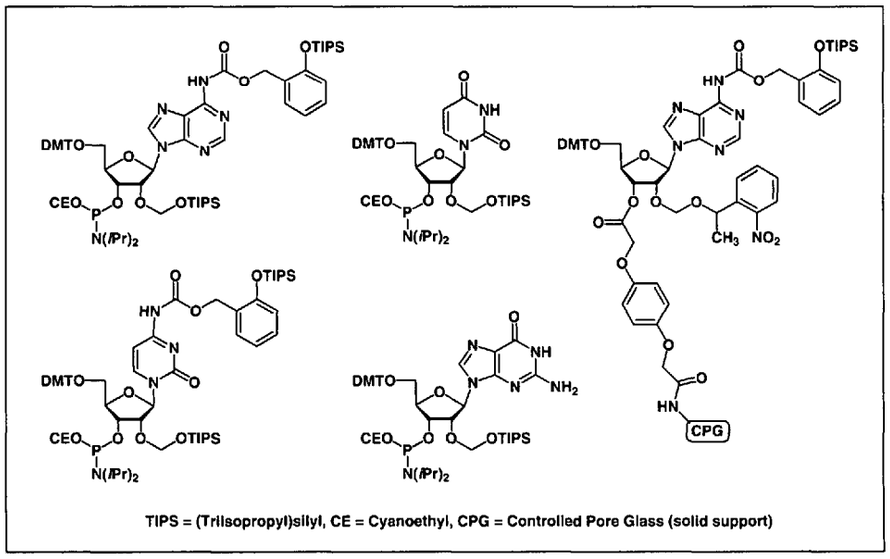


Fig. 4. Structure of the building blocks employed for the synthesis of aminoacylated RNA-fragments.

2.2. *Preparation of Aminoacylated RNA-Sequences*

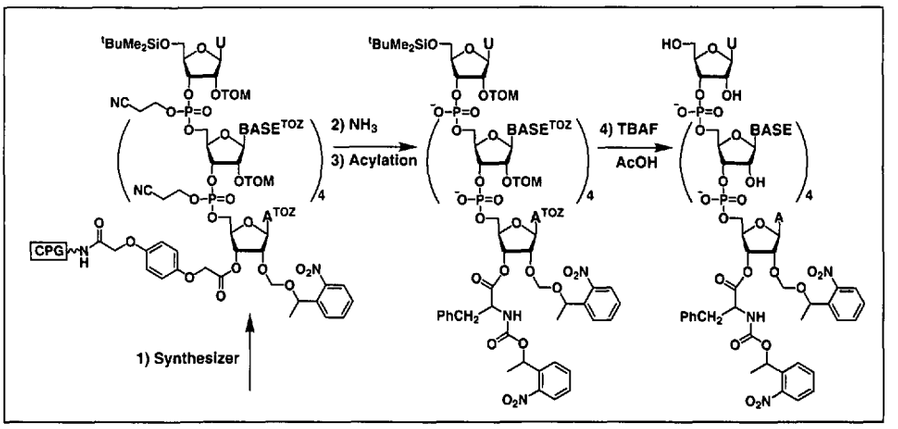
Our approach to the synthesis of aminoacylated RNA-sequences has been published recently [7]. In this context, novel RNA phosphoramidites with (fluoride-labile) 2'-O-[(triisopropylsilyl)oxy] methyl (= tom) sugar-protecting and (fluoride-labile) N-{{2-[(triisopropylsilyl) oxy]benzyl}oxy}carbonyl (= toz) base-protecting groups and a solid support containing an immobilized N^6-toz-protected adenosine with an orthogonal (photolabile) 2'-O-[(*S*)-1-(2-nitrophenyl) ethoxy] methyl (= (*S*)-npeom) protecting-group were prepared (Fig. 4).

From these building blocks, a hexameric oligoribonucleotide could be prepared efficiently by automated synthesis under standard conditions. After mild detachment from the solid support, the resulting fully protected sequence was aminoacylated with L-phenylalanine-derivatives, carrying photolabile N-protecting groups. Upon removal of the fluoride-labile sugar and nucleobase protecting groups, a partially photolabile-protected, stabilized precursor of an aminoacylated RNA-sequence was obtained (Scheme 2). In Fig. 5, the RP-HPLC trace of the reaction mixture obtained with the N-[(*RS*)-1-(2-nitrophenyl)ethoxy]carbonyl-protected L-phenylalanine is presented. According to this analysis, aminoacylation occurred to an extent of *ca.* 80% (splitting of the product peak results from employing both diastereoisomers of the protected amino acid). The presence of the two stabilizing (photolabile) N- and 2'-O-protecting groups allowed a straightforward purification of this crude product by RP-HPLC.

Its final photolysis under mild conditions resulted in the efficient formation of a 3'(2')-O-aminoacylated RNA-sequence (Fig. 6)

2.3. *Chemical Ligation of RNA-Fragments*

In the context of the presented project and in order to extend the length of chemically synthesized RNA-sequences beyond 100mers, we are about to develop a general method for the chemical, template-directed ligation of RNA-fragments [8]. In order to unambiguously obtain the desired 3'-5' linkage (in contrast to a 2'-5' linkage) and in order to avoid the formation of 2',3'-cyclophosphates, we decided to block the 2'-OH-group at the ligation-site by a photolabile protecting group which at the end can be removed under mild conditions (Scheme 3). Fortunately, the presence of this pro-



Scheme 2. Synthesis of stabilized aminoacylated RNA-fragments. 1) Assembly of the building blocks from Fig. 4 under DNA-coupling conditions. 2) Detachment of the sequence from the solid support and removal of the cyanoethyl protecting groups with aqueous NH_3. 3) Acylation of the terminal 3'-OH group with a N-protected L-phenylalanine under peptide coupling conditions. 4) Removal of the fluoride-labile sugar and nucleobase protecting groups.

tecting group did not interfere with the ligation reaction and we soon were able to develop very efficient reaction conditions. Specifically, we found that a 3'-O-phosphate group was much better than a 5'-O-phosphate group, that EDC (N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimid hydrochloride) in the presence of LiCl and 1,2-dimethoxyethane (DME) was the condensing agent of choice and that 2'-O-methylated RNA sequences were the best templates. Under these conditions, quantitative ligation reactions were observed within 8–24 h at 25 °C.

In Fig. 7 the polyacrylamide gel (PAGE) analysis of a ligation reaction under such conditions is presented. A 36meric RNA sequence and a modified 27meric RNA sequence containing (at the 3'-end) a photolabile 2'-O-[(*S*)-npeom]-group and a 3'-O-phosphate-group, and (at the 5'-end) a fluorescein moiety were incubated with an equal amount of a short 2'-O-methylated RNA-template in the presence of LiCl and EDC in a mixture of H_2O and DME. The PAGE analysis (visualizing the fluorophoric group present in one of the fragments and in the product) shows clean and quantitative formation of the ligation product after 24 h at 25 °C.

Presently, we are about to explore the scope and the limitations of this novel method for the preparation of long RNA-sequences by ligation of RNA-fragments in the presence of suitable, short templates.

3. Outlook

We have developed a spectrum of novel photo- and fluoride-labile protecting groups for the straightforward synthesis of relatively long RNA-sequences, the preparation of stabilized aminoacylated RNA-sequences and the template-di-

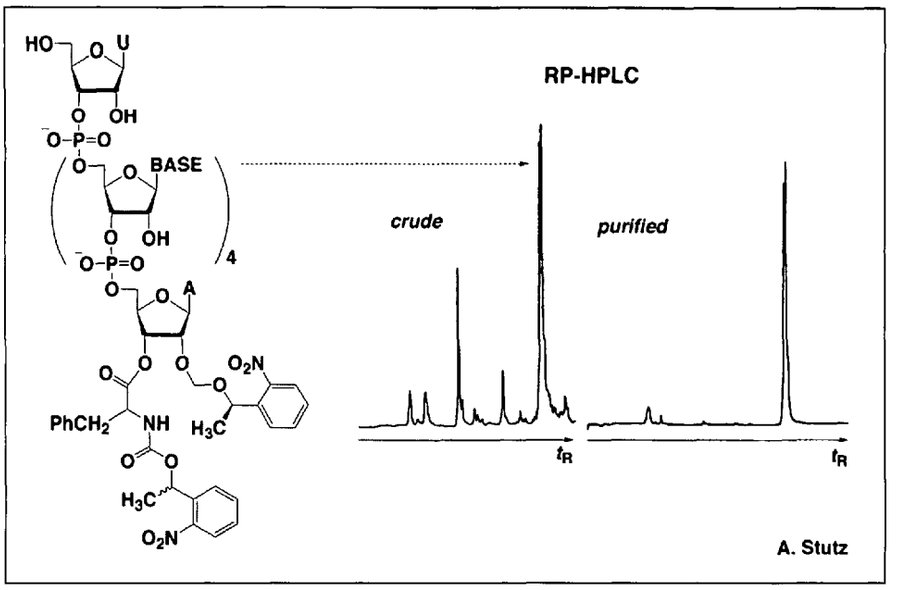


Fig. 5. Reversed phase HPLC traces of the crude and purified N- and 2'-O-protected aminoacylated RNA-sequence from Scheme 2.

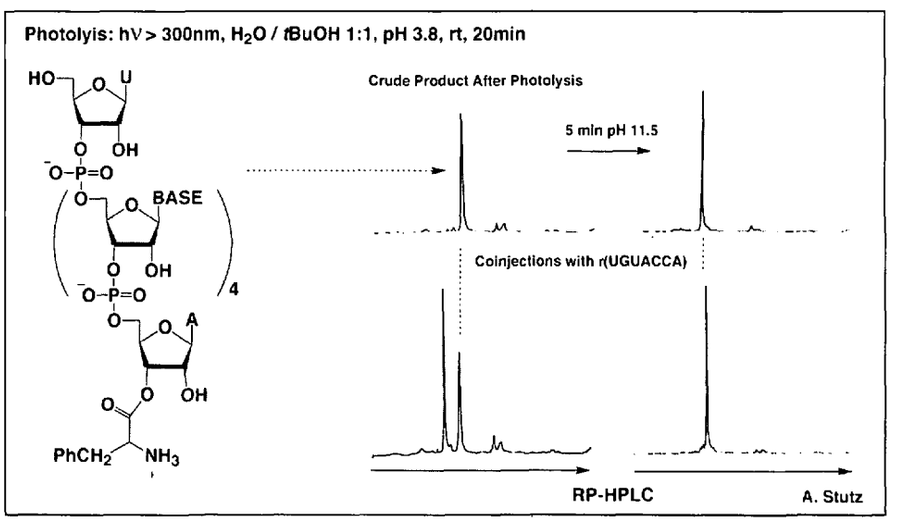
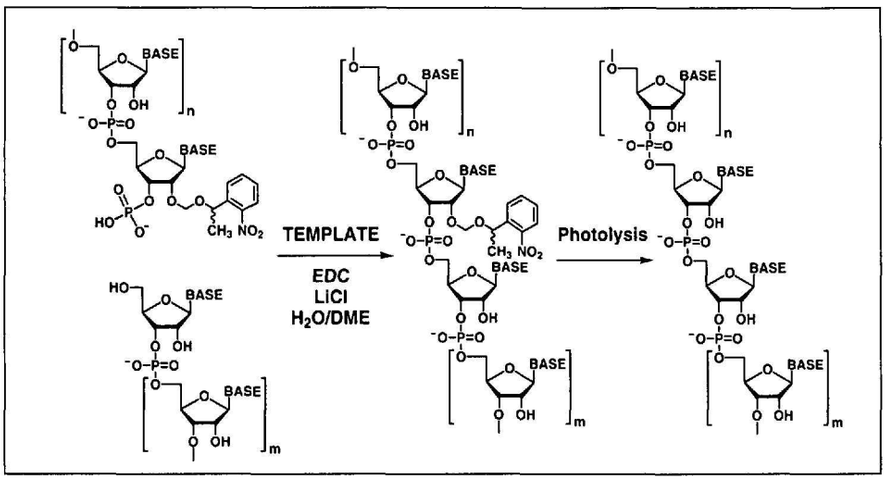


Fig. 6. Short photolysis of the N- and 2'-O-protected aminoacylated RNA-sequence (Scheme 2 and Fig. 5) gave the fully deprotected aminoacylated RNA-sequence (top left). Short incubation of this product at pH 11.5 resulted in a complete cleavage of the labile ester-bond with subsequent liberation of the corresponding RNA-sequence (top right).



Scheme 3. Template-directed ligation of RNA-fragments. The presence of a photolabile 2'-O-protecting group prevents formation of a terminal 2',3'-cyclophosphate group. After ligation, the protecting group is removed by photolysis. EDC = (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimid hydrochlorid), DME = 1,2-dimethoxyethane.

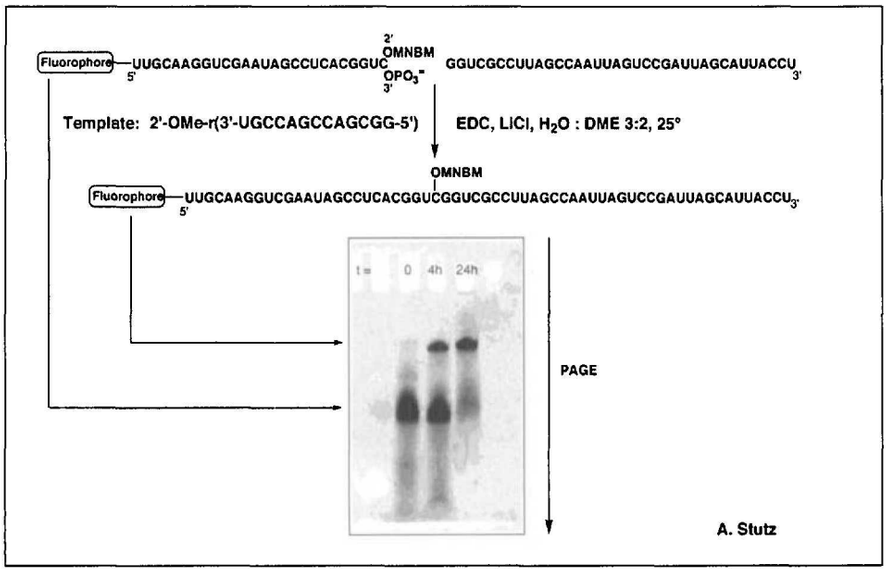


Fig. 7. Ligation of two RNA-fragments with the help of a short 2'-O-methyl-RNA template. The reaction was followed by PAGE analysis (fluorometric detection) which indicates quantitative ligation after 24 h.

rected ligation of RNA-fragments. Now we are about to combine these methods in order to synthesize a variety of aminoacylated t-RNAs and analogues.

Our approach relies exclusively on organic synthesis and contains no enzymatic steps. Therefore it should allow a clear distinction of the two major processes in which t-RNAs are involved: first, the loading with the correct amino acid (catalyzed by aminoacyl-t-RNA-synthetases), and second, the interaction of the aminoacylated t-RNAs with the ribosome, the codon of the m-RNA and the elongation factors, respectively. We are planning to synthesize a large number of different aminoacylated t-RNA analogues and to determine in which of these two major processes the conserved structural elements and the modified nucleosides, respectively, are involved.

Additionally, we are aiming at the development of chemically and enzymatically stable, ready available 'minimal' aminoacylated t-RNA-analogues which, in respect to the *in vitro* synthesis of proteins, would still be efficient carriers of amino acid building blocks. This technology would allow the routine – and even combinatorial – introduction of unnatural amino acids into proteins. Such protein analogues could have altered catalytic properties or could be used as specific probes for biological processes and for structure determination.

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