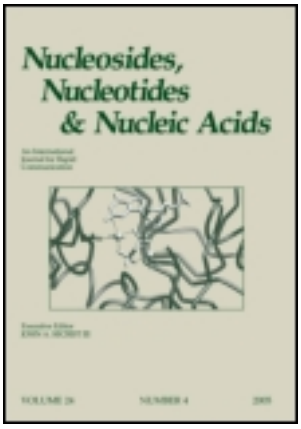

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Aminoacyl-tRNA Analogues; Synthesis, Purification and Properties of 3′-Anthraniloyl Oligoribonucleotides

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AMINOACYL-tRNA ANALOGUES; SYNTHESIS, PURIFICATION AND PROPERTIES OF 3'- ANTHRANILOYL OLIGORIBONUCLEOTIDES[1]

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ABSTRACT: Reaction of isatoic anhydride with adenosine, adenosine 5'-phosphate, oligoribonucleotides or with the *E. coli* $tRNA^{Val}$ led to attachment of an anthraniloyl residue at 2'- or 3'-OH groups of 3'-terminal ribose residue. No protection of the 5'-hydroxyl group or internal 2'-hydroxyl groups is required for this specific reaction. Anthraniloyl-tRNA which is an analogue of aminoacyl-tRNA forms a ternary complex with EF-Tu*GTP. The anthraniloyl-residue is used as a fluorescent reporter group to monitor interactions with proteins.

Modification of the ribose moiety of adenosine, guanosine or their 5'-phosphates with isatoic anhydride was reported to yield fluorescent 3'-*O*-anthraniloyl derivatives [1]. A similar reaction on terminal adenosine residue of $tRNA^{Phe}$ was found to provide exclusively a 3'-*O*-anthraniloyl-tRNA [2]. A stable fluorescent analogue of aminoacyl-tRNA is of interest as a spectroscopic label to study the interaction with enzymes of protein-synthesizing machinery. For such studies it is essential to know the precise position of the attachment of the fluorescent group on the utilized RNA substrate. From the previous work [2] it was not entirely clear if, and why the reaction of isatoic anhydride with tRNA takes place only on the 3'-terminal ribose, leaving the 5'-terminal hydroxyl group and the 2'-internal hydroxyl groups unmodified.

It also remains unclear why the anthraniloyl residue should be attached selectively to the 3'-position of the terminal ribose [1,2] when a mixture of both 2'- and 3'-isomers are the products of similar acylation reactions [3]. In order to clarify these questions we used; adenosine,

[1] This paper is dedicated to the late Professor Tsujiaki Hata

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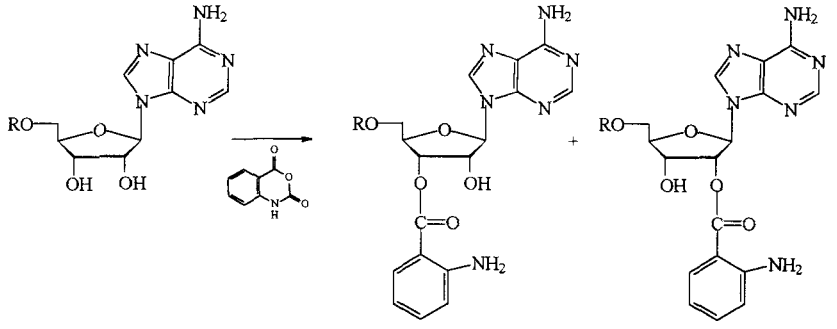
AMP, trimer CpCpA, 14mer $^{5'}$CpApGpApApUpUpCpGpCpApCpCpA$^{3'}$, a duplex derived from the acceptor stem of yeast tRNA^{Phe} composed of the 14mer $^{5'}$CpApGpApApUp UpCpGpCpApCpCpA$^{3'}$ and 10mer $^{3'}$GpUpCpUpUpApGpGpCpGp$^{5'}$, and *E. coli* tRNA^{Val} as substrates for RNA-modification with isatoic anhydride. The formed products were analyzed.

RESULTS

1. Reaction of isatoic anhydride with nucleosides, nucleotides and oligoribonucleotides.

As described in our previous communication [4], treatment of adenosine or adenosine 5'-phosphate with isatoic anhydride at pH 9.6 leads to formation of a mixture of monosubstituted 2'- and 3'-*O*-anthraniloyl-derivatives. The crude mixture of anthraniloyladenosine consists of 33 % 2'- and 67 % 3'-isomer (FIG. 1). Crystallization of this mixture from ethanol / diethyl ether (1/1, v/v) leads to the enrichment with 3'-isomer up to 98 %. In aqueous solutions however, both positional isomers reach equilibrium at the rate $k=1.15\times10^{-4}$ s^{-1} (p^2H 7.2, 4 °C) with a constant of K=0.43 [4]. A similar distribution of 30 % of the 2'-isomer and 70 % of the 3'-isomer was obtained for anthraniloylation of adenosine 5'-phosphate. The proportion of the isomers in the crude reaction mixtures reflects either the rate of substitution on 2'- or 3'-hydroxyl groups by isatoic anhydride, or the equilibrium of the transacylation reaction which involves the vicinal hydroxyl groups. Although the reaction of nucleosides, nucleotides or tRNA with isatoic anhydride was described as a selective acylation reaction yielding exclusively in 3'-*O*-anthraniloyl-derivatives [1,2], Hiratsuka [1] reports that in early stages of the reaction of isatoic anhydride with adenosine a minor fluorescent component with high R_f value on silica gel TLC is formed. It was concluded that this by-product was the 2'-isomer [1]. We also observed the formation of this more hydrophobic fluorescent product, especially when an excess of isatoic anhydride was used for the reaction with adenosine or adenosine 5'-phosphate. We identified this compound as a 2',3'-bis-*O*-anthraniloyladenosine or its 5'-phosphate. We did not find any of 5'-*O*-anthraniloyl-modified nucleoside when adenosine or guanosine were reacted with an excess of the reagent.

The anthraniloylation of 2'-deoxyadenosine with isatoic anhydride (FIG. 2) proceeded, under comparable conditions, with lower yield (33%) than the anthraniloylation of adenosine (42%). The crude reaction mixture provided 70 % of 3'-*O*-anthraniloyl-2'-deoxyadenosine and 30 % of 5'-*O*-anthraniloyl-2'-deoxyadenosine, as was determined by ^{1}H NMR analysis. This observation suggests that 2',3'-cis-diol function of the ribose facilitates the anthraniloylation, compared to the reaction on a better accessible 5'-hydroxyl group of the ribose residue.



R	Abbreviated names (% of isomer)		yield*(%)
H	3'-ant-Ado (67)	2'-ant-Ado (33)	42
PO_3^{-2}	3'-ant-AMP (70)	2'-ant-AMP (30)	36
CpCp-	3'-ant-CCA (75)	2'-ant-CCA (25)	18.0
$^{5'}$CpApGpApApUpUpCpGpCpApCpCp$^{3'}$-	3'-ant-14mer (70)	2'-ant-14mer (30)	40.0
-$^{3'}$UpCpUpUpApGpGpCpGp$^{5'}$§ $^{5'}$CpApGpApApUpUpCpGpCpApCpCp$^{3'}$-	3'-ant-10mer (63)/ 3'-ant-14mer (70)	2'-ant-10mer (37)/ 2'-ant-14mer (30)	20.0
$tRNA^{Val}$-CpC^{75}	3'-ant-tRNA (72)	2'-ant-tRNA (28)	14.3
$^{3'}$GpUpCpUpUpApGpGpCpGp$^{5'}$ $^{5'}$CpApGpApApUpUpCpGpCpApCpCp$^{3'}$-#	3'-ant-duplex	2'-ant-duplex	

* yield after chromatographic purification

§ guanosine residue present at 3'-end of 10mer is modified by isatoic anhydride

obtained by incubation of 3'(2')-ant-14mer with 10mer

FIG. 1. Reaction scheme for modification of ribose moiety with isatoic anhydride. The different functional groups R- of synthesized anthraniloyl derivatives are summarized.

To achieve modification of oligoribonucleotides and tRNA with isatoic anhydride the reaction was performed at low concentrations of nucleic acids in aqueous acetonitrile solutions (0.2 mM) using 50-fold excess of the reagent at pH 8.6. Examples for chromatographic purification of anthraniloyl-modified oligoribonucleotides are shown in FIG 3. Ion exchange chromatography on DEAE Sephadex separated anthraniloyl-CCA (peak II) form isatoic anhydride (peak I) and anthranilic acid (peak III, FIG. 3A). Remaining anthraniloyl-modified

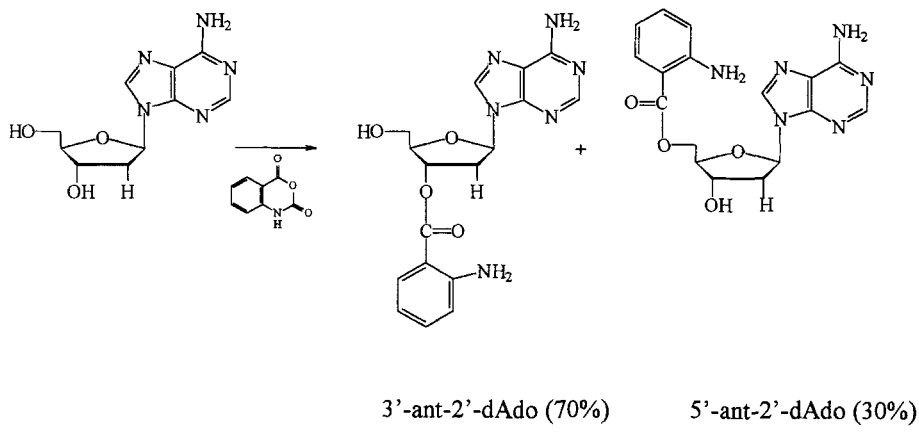


FIG. 2. Modification of 2'-deoxyadenosine with isatoic anhydride. Abbreviated names are given under the structures and the ratio of the isomers is in brackets.

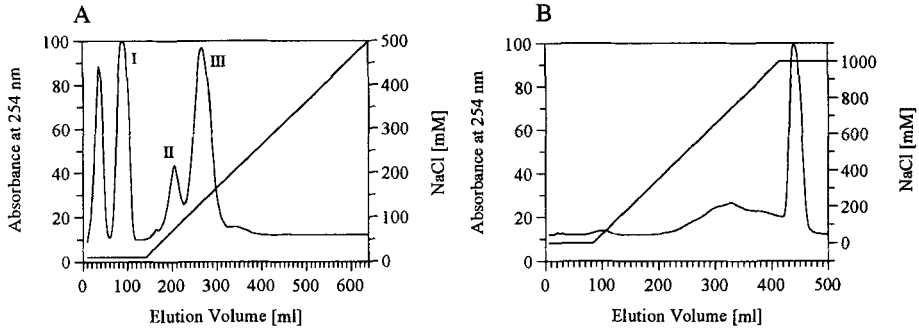


FIG. 3. Chromatographic purification of 2'(3')-ant-CCA (A) and 2'(3')-ant-14mer (B) on a DEAE Sephadex A-25. (A) fraction I - isatoic anhydride, fraction II - 2'(3')-ant-CCA, fraction III - anthranilic acid. 2'(3') ant-14-mer in (B) was eluted with 25% ethanol added to the final buffer containing 1 M NaCl.

oligomers and anthraniloyl-tRNA were precipitated with ethanol and then purified by hydrophobic chromatography on BD-cellulose (FIG. 3B). Traces of unmodified oligomers were eluted by salt gradient at pH 5.2 and the anthraniloyl-modified oligomers and anthraniloyl-tRNA were eluted by the same buffer containing 25 % of ethanol (FIG. 3B). Yields of the labelling reactions after isolation of products are summarized in FIG. 1.

2. Selectivity of anthraniloylation

For nucleoside composition analysis the purified anthraniloyl-RNAs were digested with nuclease P1 and alkaline phosphatase. The formed nucleosides mixtures were analyzed by

HPLC [5] with simultaneous detection by UV absorbance at 254 nm and by fluorescence emission at 427 nm (excitation for anthraniloyl residue was at 335 nm). Fluorescent nucleosides were identified by comparison of the retention times with model 2'- and 3'-*O*-anthraniloyl-nucleosides (TABLE 1). These models were prepared as described[1,4].

Chromatograms of nucleosides obtained by enzymatic digestion of 2'(3')-ant-CCA (A), 2'(3')-ant-14mer (B), 2'(3')-ant-14mer/2'(3')-ant-10mer (C) and 2'(3')-ant-tRNA (D) are shown in FIG 4. The main peaks correspond to the naturally occurring nucleosides C, U, G and A. Fluorescent peaks (market by asterisks) correspond to 2'-ant-Ado and 3'-ant-Ado. 2'-*O*-Anthraniloyladenosine is eluted at the retention time of 47 min, 3'-*O*-anthraniloyladenosine is eluted at the retention time of 56 min. In the chromatogram of 2'(3')-ant-14mer / 2'(3')-ant-10mer (FIG. 4C) there are two additional fluorescent peaks at 42 and 52 min. These correspond to 2'- and 3'-*O*-anthraniloylguanosine. Fluorescent nucleosides, originating from modification of the 2'-hydroxyl groups at the internal positions of the ribonucleotide chain were not detected. This can be used as evidence that isatoic anhydride under given conditions does not react with internal 2'-hydroxyl groups of RNA.

To estimate quantitatively the amount of anthraniloyl residues introduced into labelled RNAs, we used two different methods. First, the quantitative analysis of HPLC chromatograms was done by integration of the peaks using molar extinction coefficients given in TABLE 2.

Secondly, absorption spectra of ant-derivatives exhibit two maxima; i) at the range of base absorption at 260 nm and ii) at the range of anthraniloyl residue absorption at 335 nm. Thus, the extent of the anthraniloylation can be also estimated from the UV spectra by the ratio of A_{260}/A_{335}. Molar extinction coefficients of ant-derivatives at 260 nm and at pH 8.0 are given in TABLE 3. Molar extinction coefficient for anthraniloyl chromophore at 335 nm ($\varepsilon = 4.6 \times 10^3$) was taken from the literature[1].

In the case of 2'(3')-ant-CCA, where the single 3'-terminal adenosine is anthraniloylated, no adenosine peak at 25 min is present after the reaction with isatoic anhydride (FIG. 4A). Instead, in this sample two peaks belonging to 2'-ant-Ado and 3'-ant-Ado appear. The calculated proportion of cytidine residues to the sum of 2'- and 3'-ant-Ado residues is 2.2 : 1.0. The obtained ratio of A_{260}/A_{335} is in agreement with estimated value (TABLE 3) and confirms the presence of one anthraniloyl residue per one ant-CCA molecule. As determined by HPLC analysis of the 2'(3')-ant-14mer digest (FIG. 4B) a ratio of adenosine residues to the sum of 2'- and 3'-*O*-anthraniloyladenosine residues present in the molecule is 4.0 : 0.9,

TABLE 1. HPLC retention times, R_t and 2'- and 3'-isomer composition for anthraniloyl-derivatives of adenosine, cytidine, uridine and guanosine. Experimental conditions are described in Materials and Methods.

	R_t (min.) / proportion (%)*	
nucleoside, R_t (min.)	2'-ant-nucleoside	3'-ant-nucleoside
adenosine 25	47 (33)	56 (67)
cytidine 11	40 (33)	45 (67)
uridine 12	48 (23)	43 (77)
guanosine 19	42 (27)	52 (73)

* Retention times (R_t) were determined as described in Materials and Methods. Proportion of isomers in the isolated reaction product was calculated by peaks numeric integration.

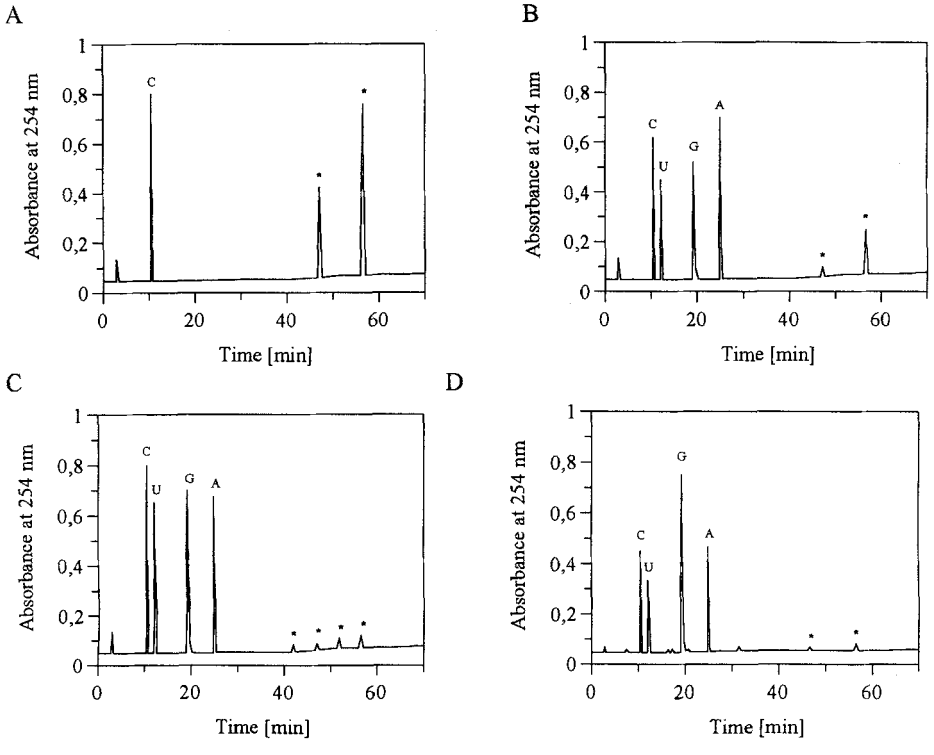


FIG. 4. HPLC chromatogram of the nucleosides mixture obtained by nuclease P1 and alkaline phosphatase digestion of; 2'(3')-ant-CCA (A); 2'(3')-ant-14mer (B); 2'(3')-ant-10mer / 2'(3')-ant-14mer (C); 2'(3')-ant-tRNA (D). Nucleosides were identified by retention times given in TABLE 1. Fluorescent peaks are indicated by asterisks.

TABLE 2. Molar extinction coefficients (ε) at λ 254 nm for A, C, U, G, 2'(3')-ant-Ado and for 2'(3')-ant-guanosine at pH 3.5.

Nucleoside	ε_{260} x 10^3 (M^{-1} cm^{-1})	ε_{254} x 10^3 (M^{-1} cm^{-1})
adenosine	14.24	13.97
cytidine	6.81	5.50
guanosine	13.35	16.00
uridine	10.17	9.24
2'(3')-ant-Ado	19.83	23.30
2'(3')-ant-guanosine	12.82	18.40

TABLE 3. Molar extinction coefficients at pH 8.0 (50 mM TRIS/HCl) and A_{260}/A_{335} ratio at UV spectra of anthraniloyl-modified derivatives.

Nucleoside, oligoribonucleotide	ε_{260} x 10^3 (M^{-1} cm^{-1})	estimated A_{260}/A_{335}	obtained A_{260}/A_{335}
ant-Ado	20.3 [1]		
ant-guanosine	20.7 [1]		
ant-CCA	32.3	6.9	6.0
ant-14mer	106.0	23	21
ant-14mer/ant-10mer	174.0	19	22
ant-tRNA	500.0 [2]	110	98

respectively. This indicates introduction of one anthraniloyl residue per one 14mer molecule and is compatible with the data given in TABLE 3.

Modification of the 14mer / 10mer duplex with isatoic anhydride leads to modification of 3'-ends of both strands (FIG. 4C). Quantitative analysis of both HPLC and A_{260}/A_{335} ratio confirms introduction of two anthraniloyl residues per one 14mer / 10mer duplex molecule and demonstrates a selective reaction exclusively on 3'-terminal nucleotide of each RNA strand. *E.coli* $tRNA^{Val}$ contains several naturally occurring modified nucleosides: s^4U, D, V, me^6A, me^7G, me^5U and ψ [6]. Thus, its modification with isatoic anhydride can lead to a more complex mixture of products. However, a chromatogram of the exhaustive enzymatic digest of ant-tRNA shows the only two fluorescent peaks at retention times corresponding to 2'- and 3'-ant-Ado. Several low intensity peaks with shorter retention times are characteristic for modified nucleosides (FIG. 4D). Spectroscopic analysis confirms introduction of one anthraniloyl residue per one molecule of tRNA (TABLE 3). However, the ratio of peaks area for adenosine

and for the sum of 2'- and 3'-ant-Ado suggests that less than one anthraniloyl residue per tRNA molecule is introduced. This discrepancy can be a result of an error due to the very low absorbance intensity of both 2'- and 3'-anthraniloyladenosine isomers in HPLC chromatograms (FIG 4D).

3. Properties of anthraniloyl-modified derivatives

Ternary complex formation between 2'(3')-ant-tRNA and *Thermus thermophilus* EF-Tu*GTP was monitored by polyacrylamide gel electrophoresis (FIG. 5). Formation of the complex between ant-tRNA and elongation factor Tu, indicates that anthraniloyl residue mimics aminoacyl residue when introduced to tRNA. The formation of the complex between shorter aminoacyl-tRNA analogues (2'(3')-ant-duplex, 2'(3')-ant-CCA) could not be detected by polyacrylamide gel electrophoresis.

Anthraniloyl residue is fluorescent with an excitation maximum at 335 nm and emission maximum at 427 nm. The quantum yield in water is relatively low (0.12) in comparison to that in ethanol (0.71) or in N,N-dimethylformamide (0.88) [1]. However, an aqueous buffer has to be used for fluorescence measurements in biochemical systems. The fluorescence spectrum of 2'(3')-ant-tRNA^{Val} is shown in FIG. 6. Addition of 2-fold excess of elongation factor Tu causes an increase of the relative fluorescence by about 85 % (c). This is an indication of a specific binding of 2'(3')-ant-tRNA^{Val} to EF-Tu*GTP.

Fluorescence spectra of 2'(3')-ant-Ado, 2'(3')-ant-AMP, 2'(3')-ant-CCA and 2'(3')-ant-duplex show the maximum at 427 nm as well, with only a small increase of the relative fluorescence (up to 10 %), which can be observed at μM concentrations of the fluorescent reporter upon addition of 2-fold excess of EF-Tu*GTP. This is an indication of a lower affinity of anthraniloyl-oligoribonucleotides to EF-Tu*GTP as compared to 2'(3')-ant-tRNA.

The relative fluorescence of 2'(3')-ant-tRNA does not increase neither in the presence of nucleotide free EF-Tu nor in that of EF-Tu*GDP (data not shown). Addition of an excess of Val-tRNA^{Val} into the ant-tRNA*EF-Tu*GTP ternary complex causes competitive formation of Val-tRNA^{Val}*EF-Tu*GTP ternary complex, thus fluorescence of the free substrate in the absence of EF-Tu*GTP drops to the level of 2'(3')-ant-tRNA (data not shown).

Fluorescence-monitored titration of 2'(3')-ant-tRNA solution with EF-Tu*GTP allows determination of dissociation constant (K_d) [7]. The relative fluorescence F/Fo of 2'(3')-ant-tRNA is plotted against concentration of EF-Tu*GTP (FIG. 7). Scatchard analysis of the binding data shows linear dependence of binding of one 2'(3')-ant-tRNA molecule per one EF-

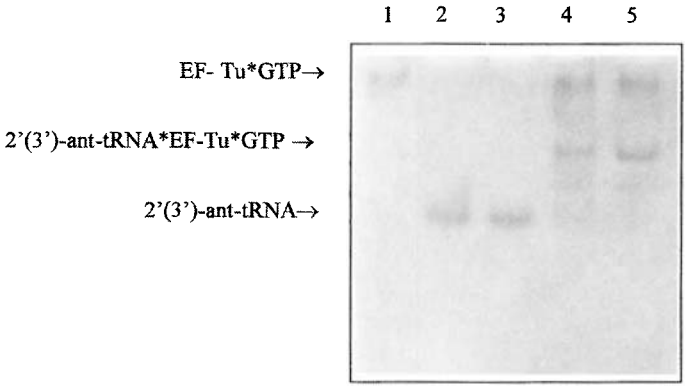


FIG. 5. Ternary complex formation between 2'(3')-ant-tRNA and EF-Tu*GTP monitored by polyacrylamide gel electrophoresis. EF-Tu*GTP (lane 1), 2'(3')-ant-tRNA (lane 2), Val-$tRNA^{Val}$ (lane 3). Incubation of EF-Tu*GTP with 2'(3')-ant-tRNA (lane 4) or with Val-tRNA (lane 5) was done at 37°C for 20 min.

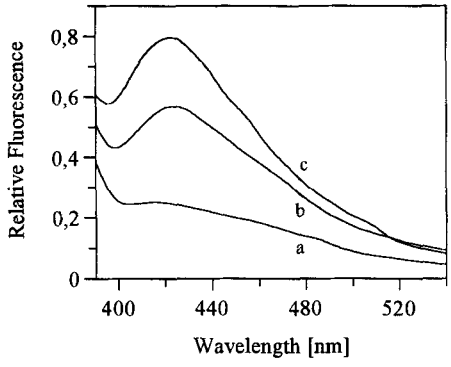


FIG. 6. Fluorescence spectrum of 2'(3')-ant-tRNA taken in 50 mM Tris/HCl pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$ at 4 °C at the excitation wavelength of 335 nm and emission wavelength of 390-540 nm. The relative fluorescence of 10 nM ant-tRNA (plot b) increases upon addition of 20 nM EF-Tu*GTP (plot c). Plot a - base line.

Tu*GTP complex. The value of the dissociation constant K_d for binding of 2'(3')-ant-tRNA to EF- Tu*GTP is 2.5 x 10^{-9} M. This K_d falls in the range of the values for binding of naturally aminoacylated tRNAs to EF-Tu*GTP [8].

DISCUSSION

We demonstrate in this work that specific anthraniloylation of RNA at the 3'-terminal cis-diol can be accomplished. The internal 2'-hydroxyl groups are not modified under the

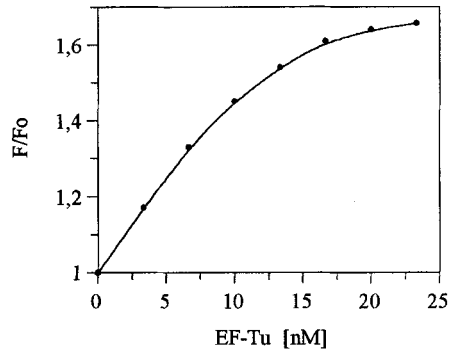


FIG. 7. Fluorescence-monitored titration of 10 nM 2'(3')-ant-tRNA with EF-Tu*GTP.

applied conditions. Chemical aminoacylation of tRNA was previously investigated by Gottikh and coworkers[9]. These authors activated the natural, protected amino acids with N,N dicyclohexylcarbodiimide and always obtained a substantial amount of a product aminoacylated at the internal 2'-hydroxyls besides 3'-terminal aminoacylated RNA [10]. The lack of methods for direct specific chemical aminoacylation of tRNA stimulated the development of a procedure in which an adenosine 5'-phosphate or a pCpA is first aminoacylated chemically and then linked to the 3'-end of the shortened tRNA by RNA ligase to reconstitute the complete aminoacylated CCA end [11]. This method is now generally used for preparation of tRNAs aminoacylated by unnatural amino acids [12]. RNAs esterified with natural α-amino acids are difficult to handle due to lability of the aminoacyl ester bond [13]. The method presented here provides an alternative for such applications in which a stable 3'-aminoacylated RNA is required and the structure or the aminoacyl side chain is not crucial.

In light of our present investigations it is remarkable, that isatoic anhydride is more specific for the 3'-terminal modification as compared to other activated amino acids which were used by Gottikh and coworkers [9]. The mechanism proposed in FIG. 8. depicts the possible role of the proximal hydroxyl group in the aminoacylation of nucleosides. The preference for the vicinal 2'- or 3'-hydroxyls as a site of aminoacylation, is primarily determined by their rather low pKa values [14]. Although sterically less hindered than the 2'(3')-hydroxyls, the 5'-hydroxyl is therefore less reactive in basic aqueous medium. Furthermore, after the attack of the alkoxy anion (2'or 3') to the activated carbonyl of the amino acid, the vicinal OH group can intramoleculary provide the proton to stabilise the orthoester transition intermediate. This intermediate then decomposes to either the 3'-isomer (a) or 2'- (b) of the aminoacylated

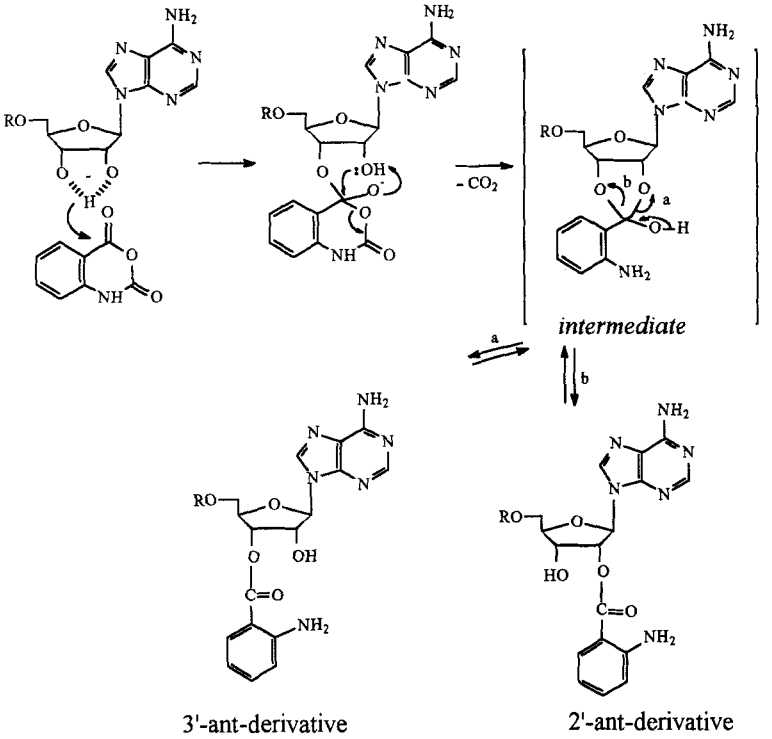


FIG. 8. Proposed mechanism of the reaction between the 2'(3')-cis-diol function of the ribose moiety of nucleotides or nucleosides and isatoic anhydride in basic aqueous medium.

ribose. In accordance with this mechanism we observe a higher reactivity of the cis-diol function as compared to the 5'-hydroxyl of adenosine, the 3'-hydroxyl of 2'-deoxyadenosine or the 2'-internal hydroxyls in oligoribonucleotides or tRNA. In variance with the previous reports[1,2] we further show that the product of the reaction of isatoic anhydride with ribonucleotides is a mixture of the 2'- and 3'-anthraniloylated isomers. However, the distribution of the isomers presented in FIG. 1, reflects the equilibrium of isomers formed by transacylation (FIG. 8)

The anthraniloylated tRNA forms ternary complexes with *Thermus thermophilus* elongation factor Tu, which leads to a change in fluorescence intensity of the anthraniloyl residue. This feature can be used to determine the dissociation constants of aminoacyl-tRNA*EF-Tu*GTP ternary complex. Although several methods were described to monitor this interaction [7,8,15,16] only methods based on spectroscopic measurements [7,16] provide Kd values, which were determined under true equilibrium conditions. Preparation of fluorescence-

labeled tRNAs for such measurements is rather tedious. The chemical aminoacylation of tRNAs with isatoic anhydride provides now an universal fluorescent reporter molecule for investigation of aminoacyl-tRNA function in protein biosynthesis.

MATERIAL AND METHODS

Analytical grade chemicals were obtained from Merck (Darmstadt, Germany. Adenosine, guanosine and uridine were modified with isatoic anhydride at pH 9.6 at 38 °C as described in [1,4]. Cytidine was modified analogously to [1]. Adenosine 5'-phosphate was modified with isatoic anhydride at similar conditions to give 2'(3')-*O*-anthraniloyladenosine 5'-phosphate isolated from the reaction mixture by means of DEAE Sephadex A-25 column chromatography as described previously (42 % yield) [4]. Elution of the column with higher salt concentration (55-110 mM) yielded 2',3'-bis-*O*-anthraniloyladenosine 5'-phosphate (16 % yield). LSI MS 584 (M-H); ^{1}H NMR δ ppm (D_2O/MeOH 60/40, at 23 °C): 8.70 (s, 1H, H-8), 8.14 (s, 1H, H-2), 7.92(d), 7.51(d), 7.30 (t), 7.18(t), 6.75(d), 6.64(d), 6.62(t), 6.36(t) (8H, anthraniloyl residues protons), 6.52 (d, $J_{1',2'}$=6.82 Hz, H-1'), 6.05 (t, J=6.13 Hz, H-2'), 5.93 (br s, 1H, H-3'), 4.72 (br s, 1H, H-4'), 4.20 (br s, 1H, H-5',5''); TLC (n-propanol / NH_4OH / water, 6/3/1, (v/v/v): R_f= 0.46 (AMP R_f= 0.22, 2'(3')-ant-AMP R_f= 0.40).

Oligoribonucleotides were synthesized by H-phosphonate approach on Gen Assembler Plus (Pharmacia, Sweden) [17,18]. *E.coli* $tRNA^{Val}$ was obtained as described in [19]. Nucleotide-free EF-Tu, EF-Tu*GDP and EF-Ts from *Thermus thermophilus* (EF-Ts) were prepared according to [20]. EF-Tu*GTP was obtained by incubation of EF-Tu*GDP in 50 mM Tris/HCl pH 7.5, 50 mM NH_4Cl and 10 mM $MgCl_2$ with pyruvate kinase (1μl), EF-Tu*GDP (100 μM), phospho(enol)pyruvate (5mM), GTP (1 mM) and EF-Ts (10 μM) for 20 min. at 37 °C. *Rabbit muscle* pyruvate kinase (1 mg / 1 ml glycerol) and *Penicillium citrinum* nuclease P1 (1mg / 500 μl 25 mM Tris/HCl pH 4.7) were obtained from Boehringer (Mannheim, Germany). Alkaline phosphatase (4.5 *units* / ml 200 mM Tris/HCl pH 7.6) was from Sigma, Israel. Valyl-tRNA synthetase was isolated as described in [19].

Modification of oligoribonucleotides with isatoic anhydride was performed in water / acetonitrile solution at 38 °C. Typically, 0.2 mM aqueous solution of oligoribonucleotide was adjusted to pH 8.6 with 0.1 N NaOH, then solution of isatoic anhydride in acetonitrile (15 mg/ml) was added up to the concentration of 10 mM. The pH was maintained at 8.6 by titration with 0.1 N NaOH and the reaction was allowed to proceed for 3 h at 38 °C. After the cooling in water / ice bath the reaction mixture was acidified to pH 5 with 0.1 N HCl. The products of anthraniloylation were then isolated and purified as follows:

2'(3')-ant-CCA reaction mixture was diluted twice with water and then loaded on DEAE Sephadex A-25 column (4 cm x 1 cm). Elution was performed with buffer A (20 mM sodium acetate pH 5.2, 10 mM $MgCl_2$ and 10 mM NaCl), until isatoic anhydride was eluted from the column (150 ml). Then 2'(3')-ant-CCA was eluted with the gradient from 10 mM to 500 mM NaCl. Fractions eluted with 60-90 mM salt concentration were desalted on Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA, USA). Thus sample was suspended on the cartridge, the salts were eluted with water and then the product was released from the cartridge by washing with 50 % aqueous methanol. ^{1}H NMR spectrum was recorded at 500 MHz on a Bruker DRX 500 spectrometer (Bruker, Karlsruhe, Germany) in 50 mM borate buffer p^{2}H 7.5, 50 mM KCl, 10 mM $MgCl_2$ at 3 °C. Resonances indicating the ant-residue presence are as follows: for 3'-ant-CCA 8.42 (H8 of A), 8.07 (H2 of A), 6.10 (H1' of A, $J_{1',2'}$ = 6.4 Hz), 4.69 (H2' of A), 5.45 (H3' of A), 4.49 (H4' of A); for 2'-ant-CCA the respective

values are as follow: 8.32, 8.02, 6.26 ($J_{1',2'}$ = 3.4 Hz), 5.52, 4.67 and 4.32. For comparison the following values were collected for CCA: 8.55, 8.20, 6.15 ($J_{1',2'}$ = 4.5 Hz), 4.53, 4.43, 4.34, respectively. The ratio of 3'- to 2'-ant-CCA isomer was 1.9. The assignment was done with the help of 2D COESY and NOESY spectra described elsewhere.

2'(3')-ant-14mer, 2'(3')-ant-14mer / 2'(3')-ant-10mer or 2'(3')-ant-tRNA were isolated from the reaction mixture by ethanol precipitation. The reaction mixture was treated with 2.5-fold volume of cold ethanol, left for one hour at -20 °C and centrifuged at 10000 g at 0 °C. The pellet was washed with 70 % aqueous ethanol, dried under vacuum, diluted with water and desalted by gel filtration on Bio-gel P6 column (3 cm x 50 cm). Fractions with UV absorption at 254 nm were pooled, concentrated and loaded onto BD-cellulose column (4 cm x 1 cm). Elution of parent oligomers was performed with gradient of salts from 0 M to 1 M NaCl in 20 mM sodium acetate pH 5.2 and 10 mM $MgCl_2$, except for 2'(3')-ant-tRNA, where the preliminary elution was done isocratically with 1 M NaCl. Elution of hydrophobic anthraniloyl-modified oligoribonucleotides and 2'(3')-ant-tRNA was achieved with 1 M salts in 20 mM sodium acetate pH 5.2 and 10 mM $MgCl_2$ and ethanol mixture 3/1 (v/v). Pooled fractions were desalted on Bio-gel P6 column, concentrated and kept at -20 °C.

For enzymatic digestion anthraniloyl-modified oligoribonucleotides or 2'(3')-ant-tRNA (0.2 A_{260}) were dissolved in 100 mM NH_4Cl pH 5.3 (5 µl), kept at 100 °C for 2 min and in water / ice bath for 5 min. Reaction mixture was treated with 20 mM zinc acetate (0.4 µl) and nuclease P1 (1 µl) then incubated for 1 hour at 37 °C. Then alkaline phosphatase (1 µl) and 500 mM Tris/HCl pH 7.9 (2.6 µl) were added and the digest was further incubated for 1 hour.

HPLC analysis was performed with a Beckman HPLC System Gold using Supelcosil LC-18S column, 250 mm x 4.6 mm, with a Supelcosil guard column, (Supelco, Bellafonte, USA). Elution was done at 9 °C, with a flow rate of 1 ml / min, with the gradient of 5 mM sodium phosphate buffer pH 3.5 and methanol as follows: 0 min to 5 min - 2.5 % methanol, 5 min to 10 min -10 % methanol, 10 min to 65 min - gradient of methanol from 10 % to 70 %, 65 min to 70 min - 70 % methanol, then 70 min to 80 min -70 % to 2.5 % methanol. Detection was monitored by UV at 254 nm and additionally by fluorescence at excitation wavelength of 335 nm and emission wavelength of 427 nm on HPLC monitor (Simadzu RF-535, Kyoto, Japan). Proportion of nucleosides was calculated from peaks area. Corresponding absorption coefficients were corrected for pH 3.5 and the composition of the solvent.

Fluorescence measurements were performed at 4 °C with Aminco SPF-500 (SLM 8000) spectrofluorometer (Travenol Laboratories, Silver Spring, USA) equipped with a 250-W xenon lamp and controlled by Lambdac software from Sopra (Büttelborn, Germany). Fluorescence emission spectra were monitored between 390 and 540 nm, band-pass 10 mm, with the excitation wavelength of 335 nm, band-pass 5 nm. Samples of anthraniloyl-derivatives were used at 1 µM concentration, EF-Tu*GTP at 2 µM concentration, and 2'(3')-ant-tRNA at 10 nM concentration in 50 mM Tris/HCl buffer pH 7.5, 50 mM NH_4Cl and 10mM $MgCl_2$. 2'(3')-ant-duplex was obtained by incubation of the mixture of 2'(3')-ant-14mer and 10mer at 60 °C for 2 min and slowly cooled down to room temperature.

The dissociation constant K_d of 2'(3')-ant-tRNA for its interaction with EF-Tu*GTP was determined by fluorescence spectroscopy [7], using an AMINCO SPF-500™ (Aminco Silver Spring) ratio spectrofluorometer with an excitation wavelength of 335 nm and emission wavelength of 427 nm. The titrations of 2'(3')-ant-tRNA (10 nM) were performed at 5 °C by stepwise addition of 3.3 - 23.1 nM of EF-Tu*GTP in 50 mM sodium borate, pH 7.5, 50 mM NH_4Cl and 10 mM $MgCl_2$. Based on the change of the relative fluorescence upon addition of EF-Tu*GTP, the K_d value was calculated [7].

Ternary complex formation between EF-Tu, GTP and 2'(3')-ant-tRNA was observed by polyacrylamide gel electrophoresis. 2'(3')-ant-tRNA (2.5 μM) was incubated with EF-Tu (0.5 μM) and GTP (200 μM) in 50 mM Tris/HCl pH 7.5, 50 mM KCl, 50 mM NH_4Cl and 7 mM $MgCl_2$ for 20 min at 37 °C. PAGE was performed on 6% polyacrylamide gel in 25 mM Tris acetate buffer pH 7.2 containing 5 mM magnesium acetate on Mighty Small vertical Slab Unit (Hoefer Scientific Instruments, San Francisco, USA) at room temperature for 1h at 100 V. The running buffer was 25 mM Tris acetate buffer pH 7.2 containing 5 mM magnesium acetate. Staining of the gel was achieved by subsequent Coomassie and toluidine blue dyes. As a control the same experiment was performed with [^{14}C] Val-tRNA^{Val} prepared as described elsewhere.

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