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Chemical synthesis of a biologically active natural tRNA with its minor bases

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ABSTRACT

The complete chemical synthesis of an *E.coli* $tRNA^{Ala}$ with its specific minor nucleosides, dihydrouridine, ribothymidine and pseudouridine, is reported. The method makes use of protected 2'-O-tertiobutyldimethylsilyl-ribonucleoside-3'-O-(2-cyanoethyl-N-ethyl-N-methyl)phosphoramidites. The exocyclic amino functions of the bases were protected by the phenoxyacetyl group for purines and acetyl for cytosine. The assembling has been performed on a silica support with coupling yield better than 98% within 2 min of condensation. Triethylamine tris-hydrofluoride allowed a clean and complete deprotection of the tBDMS groups. The synthetic $tRNA^{Ala}$ has been transcribed into cDNA by reverse transcriptase and sequenced. With *E.coli* alanyl-tRNA synthetase the alanyl acceptance activity and k_{cat}/K_m were 672 pmol/A_{260} and $6 \times 10^4 M^{-1}s^{-1}$, respectively.

INTRODUCTION

The importance of RNA in cellular processes has long been recognized and recently much attention has been focused on problems concerning antisense RNA and biological function of RNA (1). RNA oligomers are currently obtained by using phage RNA polymerase and ribonucleoside triphosphates but the method is template sequence dependent and only the natural bases can be inserted at will (2).

Another approach is the chemical synthesis using protected ribonucleoside phosphoramidites (3–9). The chemical synthesis of DNA fragments on solid support is performed with great efficiency. However similar, rapid and high yield RNA synthesis giving a pure product is lagging behind. The difficulty arises mainly from the presence of the additional 2'-hydroxyl function on the ribose moiety and the key of the success is a suitable selective protection. The protecting group chosen influences the course of the internucleotide coupling reaction and modifies the stability of the protected ribonucleoside phosphoramidites. It must remain stable throughout chain assembling and final deprotection steps, and it must be totally removed without damaging the 3'–5' phosphodiester linkage. In addition, natural transfer RNAs have fragile minor bases and the structural integrity of these bases must be kept.

The tetrahydropyranyl (Thp) and achiral 4-methoxy-tetrahydropyranyl (Mthp) were used for a number of years as 2'-hydroxyl protecting groups (10, 11). They are not prone to 2'→3' migration and can be removed at pH 2 at room temperature. Nevertheless, it has been reported that they are not stable enough in the usual acidic conditions required for the hydrolysis of the pixyl or the dimethoxytrityl groups used to protect the 5'-hydroxyl (12). The use of 4-methoxypiperidinyl type of protection avoids this problem (13,14). Another improvement is the fluorene methoxy carbonyl group (FMOC) to replace the dimethoxytrityl group for the 5'-OH protection (15). The 2-nitrobenzyl group which has been proposed as a 2'-OH protecting group does not migrate, is stable in mild acidic conditions and can be cleaved by photolysis (16). Precautions have to be taken to exclude light during storage and synthesis until the last deblocking step. We have selected the tert-butyldimethylsilyl group (tBDMS) proposed by Ogilvie (9). This group is stable in the acidic detritylation medium and also in the alkaline conditions used to deprotect the phenoxyacetyl group (PAC) (17). However, we experienced that in the conditions previously described this group has serious inconveniences: hindrance of the internucleotide coupling reaction and incomplete elimination by fluoride ions.

So far the total chemical synthesis of a natural tRNA has not been achieved. The difficulties arise from the length of the chain and from the presence of alkali labile modified bases. The first synthesis of a natural tRNA has been successfully realised in 1981 after thirteen years of joint effort of six laboratories (18). This tRNA was obtained by enzymatic ligation of short oligonucleotides ranging from three to eight nucleotides and obtained by either chemical synthesis or hydrolysis of other natural tRNA. This hemisynthesis method based on ligation of natural tRNA fragments has been used by different laboratories

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to study the 'second genetic code'. There is a need for a more efficient way. Ogilvie described the first total chemical synthesis of a 77 nucleotide long RNA sequence having a low methionine acceptance activity but this product lacked natural modified nucleotides (3).

In this work we report new reagents for oligoribonucleotide synthesis and their application to the first total chemical synthesis of a natural tRNA with its modified bases. This $tRNA^{Ala/GGC}$ has been isolated by Mims (19) from *E.coli* K12 and contains the anticodon sequence GGC, dihydrouridine, ribothymidine and pseudouridine (Fig. 1). The full length tRNA has been synthesised, isolated, sequenced and it exhibited a good aminoacylacceptance activity. In addition, we propose a new method to confirm the synthetic $tRNA^{Ala/GGC}$ sequence : the tRNA was copied by a reverse transcriptase and the obtained cDNA sequenced.

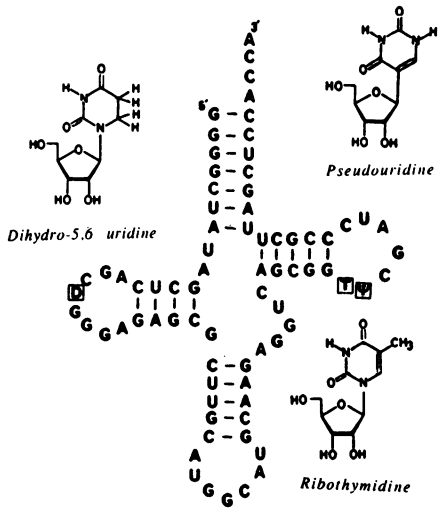

Figure 1. Primary structure of *E.coli* $tRNA^{Ala/GGC}$ with its modified nucleosides.

RESULTS AND DISCUSSION

Use of labile amino protecting groups

In the search of a practical way to synthesize a natural tRNA sequence with its modified bases, we experienced that the previously reported chemistry was not adapted. The removal of amino protecting groups such as isobutyryl for guanine required too drastic ammonia treatment. We proposed the phenoxyacetyl group for adenine and guanine and phenoxyacetic anhydride as the capping reagent (20–24). In this way we performed the solid phase synthesis of the 5'-half initiator tRNA from *B.subtilis* (20). However in spite of many efforts we were not able to extend this result to the synthesis of a pure complete natural tRNA molecule having an alkaline hydrolysis PAGE ladder without smears, a correct sequencing and a significant aminoacyl acceptance activity.

Now we have solved the problem by the introduction of new reagents and new methods:

a) in the assembling step, protected 2'-O-tBDMS ribonucleoside-3'-O-(2-cyanoethyl-N-ethyl-N-methyl) phosphoramidites which gave much better coupling results than protected 2'-O-tBDMS ribonucleoside-3'-O-(2-cyanoethyl-N-diisopropyl) phosphoramidites.

b) in the tBDMS deprotection step, triethylamine tris-hydrofluoride (TEA,3HF) which is a more efficient deprotecting reagent than tetrabutylammonium fluoride in tetrahydrofuran.

Protected 2'-O-tBDMS ribonucleoside-3'-O-(2-cyanoethyl-N-ethyl-N-methyl) phosphoramidites

The protected nucleoside 3'-O-(cyanoethyl-N,N-diisopropyl) phosphoramidites are nowadays universally used for DNA and RNA synthesis but, owing to the presence of the 2'-O-protecting group, the coupling yield (95%) and the condensation rate (15 minutes) for RNA synthesis are significantly lower than in DNA synthesis (98% and 1 min) (25–29).

In a preliminary study, we synthesized a series of protected uridine-2'-O-tBDMS-3'-O-(2-cyanoethyl-N,N-dialkyl) phosphoramidites and estimated their efficiency in RNA synthesis (30). We showed that 5'-O-dimethoxytrityl –2'-O-tBDMS-uridine-3'-O-(2-cyanoethyl-N-ethyl-N-methyl) phosphoramidite **2d** (Fig.2) and 5'-O-dimethoxytrityl –2'-O-tBDMS-uridine-3'-O-(2-cyanoethyl-N-diethyl) phosphoramidite gave an average coupling yield of 98% within 4 min reaction time and that the products were stable for several months when stored under argon at –20°C. We have extended this method to the nucleosides found

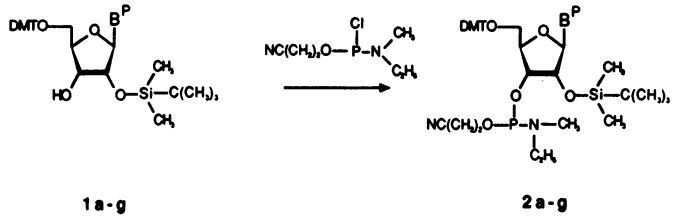

Figure 2. Preparation of fully protected ribonucleoside 3'-O-phosphoramidites. **a**: $B^P = N^6$-phenoxyacetyl-adenosin-9-yl, **b**: $B^P = N^2$-phenoxyacetyl-guanin-9-yl, **c**: $B^P = N^4$-acetyl-cytosin-1-yl, **d**: B^P=uracil-1-yl, **e**: B^P=uracil-5-yl, **f**: B^P=thymin-1-yl, **g**: B^P=5-6-dihydrouracil-1-yl.

in the synthetic $tRNA^{Ala/GGC}$. No traces of the 3' O-tBDMS could be detected by reverse phase HPLC, silicagel TLC, ^1H NMR and ^{31}P NMR analyses. Surprisingly, protected ribonucleoside N-ethyl-N-methyl -phosphoramidites were purified much more easily and gave better results than the N-diethyl derivatives. Thus we prepared the 2'-O-tBDMS-nucleoside-3'-O-(2-cyanoethyl-N-ethyl-N-methyl) phosphoramidites corresponding to adenosine, guanosine, cytidine, ribothymidine, dihydrouridine and pseudouridine (**2a–g**) from their corresponding precursors (**1a–g**) with the phosphitylating reagent (2-cyanoethyl)-(N-ethyl-N-methyl) phosphonamidic chloride.

These phosphoramidites were purified by HPLC giving 55 to 80% yield. ^1H NMR and ^{31}P NMR analyses of the purified products revealed the presence of 3'-phosphoramidites as sole products and no traces of 2'-phosphoramidites could be detected (table 1).

We have already shown that in the deprotecting conditions used for the synthesis of RNA fragments with phenoxyacetyl base protecting groups, 5,6-dihydrouridine was stable enough (20). Ribothymidine is the RNA homologue of thymidine and consequently, it should be stable too. In contrast, pseudo-uridine was reported to be isomerized in a mixture of α and β anomers of both pyranose and furanose forms in acid and alkali and therefore stability tests were necessary before incorporating this nucleoside in synthetic RNA fragments. When pseudouridine was

Table 1. Proton NMR chemical shifts of protected nucleoside phosphoramidites in CD_3COCD_3.

Protons	Compounds						
	2a	2b	2c	2d	2e	2f	2g
H1′	6.27 & 6.24 d	6.05 & 6.08 d	5.97 & 5.91 d	6.05 & 6.00 d	4.85 m	6.16 & 6.11 d	6.05 m
H2′	5.40 m	5.00 m	4.61 m	4.64 m	4.58 m	4.69 m	4.53 m
H3′	4.77 m	4.60 & 4.52 m	4.61 m	4.60 m	4.45 m	4.54 m	4.40 m
H4′	4.57 & 4.47 m	4.51 & 4.41 m	4.45 m	4.41 & 4.35 m	4.27 m	4.31 m	4.23 m
H5′& H5″	3.71 & 3.53 m	3.57 m	3.72 m	3.63 m	3.57 m	3.55 m	3.45 m
H2	8.70 & 8.69 s						
H8	8.63 s	8.18 s					
H5			7.22 & 7.18 d	5.38 & 5.36 d			2.65 m
H6			8.65 & 8.64 d	8.06 & 8.04 d	7.71 d	7.81 q	3.81 m
CH_3			2.35s (Ac)			1.53 d (Thy)	
CH_2-pac	5.29 s	5.01 s					
CH_2-O-P	4.07 m	4.05 m	3.86 m	3.98 m	3.83 m	3.99 m	3.96 m
O-CH_3	3.91 s	3.92 s	3.92 s	3.92 s	3.90 s	3.90 s	3.91 s
CH_2 N-ethyl	3.16 m	3.13 m	3.05 m	3.11 m	3.06 m	3.11 m	3.06 m
CH_2-CN	2.90 & 2.75 t	2.91 & 2.70 t	2.83 & 2.72 d	2.87 & 2.73 t	2.83 & 2.71 t	2.89 & 2.70 t	2.87 & 2.64 t
N-CH_3	2.76 & 2.68 d	2.75 & 2.67 d	2.67 & 2.56 d	2.72 & 2.61 d	2.66 & 2.56 d	2.71 & 2.61 d	2.68 & 2.59 d
CH_3 N-ethyl	1.22 & 1.13 t	1.23 & 1.15 t	1.18 & 1.13 t	1.20 & 1.12 t	1.16 & 1.08 t	1.19 & 1.11 t	1.19 & 1.11 t
tbu silyl	0.93 & 0.91 s	0.94 s	1.07 & 1.06 s	1.04 s	1.04 s	1.04 s	1.05 s
CH_3 silyl	0.15 psd; −0.03 psd	0.185 s; 0.05 psd	0.38 s; 0.32 psd	0.30 & 0.29 s	0.31 & 0.27 s	0.29 & 0.26 s	0.30 s

treated in 28% aqueous ammonia, no trace of isomerization was detected even after 24 hours at 55°C. When treated with fluoride ion, pseudo-uridine showed no degradation. Acid treatment led to less than 10% of α-furanoside when pseudouridine was reacted in 80% aqueous acetic acid for 24 hr at 65°C. These conditions are very drastic in comparison to those used in the tRNA synthesis.

These phosphoramidites (**2a−g**) were stable in acetonitrile solution during more than one month and were fully compatible with automated synthesis. The protected cyanoethyl-N-ethyl-N-methylphosphoramidites **2a−g** (15 eq.) exhibited a high coupling yield (> 98%), with a reduced coupling time (2 min) on a 1 μmol CPG column. The RNA fragments were cleaved from the solid support upon treatment with a 3 : 1 mixture of 28% ammonia and ethanol for 1 hr at room temperature. The resulting solution was then heated for one hour at 55°C to eliminate the base protecting groups and gave 128 A_{260} of crude 2′-hydroxyl protected tRNA.

Triethylamine tris-hydrofluoride as a new reagent for the deprotection of tBDMS groups

When treated with tetrabutylammonium fluoride (TBAF) in THF we observed that 1 min was sufficient for a complete desilylation of a tBDMS protected nucleoside and that 24 hr were necessary to deprotect 20−35 mer oligoribonucleotides. For longer RNAs, we did not obtain a complete deprotection. This can be due in part to their solubilities in apolar solvent and a first improvement was the use of dimethylsulfoxide (31). However, much better results were obtained with a new deprotection reagent : neat triethylamine tris-hydrofluoride (TEA,3HF).

To compare the rate of the deblocking reaction, oligodeoxynucleotides including a single ribonucleotide in the middle of their sequence were used as model compounds. In figure 3A, the deprotection yield of a 42-mer polythymidylic acid containing a tBDMS protected uridine in central position is plotted versus time in various conditions. The curves show that the reaction is complete after 6 hr at room temperature in pure TEA,3HF. The results extended to tBDMS protected RNA fragments showed the validity of the model and confirmed the high efficiency of TEA,3HF.

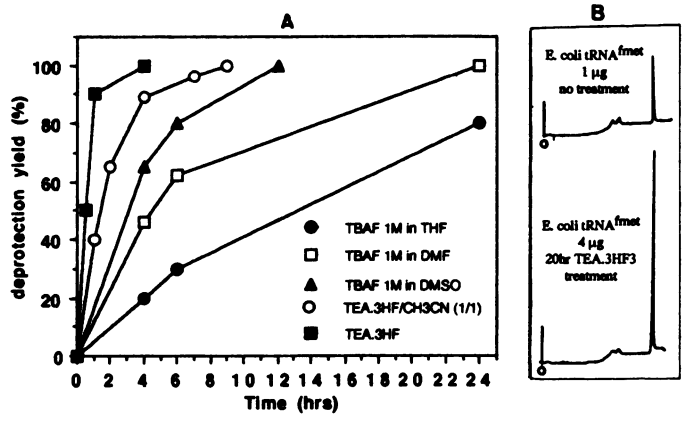

Figure 3. A) Deprotection yield of dT_{21}-U_{tBDMS}-dT_{21} against reaction time at room temperature. B) Natural *E.coli* $tRNA^{fMet}$ stability during TEA,3HF treatment. Analyses were performed on a monoQ (*Pharmacia*) column using a linear gradient: 0 to 1 M KCl, in 20 mM phosphate buffer (pH 6.0)/20% acetonitrile.

To check the absence of side reactions, such as base release or adduct formation on the natural and modified nucleosides during the TEA,3HF deprotection, each nucleoside was dissolved in the reagent and kept for two days at room temperature. The HPLC and proton NMR analysis showed no modification of the substrates (data not shown). In the same way, deprotected RNA fragments (synthetic 16 and 18 mers and natural $tRNA^{fMet}$ from *E.coli*) have been treated with TEA,3HF during 20 hr (Fig. 3B). Chromatographic analysis of the crude reaction mixtures showed no degradation of the fragments : in the case of tRNA only one peak was observed as expected. This weakly acid reagent is very convenient since no breakage of RNA was detected.

We have determined whether the modified nucleosides of alanine tRNA were deprotected without damage on shorter oligonucleotides. To demonstrate the integrity of the modified bases, shorter oligomer models were prepared : $^{5'}\psi CGA^{3'}$, $^{5'}T\psi CGA^{3'}$ and $^{5'}DGGGA^{3'}$. They were deprotected according to the procedure described for the $tRNA^{Ala/GGC}$ and analysed by

FAB mass spectrometry. This analysis shows the expected molecular weight and the sequence for all oligomers (data not shown). The integrity of the modified bases was also determined by an other way. The same oligonucleotides containing the modified nucleoside located at the 5'-end were ^{32}P-end labelled, treated by nuclease P1 and analysed on PEI plates. Two dimensional TLC showed that the 5'-^{32}P-nucleotide exhibited the mobility of authentic reference samples (data not shown).

Finally, the crude tRNA (128 A_{260}) resulting from ammonia deprotection was treated with TEA,3HF during 16 hr at room temperature. After a desalting step on G-25 Sephadex columns, the crude product (56 A_{260}) was purified using preparative polyacrylamide gel electrophoresis (PAGE) with 8 M urea and a long migration to have a better resolution. The tRNA$^{Ala/GGC}$ was observed by UV shadowing at the expected position (comparatively to the natural tRNA^{fMet} 77 mer marker) giving after elution, desalting and dialyses 2 A_{260} units of pure synthetic tRNA.

Subsequent HPLC analyse of the synthetic tRNA give one peak having the same retention time that the natural 77 mer tRNA^{fMet} taken as reference (data not shown).

Treatment with ribonuclease P1 and alkaline phosphatase of fully deprotected tRNA$^{Ala/GGC}$ gave rise to a complete digestion as shown by reverse phase HPLC of the resulting nucleoside mixture (data not shown). The peaks of the three minor bases are not clearly apparent from the base line but the tRNA$^{Ala/GGC}$ sequencing (see below) shows clearly that they are inserted at the right position.

3' and 5'-end labelling of the synthetic tRNA and nature of the terminal bases

To evaluate the efficiency of the synthesis, an aliquot of the crude deprotection mixture of tRNA was 5'-labelled with polynucleotide kinase and γ^{32}P-ATP, and analysed by PAGE. The autoradiography showed a band with the correct mobility but with less intensity than expected in regard of the yields obtained during the assembling step. This phenomenon was attributed to the particular sequence of the tRNA. Strong secondary interactions in the acceptor stem led to a double stranded self complementary fragment which is difficult to denature. The protruding 3'-end resulting from this secondary structure inhibits the action of polynucleotide kinase on the 5'-end. To confirm this hypothesis and consequently the structure of the synthetic tRNA, 3'-end labelling with RNA ligase and [^{32}P] pCp was used. This labelling was easily performed and the autoradiographies showed a major band with the expected mobility comparatively to labelled RNA length markers (Fig. 4A and 4B).

Analysis of the 3' and 5' terminal nucleosides of the tRNA was performed as previously described (20) and confirmed the presence of adenosine and guanosine, respectively.

Sequence analysis of the synthetic tRNA

Enzymatic sequencing

Figure 5A shows the PAGE autoradiography of the enzymatic sequencing of the synthetic tRNA. The quality of the alkaline degradation ladder (lane OH^-) gave a good indication of the efficiency of the deprotection treatment. Indeed, the results obtained with the synthetic fragment are similar to those obtained with RNA from natural source. The absence of blurry bands between the expected breaks of the molecule indicates that the 2' hydroxyl function was correctly deprotected. The tRNA structure obtained by the specific ribonucleases T_1, U_2, Phy M and *Bacillus cereus* was in agreement with the sequence. This direct RNA sequencing by specific enzymes is not easy because the hydrolysis is highly dependent on the concentration of the substrates and the experimental conditions. To obtain good results large quantities of tRNA are required to determine the best experimental conditions. Thus, to confirm the tRNA$^{Ala/GGC}$ sequence we searched for a more convenient method.

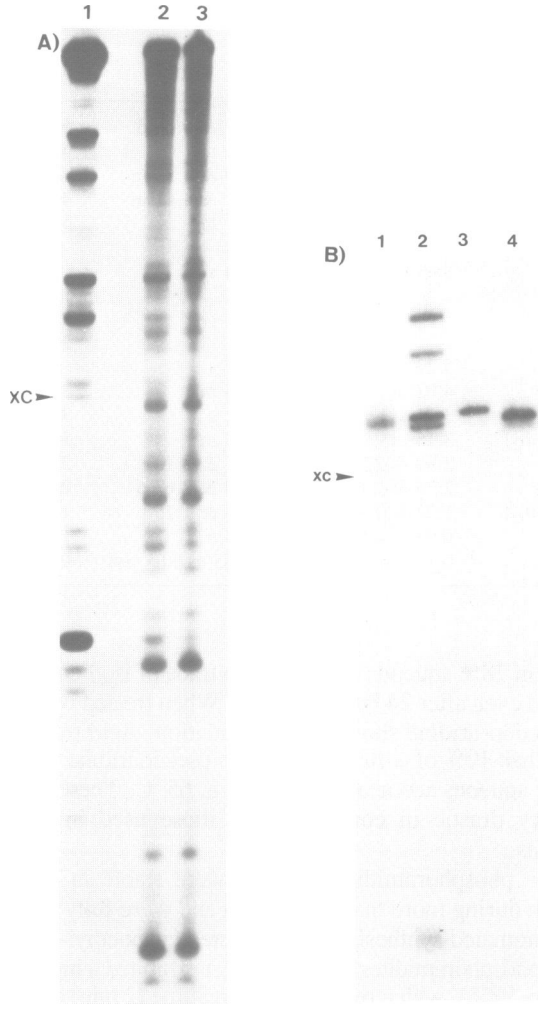

Figure 4. Gel electrophoresis of 3' end ^{32}P labelled products. A) 15% PAGE autoradiography. Lanes: 1: natural tRNA^{fMet}, 2–3: crude deprotected synthetic tRNA^{Ala}. B) 8% PAGE autoradiography. Lanes: 1: purified synthetic tRNA^{Ala}, 2: mixture of natural tRNA markers (90, 85, 77 and 76 mer corresponding to serine, tyrosine, f-methionine and valine *E.coli* tRNA respectively) and 35 mer RNA, 3: 77 mer *E.coli* tRNA^{fMet}, 4: 76 mer *E.coli* tRNA^{Val}.

Reverse transcription of the synthetic tRNA and chemical sequencing of the cDNA fragment

We found that the synthetic tRNA$^{Ala/GGC}$ could be transcribed into cDNA. A small amount was hybridized at its 3'-end with a complementary 16 mer oligodeoxynucleotide and copied with reverse transcriptase. The cDNA substrate was then amplified using polymerase chain reaction (PCR) and both chains were sequenced according to the Maxam and Gilbert method (Fig. 5B). Complete and reliable reverse transcription of the synthetic fragment was observed showing the integrity of the sequence which can be correctly read. Dihydrouridine, ribothymidine and

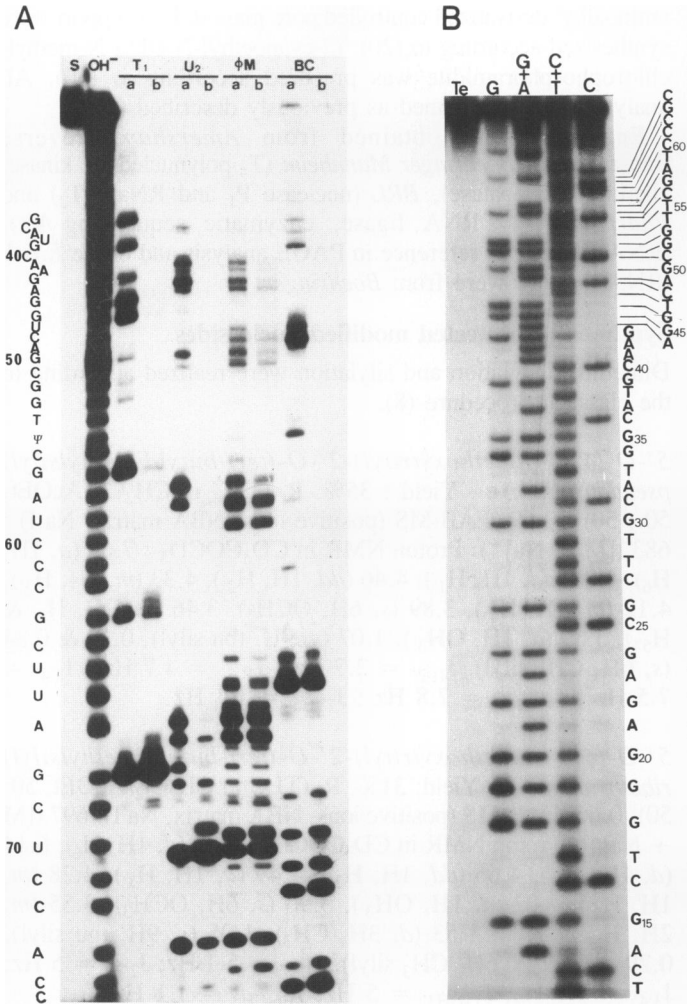

Figure 5. A) PAGE autoradiography of limited enzymatic digests of the 3′ end-labelled synthetic $tRNA^{Ala/GGC}$ performed on a 18% gel. Lanes: S: control sample, OH^- : alkaline hydrolysis ladder, nuclease T_1 : G specific, nuclease U_2 : A, nuclease Phy M : A + U, nuclease BC : U+ C. Nucleases were used at 0.25 unit (a) or 0.5 unit (b) per 0.05 μg tRNA in the standard conditions described in the experimental section. B) Chemical sequencing of 5′-end labelled DNA chain obtain by reverse transcription and PCR amplification of the $tRNA^{Ala/GGC}$. In this DNA chain, having the same sequence of the $tRNA^{Ala/GGC}$, dihydrouridine, ribothymidine and pseudouridine appear as a thymidine at the expected positions (number 17, 54, 55 respectively).

pseudouridine directed the synthesis of adenosine. This insertion is in agreement with the Watson−Crick hydrogen bonding between these modified bases and adenine.

Determination of the aminoacyl acceptance activity

The biological activity of the chemically synthesized $tRNA^{Ala/GGC}$ was compared to that of an *E.coli* tRNA mixture. The aminoacyl acceptance activity (672 pmol/A_{260}) was only obtained after strong denaturation of the synthetic product at 95°C for 1.5 min and renaturation at room temperature in the presence of KCl. In these reactions, the tRNA was present at the concentration of 0.2 μM (0.125 A_{260}). The alanyl-tRNA synthetase was added at different concentrations and the aminoacylation reaction was performed at 25°C for 30 min with ^3H alanine (32). At low enzyme concentration, the aminoacylation rate of the synthetic $tRNA^{Ala/GGC}$ was two times lower than the natural *E.coli* tRNA one's: the k_{cat}/K_m were

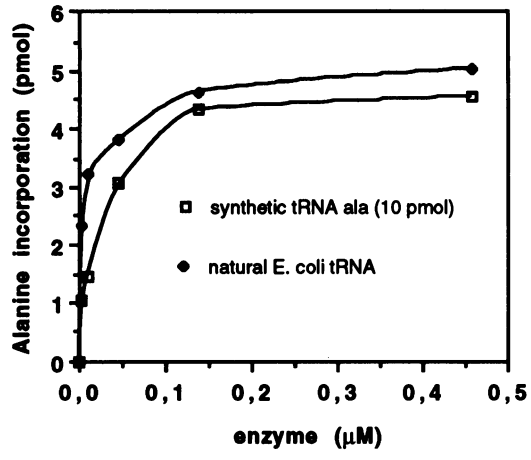

Figure 6 : Aminoacylation of synthetic $tRNA^{Ala/GGC}$ and natural crude *E.coli* tRNA. The synthetic substrate concentration was 0.2 μM (6.2 10^{-3} A_{260} unit in 50 μl) and the alanine-tRNA-synthetase were added to a final concentration ranging from 2.2 nM to 0.45 μM. The reaction was carried out for 30 min at 25°C.

estimated to be $6\times10^4M^{-1}s^{-1}$ and $12\times10^4M^{-1}s^{-1}$, respectively (Fig. 6). The maximum extent of aminoacylation was obtained for both assays at the same enzyme concentration (about 0.2 μM) for 30 min. The measured activity for the synthetic $tRNA^{Ala/GGC}$ was 672 pmol/A_{260} (42% of the theoretical activity, in our experimental conditions).

These biological results can be compared to those reported for synthetic RNA fragments. The 77 mer RNA chemically synthesized by Ogilvie had the sequence of an *E.coli* Ado-47 containing $tRNA^{fMet}$ species in which the modified nucleosides were substituted by their parent nucleosides (3). It exhibited a low aminoacyl acceptance activity (60 pmol/A_{260}). No kinetic data were given and surprinsingly this activity decreased after purification. Ohtsuka et al.(33) have reported the synthesis of a 77 nucleotide-long sequence related to the $tRNA^{fMet}$ from *E.coli*. This approach involved the enzymatic joining of short, chemically synthesized oligomers (3−10 nucleotides) and the synthetic molecules possessed 5% of the aminoacyl activity of the natural $tRNA^{fMet}$. It should be noted that so far only these two tRNAs-like molecules (3, 33) result from oligonucleotides totally prepared by chemical synthesis. However comparison of aminoacyl acceptance activities is difficult since these tRNA-like molecules have no modified nucleosides and they are aminoacylated by an enzyme of class I.

$tRNA^{Ala/GGC}$ presents recognition signals that are proximal to the aminoacid attachment site of alanyl-tRNA synthetase (class II enzyme). Alanyl-tRNA synthetase can aminoacylate a hairpin microhelix of seven pairs as well as small helices including disconnected complementary strands provided that these molecules contain a G3-U70 base pair located in the aminoacceptor helix of alanine tRNA (34,35). The aminoacylation level of the synthetic $tRNA^{Ala/GGC}$ is lower than that reported for the 24 mer synthetic microhelix (1160 pmol/A_{260}; corrected value against the length) but it is higher than that of the same microhelix obtained by transcription (580 pmol/A_{260}; corrected value) (6). However, these helices have a lower rate of aminoacylation than our synthetic $tRNA^{Ala/GGC}$ (relative to natural *E.coli* tRNA). The relative k_{cat}/K_M were reported to be 0.29, 0.02 and 0.03 for transcribed 35 mer minihelix, transcribed 24 mer microhelix and synthetic 24 mer

microhelix, respectively, against 0.5 for the synthetic $tRNA^{Ala/GGC}$ (6,36). It can be noted that the $tRNA^{Ala/GGC}$ charge is lower than that of the synthetic microhelix but the aminoacylation rate is much higher. With a strong denaturation followed by a rapid renaturation we obtain a good alanyl acceptance activity. At a lower temperature, below 80°C only a charge of 5% was obtained. This suggests that multimeric structures could be formed and partially recovered during renaturation. Consequently, the possibility that the conformation of tRNA was not optimal cannot be ruled out.

CONCLUSION

The methods reported herein may be considered as a very significant contribution to the problem of RNA chemical synthesis. The sequence was assembled on controlled pore glass support in a stepwise manner with an automated DNA synthesizer. The protected ribonucleotide building blocks used were N-acyl-5'-O-dimethoxytrityl-2'(*tert*-butyldimethylsilyl)ribonucleoside-3'-O-(2-cyanoethyl-N-ethyl-N-methyl) phosphoramidites, acyl being phenoxyacetyl for adenine and guanine and acetyl for cytosine. The coupling efficiency of these blocks was similar to that of those used in DNA synthesis. The elimination of the silyl group has been greatly improved by using a new reagent TEA,3HF. The tRNA sequence analysis has been obtained by reverse transcription of the synthetic tRNA into cDNA and sequencing of this cDNA. The level and the rate of the aminoacyl acceptance activity of the tRNA obtained with its modified nucleosides are good.

The chemical synthesis has distinctive advantages on the enzymatic ligation of natural tRNA fragments: rapidity and easiness of preparation, insertion of non usual nucleotides, mixed intrachain RNA-DNA, RNA-peptide, hybrid molecules which are not available through enzymatic means. The role of modified bases in the recognition of tRNAs by aminoacyl-tRNA synthetase is not well understood (37) and synthetic tRNA can be very helpful to solve this problem. However there are many biological areas in which tRNA-like structures are involved: aminoacid transport, primers for reverse transcriptase (HIV reverse transcriptase and primer lysine tRNA), involvment in DNA replication, cell division, translation repression, recognition by RNase P, expansion of the genetic code (selenocysteine, phosphoserine) (34). Chemical synthesis of oligoribonucleotides is necessary to obtain RNA fragments which contain non natural modified ribonucleotides.

EXPERIMENTAL SECTION

General

Solvents were distilled or dried before use. Ribonucleosides, tertio-butyldimethylsilylchloride (tBDMSCl), trimethylsilylchloride (TMSCl) and hexamethyldisilazane (HMDS) were purchased from *Fluka*. Dimethoxytritylchloride (DMTCl), triethylamine tris-hydrofluoride (TEA,3HF), tetrabutylammoniumfluoride in THF were from *Aldrich* and phenoxyacetic anhydride from *Tokyo Kasei*. Pseudouridine and ribothymidine were obtained from *Sigma*. Dihydrouridine, N-protected-5'-O-(4,4'-dimethoxytrityl)-2'-O-tertio-butyldimethylsilyl ribonucleosides with labile base protection (N^6-phenoxyacetyl adenine, N^2-phenoxyacetyl guanine, N^4-acetyl cytosine and uracil) and the ribonuclosides (phenoxyacetyl adenosine, acetyl cytidine or phenoxyacetyl guanosine) grafted on a long chain aminoalkyl derivatized controlled pore glass (CPG) support were synthesized according to (20). (2-cyanoethyl-N-ethyl-N-methyl) chlorophosphoramidite was prepared according to (30). All analyses were performed as previously described (20).

Enzymes were obtained from *Amersham* (reverse transcriptase), *Boehringer Mannheim* (T_4 polynucleotide kinase, alkaline phosphatase), *BRL* (nuclease P_1 and RNase T_2) and *Pharmacia* (T_4 RNA ligase, enzymatic sequencing kit). $tRNA^{fMet}$ used as reference in PAGE analysis and crude *E.coli* tRNA mixture were from *Boehringer*.

Synthesis of protected modified nucleosides

Dimethoxytritylation and silylation were realized according to the classical procedure (8).

5'-O-(4,4'-dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl) pseudouridine **1e** : Yield : 35%. R_f (TLC in CH_2Cl_2/AcOEt, 50 : 50) : 0.59. FAB-MS (positive ions, NBA matrix, Na I) : 683 ($[M + Na]^+$). Proton NMR in CD_3COCD_3 : 7.68 (*q*, 1H, H_6), 4.84 (*dd*, 1H, $H_{1'}$), 4.46 (*dd*, 1H, $H_{2'}$), 4.33 (*m*, 1H, $H_{3'}$), 4.12 (*m*, 1H, $H_{4'}$), 3.89 (*s*, 6H, OCH_3), 3.46 (*m*, 2H, $H_{5'}$ & $H_{5''}$), 3.39 (*d*, 1H, $OH_{3'}$), 1.07 (*s*, 9H, tbu silyl), 0.29 & 0.34 (*s*, 6H, CH_3 silyl). $J_{1'-2'}$ = 2.7 Hz ; $J_{2'-3'}$ = 4.7 Hz ; $J_{3'4'}$ = 7.5 Hz ; $J_{3'-OH3'}$ = 7.8 Hz ; $J_{1'-H6}$ = 1.1 Hz.

5'-O-(4,4'-dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl) ribothymidine **1f**: Yield: 31%. R_f (TLC in CH_2Cl_2/AcOEt, 50: 50): 0.66. FAB-MS (positive ions, NBA matrix, Na I): 697 ($[M + Na]^+$). Proton NMR in CD_3COCD_3 : 7.80 (*d*, 1H, H_6), 6.14 (*d*, 1H, $H_{1'}$), 4.66 (*dd*, 1H, $H_{2'}$), 4.49 (*q*, 1H, $H_{3'}$), 4.28 (*m*, 1H, $H_{4'}$), 4.02 (*d*, 1H, $OH_{3'}$), 3.90 (*s*, 6H, OCH_3), 3.55 (*m*, 2H, $H_{5'}$ & $H_{5''}$), 1.53 (*d*, 3H, CH_3), 1.05 (*s*, 9H, tbu silyl), 0.29 & 0.27 (*s*, 6H, CH_3 silyl). $J_{1'-2'}$ = 5.1 Hz; $J_{2'-3'}$ = 5 Hz; $J_{3'4'}$ = 4.7 Hz; $J_{3'-OH3'}$ = 5 Hz; J_{H6-CH3} = 1.1 Hz.

Synthesis of N-acyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)-ribonucleoside-3'-O-(2-cyanoethyl-N-ethyl-N-methyl)-phosphoramidites (2a–g)

N-acyl-5'-O-dimethoxytrityl-2'-O-tert-butyldimethylsilyl ribonucleoside (3 mmol) was dried by two coevaporations with anhydrous pyridine (5 ml) and THF (5 ml). The residue was dissolved in anhydrous THF (15 ml) under argon atmosphere. Dimethylaminopyridine (0.2 eq.—75 mg), N,N,N-ethyldiisopropylamine (4 eq.—2.05 ml) and (2-cyanoethoxy)-ethylmethylaminochlorophosphine were added through a rubber septum. The reaction mixture was stirred two hours at room temperature. The solution was then poured in a separatory funnel and diluted with ethyl acetate (150 ml). The organic layer was washed with saturated sodium bicarbonate (150 ml) and water (2×150 ml). The combined aqueous phases were extracted with ethyl acetate (150 ml) and the organic extracts were combined, dried over sodium sulfate and evaporated to dryness. The ribonucleoside phosphoramidites were further purified by silica gel HPLC using dichloromethane-hexane with 2% triethylamine as the eluent. After evaporation of the solvent, they were obtained as white foams and stored under argon atmosphere at −20°C. TLC were developed on silica gel Merck 60 F 254 using CH_2Cl_2/AcOEt/TEA, 50 :50 : 5.

Adenosine derivative **2a**: Yield: 70%, R_f: 0.69 FAB-MS (negative ions, NBA matrix): 974.3 ($[M-H]^-$). ^{31}P NMR in CD_3COCD_3: 149.3 & 149.5. ^1H NMR (table 1).

Guanosine derivative **2b**: Yield: 58%, R_f: 0.31. FAB-MS (negative ions, NBA matrix): 990.3 ($[M-H]^-$). ^{31}P NMR in CD_3COCD_3: 149.4 & 149.6. ^1H NMR (table 1).

Cytidine derivative **2c**: Yield: 77%, R_f: 0.66. FAB-MS (negative ions, NBA matrix): 858.8 ($[M-H]^-$). ^{31}P NMR in CD_3COCD_3: 148.3 & 150.1. ^1H NMR (table 1).

Uridine derivative **2d**: Yield: 75%, R_f: 0.60. FAB-MS (negative ions, NBA matrix): 817.5 ($[M-H]^-$). ^{31}P NMR in CD_3COCD_3: 148.9 & 149.8. ^1H NMR (table 1).

Pseudouridine derivative **2e**: Yield: 53%. R_f (TLC in CH_2Cl_2/AcOEt/TEA, 50 : 50 : 5): 0.58. FAB-MS (negative ions, NBA matrix): 817 ($[M-H]^-$). ^{31}P NMR in CD_3COCD_3: 147.7 & 149.3. ^1H NMR (table 1).

Ribothymidine derivative **2f**: Yield: 54%. R_f (TLC in CH_2Cl_2/AcOEt/TEA, 50 : 50 : 5) : 0.75. FAB-MS (negative ions, THGL matrix) : 831 ($[M-H]^-$). ^{31}P NMR in CD_3COCD_3: 149.1 & 149.3. ^1H NMR (table 1).

5,6-dihydrouridine derivative **2g**: Yield : 46%. R_f (TLC in CH_2Cl_2/AcOEt/TEA, 50 : 50 : 5): 0.81. FAB-MS (negative ions, THGL matrix): 819 ($[M-H]^-$). ^{31}P NMR in CD_3COCD_3: 148.8 & 149.2. ^1H NMR (table 1).

Solid phase syntheses

All syntheses were performed on a DNA synthesizer using 1 μmol of protected nucleoside grafted on a long chain alkylamine CPG support. The syntheses were then carried out following the standard cycle used in RNA synthesis (20) with a 2 min coupling step. A 15 fold excess of ribonucleoside phosphoramidites relative to CPG bound nucleoside was used (110 μl of 0.1 M solution). Average coupling yield, estimated by colorimetric measurement of the trityl cation, was better than 98%. The capping time with 0.3 M phenoxyacetic anhydride in lutidine/THF (1 : 8) and 6% methylimidazole in THF was 1 min.

tRNA deprotection

After removal of the trityl group the CPG support-bound oligonucleotide was transferred to a Wheaton vial. Freshly 3 : 1 saturated solution of 28% ammonia in ethanol (1.5 ml) was added, left for 1 hr at RT and then heated at 55°C for 1 hr. After cooling, the ammoniacal solution was collected and the support was washed with 50% ethanol-water (0.5 ml) and with EtOH (0.5 ml). The collected solvents were evaporated to give 128 A_{260} units of product.

To remove the 2′ silyl groups, 128 A_{260} were resuspended in 1.6 ml of neat TEA,3HF and kept at RT for 14 hr under strong agitation. After addition of 1.6 ml of H_2O, the deprotection mixture was desalted using a NAP-25 Sephadex column (*Pharmacia*). Fractions containing oligoribonucleotide were collected and lyophilized to give 56 A_{260} units.

Analysis of minor bases in small RNA fragments

The short model sequences $^{5'}\psi$CGA $^{3'}$, $^{5'}$ TψCGA $^{3'}$ and $^{5'}$ DGGGA $^{3'}$ were prepared and deprotected following the same chemical procedure as that used for the synthetic tRNA. The crude oligomers were purified on a reverse phase column using a linear gradient of 5−20% CH_3CN in 25 mM TEAA, (pH 7), over 20 min. To evaluate the integrity of all bases we have performed mass spectrometry sequence analysis of these oligoribonucleotides. Negative ions FAB-MS unambiguously confirmed the complete deprotection and the integrity of all the nucleotides.

FAB-MS (negative ions, THGL/NH_4OH matrix); ψCGA: 1222.2 ($[M-H]^-$), calc. 1223.1; TyCGA: 1542.6 ($[M-H]^-$), calc. 1543.0; DGGGA: 1609.5 ($[M-H]^-$), calc. 1609.9.

The ^{32}P-5′-end labelled oligoribonucleotide was treated at 37°C with nuclease P1 (5 units) for 2 hr and analysed by 2D TLC on PEI cellulose plates (*Schleicher & Schüll*) eluted successively by isobutyric acid/0.5 M ammonia 5 : 3 and isopropanol/concentrated hydrochloric acid/water 70 : 15 : 15. The migration of the radioactive spot was compared with that of a natural authentic sample.

Purification of fully deprotected tRNA

The synthetic tRNA (56 A_{260}) was taken up in 40 μl of 7 M urea loading buffer and applied to a 1.5 mm thick 18% denaturing polyacrylamide gel. After a long migration electrophoresis, the band located by UV shadowing was cut out and eluted by 5 ml of 0.5 M NaOAc (pH 5.5) at 4°C overnight. After filtration through a 0.22 μm filter the solution was dialyzed against H_2O (2×2 hr), 5 M NaCl (2 hr) and H_2O overnight at 4°C. The tRNA was then quantified by UV spectrometry (2 A_{260} units; 3200 pmol) aliquoted and lyophilized.

tRNA characterization

Analysis of the synthetic tRNA

The oligoribonucleotide (8 pmoles) was 5′-end labelled with 100 μCi of [γ-^{32}P]ATP and polynucleotide kinase; 3′-end labelling was made with T_4 RNA ligase and 50 μCi of [5′-^{32}P]pCp on the same scale. The radioactive fragments were purified by 8M urea 15% PAGE. The autoradiography showed the same mobility as *E.coli* $tRNA^{fMet}$. The labelled products were eluted in 1 ml of 0.5 M NaOAc (pH 5.5) for 16 hr at 4°C, desalted on Nensorb column and then dried in a Speedvac concentrator. The 5′ or the 3′ ends were determined after labelling of the tRNA, digestion with respectively nuclease P1 or RNase T_2 and analysis by 2D TLC as described above. The sequencing was performed by enzymatic hydrolysis of the 3′-labelled synthetic tRNA for 12 min at 55°C with RNase T1 (G specific), RNase U2 (A specific), RNase from Phy M (A + U specific) and RNase from *Bacillus cereus* (C and U specific). The sequence ladder (OH^-) was obtained by incubation for 6 min at 90°C of the 3′-end labelled tRNA in 0.05 M Na_2CO_3/$NaHCO_3$ buffer, (pH 9.5). The reaction was stopped by cooling at 0°C and all products were analysed by PAGE.

Total enzymatic hydrolysis of the synthetic tRNA and HPLC analysis of the resulting nucleosides mixture were performed as follows. The oligoribonucleotide (0.1 A_{260} unit) was reacted with nuclease P1 (5 units) for 2 hr at 37°C. Alkaline phosphatase (10 units) was added and the reaction was incubated for 2 hr at 37°C. Water (200 μl) was added and the reaction mixture was centrifuged for 10 min at 14000 rpm. The supernatant was directly injected onto a Li-Chrospher RP 18 (5 μm—125×4 mm) column eluted by 25 mM TEAA (pH 7) for 10 min followed by a 0 to 35% linear gradient of CH_3CN over 30 min.

In vitro reverse transcription of the synthetic tRNA, PCR amplification and chemical sequencing of the cDNA

Synthetic tRNA (1 fmol) was denatured at 95°C for 1 min in 50 μl buffer containing 10 mM Tris-HCl, (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 5 pmol of DNA primer

d(TGG TGG AGC TAA GCG G). After rapid cooling at 0°C, reverse transcriptase (20 units) was added and the mixture was incubated for 1 hr at 42°C. The 5'[^{32}P] labelled primer d(GGG GCT ATA GCT AGC) and Taq polymerase (*Cetus*, 2.5 units) were added. Twenty PCR cycles were carried out (92°C—1 min, 55°C—1 min, 72°C—1 min). The 5'[^{32}P] labelled product was purified by a 8% PAGE. The 76 mer labelled DNA was extracted from the gel(5.10^6 cpm), treated on a Nensorb column and then sequenced according to the *Maxam & Gilbert* procedure.

Aminoacylation assays

The aminoacylation reactions were carried out in a buffer containing 20 mM Tris-HCl, (pH 7.8), 0.1 mM dithiothreitol, 2 mM ATP, 7 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 33 mM alanine and [2,3-^{3}H] alanine (1500 Ci/mol final) and with 10 pmol of tRNA (estimated by UV spectroscopy : theoretically 1600 pmol per A_{260} unit). Reactions were initiated by adding various concentration of alanyl-tRNA synthetase (32). The reactions were performed at 25°C for 30 min, and stopped by precipitation on a Whatman filter pretreated with 10 µl of 25% TCA solution. The filters were washed 3 times in 5% TCA and one time in ethanol, ethanol-ether (50 : 50) and ether. The bound radioactivity on the dried filters was quantified by scintillation counting. Prior to aminoacylation, each synthetic sample was heated at 95°C in water for 1.5 min., then 150 mM KCl, 20 mM Tris-HCl, (pH 7.8), 0.1 mM EDTA were added and the sample was allowed to cool at room temperature. Positive controls (total *E.coli* tRNA, *Boehringer Mannheim*) were directly submitted to aminoacylation.

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