A Total Synthesis of Yeast Alanine Transfer RNA

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Success in the synthesis of DNA by Khorana et al. was accomplished by the wise combination of organic synthesis with enzymic synthesis by DNA ligase using template techniques and double labeling for the preparation and examination of synthetic products. However, the synthesis of ribonucleic acid has not been progressing so quickly and smoothly. The presence of a 2′-hydroxyl group in the ribose moiety of ribonucleotide molecules causes great difficulty and complexity in chemical synthesis, so that only nonaribonucleotides have been prepared by the diester method.[1] With the developments of the triester method and HPLC techniques, an eicosaribonucleoside nonadecaphosphate has been synthesized.[2]

The discovery and application of T_4 RNA ligase[3] results in a marked advance in the synthesis of larger oligoribonucleotides. Ikehara and Ohtsuka claimed that they had succeeded in the synthesis of *E. coli* formylmethionine tRNA by using organic synthesis combined with enzymic synthesis with T_4 RNA ligase.[4] Nevertheless, this work was only a model synthesis because the synthetic product lacked all modified ribonucleotides present in the natural one. The synthetic product was reported able to weakly accept formylmethionine (4–6%), but its activity in a protein biosynthesis system was not reported. As we know, the amino acid accepting activity is not specific and the mere mixture of two half-molecules obtained by cleaving the tRNA by RNase T_1[5] is already able to exhibit amino acid accepting activity,[6,7]

In China, after the total synthesis of crystalline bovine insulin, our organic chemists, biochemists, and biologists decided to attack the problem of the synthesis of nucleic acid. The study on nucleic acid chemistry was begun in 1968 under the Collaboration Committee of Nucleic Acid Synthesis organized by Acadamia Sinica consisting of six collaboration groups: Shanghai Institute of Biochemistry, Shanghai Institute of Cell Biology, Shanghai Institute of Organic Chemistry and Beijing Institute of Biophysics of Acadamia Sinica, the Department of Biology of Beijing University, and Shanghai Chemical Reagent Factory No. 2. At that time, yeast alanine transfer ribonucleic acid ($tRNA_y^{Ala}$) was the smallest ribonucleic acid whose structure had been completely elucidated[5,8,9] (Figure 1). Hence $tRNA_y^{Ala}$ was chosen as the target of our synthesis. We jointly carried out the synthesis of this ribonucleic acid until November, 1981.

Synthesis of Modified Ribonucleosides and Ribonucleotides

In the yeast alanine tRNA molecule there are nine modified ribonucleotides. They are dihydrouridine phosphate (DMP) (all nucleotides mentioned in this article except otherwise stated are 3′-ribonucleotides), ribothymidine phosphate (TMP), pseudouridine phosphate (ψMP), inosine phosphate (IMP), *N*-methylinosine phosphate (m^1IMP), *N*-methylguanosine phosphate (m^1GMP), and *N*,*N*-dimethylguanosine phosphate (m_2^2GMP), among which DMP and ψMP each occur twice. Classical synthesis of all these modified ribonucleotides were known, but some of them are not suitable for preparative purposes. We had to develop modified methods for the syntheses of TMP and ψMP as well as their protected derivatives. All known methods of organic synthesis of TMP[10,11] were based upon the phosphorylation of ribothymidine but were tedious and gave low yields. A Mannich reaction had been used for the preparation of thymidine from uridine.[12] By direct application of Mannich reaction to UMP with formaldehyde and piperidine, we got a Mannich base of UMP (45% yield),[13] which was catalytically hydrogenolyzed into TMP.

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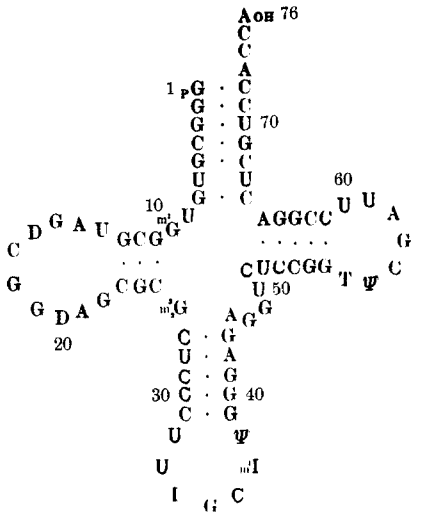

Figure 1. Structure of yeast alanine tRNA.

Pseudouridine occurs naturally in human urine from which it was isolated. Chemical syntheses of ψMP and its protected derivative were briefly mentioned by Ohtsuka et al.,[14] but no details were given. We have prepared ψMP and its derivatives by converting pseudouridine first into 5′-*O*-monomethoxytritylpseudouridine and then into 5′-*O*-monomethoxytritylpseudouridine 2′,3′-cyclophosphate on reacting with *N*-oxyphosphoryl morpholide dichloride.[15] Under the action of ribonuclease A, the cyclophospate was cleaved to produce 5′-*O*-monomethoxytritylpseudouridine phosphate, which could be either further benzoylated into 2′-*O*-benzoyl-5′-*O*-monomethoxytritylpseudouridine phosphate (overall yield 44%) or deblocked to obtain ψMP.

Protection of Functional Groups of Nucleosides and Nucleotides

Protection of 5′-OH by Tritylation and Monomethoxytritylation. In the organic synthesis of oligonucleotides or nucleic acids, a part of the functional groups of the starting material—nucleoside or mononucleotide as well as the intermediate oligonucleotide fragment—should be appropriately protected in order to prevent them from taking part in later reactions. The usual way to block the 5′-hydroxyl group of the pentose moiety is to etherify with trityl chloride (TrCl) or its mono-*p*-methoxy or di-*p*-methoxy derivative (MeOTr or $(MeO)_2$Tr derivative). Khorana and his co-workers[16] had prepared 5′-*O*-MeOTr derivatives of ribonucleotides by two ways, but both ways were tedious and not practical for larger scale preparations. We have modified Khorana's method of direct tritylation of ribonucleotides simply by purifying the crude Tr and MeOTr ribonucleotide products through repeated crystallizations from appropriate solvents. Thus pure 5′-*O*-Tr or 5′-*O*-MeOTr derivatives of ribonulceotides were easily prepared in larger batches with good yield.[17] The conditions (molecular ratios, solvents, temperature, time, etc.) of the monomethoxytritylation of CMP, UMP, and AMP, as well as the 5′-O-tritylation of *N*-acetylriboguanosine phosphate and the choice of the solvent systems for the crystallization of these protected ribonucleotides have been carefully studied.

Acylation of Ribonucleosides and Ribonucleotides with Acylimidazoles and Mechanism of the Reactions. In the synthesis of oligo- or polynucleotides, the hydroxyl and amino groups of the nucleotides are usually protected by acyl groups, among which acetyl, isobutyryl, benzoyl, and anisoyl groups are generally employed. The well known reagent for acylation is carboxylic acid anhyride with pyridine. However, it reacts too slowly; the crude product is usually dark colored; and the yield is not satisfactory because of the formation of 2′,3′-cyclophosphate in the case of ribonucleotide. Khorana et al.[18] proposed the use of *N*-tetraethylammonium salt of the carboxylic acid and anhydrous medium to depress the formation of cyclophosphate, but the process is time consuming. In 1971, Holy and Soŭcek[19] employed benzoylcyanide (BzCN) as a benzoylating agent for nucleotides. This reagent indeed reacts rapidly but is toxic. In the early sixties, Staab[20] tried acylimidazole as an acylating agent for sugars, alcohols, amines, etc. Cramer et al.[21] used benzoylimidazole (BzIm) for N-benzoylation of cytidine derivatives.[22] Since 1972 we have systematically studied the reaction of BzIm with ribonucleotides. The results are very interesting and useful.[23-27]

The reaction of acylimidazoles with ribonucleotides varies with the conditions and with the base catalyst used. With BzIm as acylating agent and UMP as a model substance to be acylated, we employed ^{31}P and ^{1}H FT NMR to follow up closely the course of the reaction in *N*,*N*-dimethylformamide (DMF) and were able to trace the paths of the benzoylation of UMP with BzIm under various conditions. The results indicate clearly that U_{pbz} is the active intermediate in these reactions. Although the mixed anhydride as a possible active intermediate in the 2′,3′-cyclophosphate formation had been suggested by earlier workers,[28,29] it was never actually detected. Now, for the first time its appearance and disappearance in the cyclophosphate formation were spectrometrically determined. The details of all these results will be published elsewhere.[30]

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It should be pointed out that in the presence of a strong base the formation rate of the mixed anhydride is depressed; the formation of the cyclophosphate is also inhibited.

We have compared the usefulness of benzoic anhydride–pyridine, benzoyl cyanide, and *N*-benzoylimidazole as benzoylating agents for blocking the functional groups of ribonucleosides and ribonucleotides and their derivatives.[23] In our experience BzIm is the best choice. In addition to its advantages of quick reaction, easy manipulation, high yield, and lower toxicity than the other reagents, it does not cause the replacement of preexisting acyl group.[31]

The Activation of the Phosphate Group of Nucleotides in the Oligonucleotide Synthesis

In the chemical synthesis of oligonucleotides by the diester method and sometimes also by the triester method, the internucleotide linking is usually achieved by using an activating reagent such as *N,N'*-dicyclohexylcarbodiimide (DCC), mesitylenesulfonyl chloride (MS), triisopropylbenzenesulfonyl chloride (TPS), or the imidazole, triazole, or tetrazole derivative of MS. The mechanisms of the activation of mononucleotides by DCC and TPS have been studied by Todd,[32] Khorana et al.,[33] Eckstein et al.,[34] and Zarytova et al.[35] Zarytova and her co-workers applied ^{31}P NMR techniques to study the kinetics and mechanism of the reactions of thymidine 5'-phosphate (pdTac) with TPS and DCC, respectively, and concluded that the first step of the reaction is the formation of a mixed anhydride of pdTac with triisopropylbenzenesulfonic acid, but this reactive intermediate has never been detected. The second step is the formation of metaphosphate or its pyridinium salt, which has also not been recognized in the ^{31}P NMR spectrum at the beginning of the reaction. Recently we have investigated the mechanism of the activation of pdTac by TPS or DCC with application of the ^{31}P NMR method to follow the course of the reaction. Our results show that the "metaphosphate" is most likely not formed directly from the hypothetical mixed anhydride at the beginning of the reaction of pdTac with TPS and DCC but rather produced at the end of the activation course when an excess of the activating agent is present.[36]

Synthesis of Yeast Alanine Transfer Ribonucleic Acid

Chemical Synthesis of Oligoribonucleotides. Holley and his co-workers had first determined the nucleotide sequence of $tRNA_y^{Ala}$ molecule.[5,8] Later on, the structure was further corrected by Merril[9a] and Penswick et al.[9b] Now it is known that $tRNA_y^{Ala}$ consists of 76 nucleotides (Figure 1). Using RNase T_1, Penswick and Holley were able under definite conditions to cleave the whole molecule at GpC of the anticodon loop into two half-molecules—3'- and 5'-terminal half-molecules consisting of 41 and 35 ribonucleotides, respectively.[5]

After some preparatory work we decided to concentrate our effort first to synthesize the 3'-terminal half-molecule. As a model, we had prepared the 3'-terminal quarter-molecule fragment AUUCCGGACUCGUCCA.[37] Then the nonadecanucleotide **6**[38] and the 3'-terminal half-molecule were synthesized. Afterwards, the 5'-terminal half-molecule and finally the whole molecule were successfully synthesized. Since total organic synthesis of the whole $tRNA_y^{Ala}$ molecule is not yet realistic at present, we decided to adopt Khorana's combination method, but with modifications using organic synthesis for smaller oligonucleotide fragments and enzymic synthesis for linking smaller fragments into larger ones. We employed the general diester method for organic synthesis of oligonucleotides ranging from dinucleotides to octanucleosideheptaphosphate, AUUCCGGA (**7**), but largely tetranucleoside triphosphates and tetranucleotides. The largest fragment prepared by the organic method and used for enzymic synthesis was the hexanucleoside pentaphosphate AGDCGG (**8**).[39]

We started from an O- and N-protected 3'-mononucleotide (called 3'-phosphate component) and a mononucleotide or a nucleoside whose 5'-OH group was free but whose other functional groups were fully protected (called 5'-OH component). A Tr or MeOTr group is usually used for the protection of 5'-OH, acetyl or benzoyl groups are used for heterocyclic amino and 2'- and 5'-hydroxyl groups; and anilido groups are used for phosphate group. After the two components are condensed by DCC or TPS, the dinucleotide or dinucleoside monophosphate product was isolated, purified, and characterized as usual. After freeing from 5'-O-protecting group or 3'-*P*-anilido group, the product was ready for the next step of the oligonucleotide synthesis. Thus, for example, trinucleoside diphosphate DAG (**9**),[40] tetranucleoside triphosphate AUUC (**10**),[15] **8**, and **7**[41] were prepared. All these synthetic products were subjected to rigid chemical and enzymic analyses for purities and composition,[42] and some had been analyzed for sequence. All the required small oligonucleotides of predefined sequences had been synthesized by the four institutes of Academia Sinica.[15,31,39,40,43]

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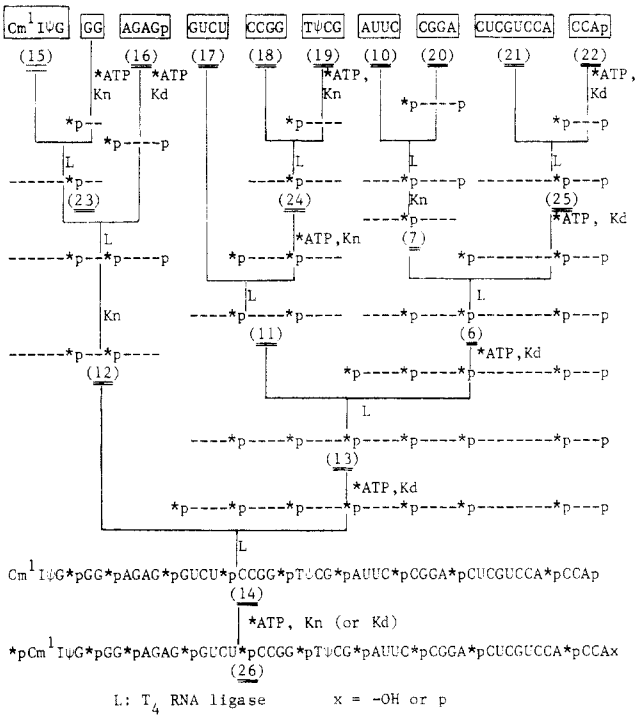
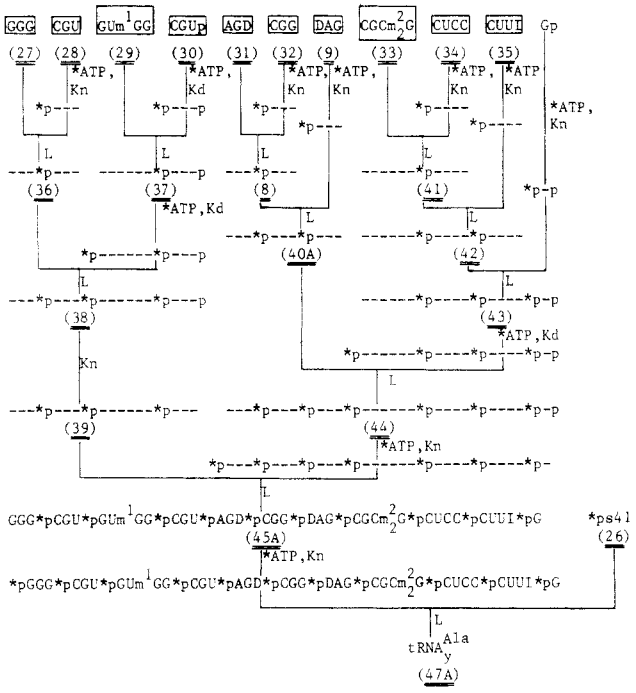
Scheme I. The Synthesis of 3′-Terminal Half-Molecule of $tRNA_y^{Ala}$



Scheme II. The Total Syntheses of 5′-Terminal Half-Molecule (by Route A) and the Whole Molecule of $tRNA_y^{Ala}$



The Synthesis of 3′- and 5′-Terminal Half-Molecules. On the basis of the findings of previous workers that T_4 RNA ligase can link oligoribonucleotides or oligoribonucleotides with ribonucleoside derivatives without using any template,[3,44-47] we had since 1976 been employing the newly discovered T_4 RNA ligase to synthesize larger oligoribonucleotides.[37,38,48-54] After some trials we adopted the step-by-step method as shown in Schemes I–III. The oligonucleotide chain was in general elongated from 3′ terminal to 5′ terminal by adding block after block of the synthetic oligonucleotide intermediates. Before joining, the 5′-OH of the donor compound was labeled with ^{32}P (denoted by *P) by phosphorylation with [γ-*p]ATP in the presence of polynucleotide kinase [normal (Kn) or double-mutated (Kd) enzyme], then mixed with an acceptor bearing a free terminal 3′-hydroxyl group and the ligase, and incubated at 5 °C.[38] The label synthetic intermediate products were in general purified by DEAE-Sephadex A-25 chromatography or by 20% polyacrylamide slab gel electrophoresis. Thus the synthetic intermediates[15,31,39,40,43] were linked stepwise by the ligase and elongated successively into various oligonucleotide fragments of the 3′-terminal half-molecule—nonadecanucleotide **6**,[38] dodecanucleoside undecaphosphate **11**,[48] decanucleoside nonaphosphate **12**,[49] and hentriacontanucleotide **13**[50]—and finally hentetracontanucleotide **14**, i.e., the 3′-terminal half-molecule.[50] The ligation yields were high (in general 67–78%), and the products were rather pure (89–92%). For example **13** (s31p) and **14** (s41p) were synthesized with 78 and 67% ligation yield of 89% and 92% purity, respectively (Scheme I).[50b]

The 5′ half molecule was similarly synthesized but via two routes, route A and route B.[51,53] They differed from each other in the following ways: (1) In route A, the nucleotide chain was extended principally by adding block by block from the 3′ terminal toward the 5′ terminal (Scheme II),[51] and in route B, the nucleotide chain from the 5′ terminal toward the 3′ terminal (Scheme III).[53] (2) In route A, the middle fragment nonanucleoside octaphosphate AGDCGGDAG (**40A**) was joined together in two steps from three intermediate trinucleotide diphosphates (**9**, **31**, and 32) by the ligase. In route B, the middle fragment

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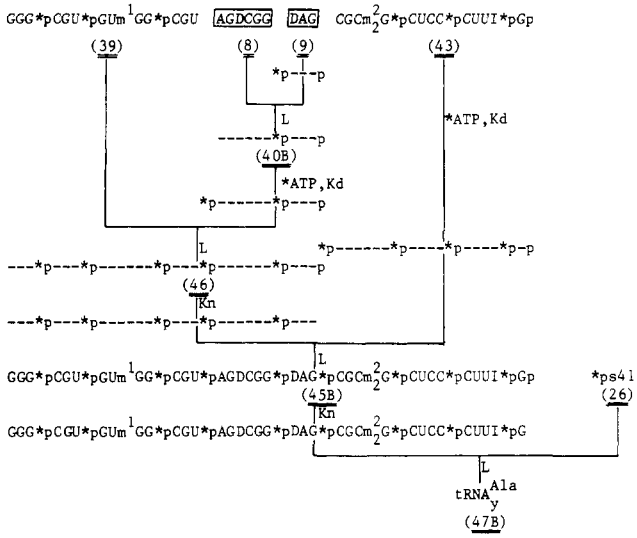
Table I.
[^{3}H]Alanine Accepting and Incorporating Activities of Partially and Totally Synthetic tRNA$_y^{Ala}$

	sample, purified						sample in ligatn mixture	
	[^{3}H]Ala accept activ			[^{3}H]Ala incorp activ			[^{3}H]Ala incorp activ, %	
		activ, %			activ, %			
tRNA$_y^{Ala}$	cpm	*a*	*b*	theor, %	*a*	*b*	*a*	*b*
nat tRNA$_y^{Ala}$	6311	100		69	100		100	
*pn35*pn41	3567	57	100	54	78	100	70	100
*pn35*ps41p[c]	3807	60	107	60	87	111		
*ps35A*ps41	3778	60	106	63	91	117		
*pn35*ps41							35	50
s35B*ps41							56	80

[a] The relative activity with respect to the natural one as 100%. [b] The relative activity with respect to the recombined one as 100%. [c] The activities were measured after the removal of 3′-phosphate group of the partially synthetic product.

Scheme III. The Total Syntheses of 5′-Terminal Half-Molecule (by Route B) and the Whole Molecule of tRNA$_y^{Ala}$



AGDCGGDAGp (**40B**) was synthesized by means of the ligase linking AGDCGG (**8**), prepared by organic synthesis, with *pDAGp.[54] In route A the 5′ terminal OH group of the final product **45A** (s35A) was labeled with a **P*-phosphate group, and in route B, it was not labelled. The yield of the last ligation step for the synthetic 5′-terminal half-molecule prepared by route A was 90% and that by route B, 47%. The isolated products were over 96% in purity, and were checked by terminal analysis, nearest-neighbor analysis, and gel electrophoresis.

Partial Synthesis of tRNA$_y^{Ala}$. Recombination of the two natural 3′- and 5′-terminal half-molecules, obtained by cleaving the natural tRNA$_y^{Ala}$ with RNase, T_1, into biologically active tRNA$_y^{Ala}$ (21–61% yield) was successfully achieved by using the ligase.[7a] For partial synthesis, the 5′-*P-phosphorylated **45A** (*ps35A) obtained from route A was annealed with 5′-**P*-phosphorylated natural 3′-terminal half-molecule (*pn41) and then incubated with the ligase at 2–5 °C. Partially synthetic tRNA$_y^{Ala}$ (*ps35*pn41) was obtained in 30–50% yield. The 5′-*P-phosphorylated 3′-terminal half-molecule **26** (*ps41x) and 5′-terminal half-molecule (*pn35) were similarly treated and joined by the ligase into the other two partially synthetic tRNA$_y^{Ala}$ (*pn35*ps41x) with 17–52% yield. It was revealed on analysis that all the three partially synthetic products and the reconstituted natural product had the identical length of the natural molecule of tRNA$_y^{Ala}$ shown on polyacrylamide gel electrophoresis. It was also proven by nearest-neighbor analysis and terminal analysis that all these ligations are correct.

Total Synthesis of tRNA$_y^{Ala}$. The 5′-*P-phosphorylated synthetic 3′-terminal half-molecule (*ps41) and the 5′-*P-phosphorylated synthetic 5′-terminal half-molecule from route A (*ps35A) were annealed together and ligated as usual. Gel electrophoresis showed that the synthetic product was identical in the length of the molecule with the natural tRNA$_y^{Ala}$. After purification on polyacrylamide slab gel electrophoresis, a totally synthetic product **47A** (s76A) of 90% purity was isolated.[52b] After RNase T_2 digestion, the 5′-terminal nucleotide was proven to be *pGp and the joining ribonucleotide G*p. The 3′ terminal still retained most of the terminal adenosine. Total synthesis was also carried out with 5′-*P-phosphorylated synthetic 3′-terminal half-molecule (*ps41) and the synthetic 5′-terminal half-molecule **45B** (s35B) obtained from route B. The molecule length of the latter synthetic product **47B** (s76B) (Scheme III) was shown by analysis to be the same as that of the natural tRNA$_y^{Ala}$.[53]

Bioassays. Very sensitive bioassay methods for the biological activities of tRNA have been worked out by biologists and biochemists of the Institute of Cell Biology and Institute of Biochemistry for picomoles of tRNA$_y^{Ala}$, employing ^{3}H and ^{32}P double-labeling technique for aminoacylation with crude rat liver aminoacyl tRNA synthetases and for [^{3}H]alanine incorporation into protein in vitro by rabbit reticulocyte lysate system.[7b] The results of bioassays for the crude and purified products are given in Table I. All partially and totally synthetic products had about the same biological activities as those of the recombined products (*pn35*pn41).[52] Their aminoacylation activity was about 60% of the natural one. The [^{3}H]alanine incorporation activities of the purified synthetic products were about 90% of that of the natural tRNA$_y^{Ala}$.[52]

Conclusion. All the synthetic products have been proven to be the expected tRNA$_y^{Ala}$. It is known for the first time that a totally synthetic ribonucleic acid having the same chemical structure with all nine modified nucleotides of the natural product is able to express the complete biological activities of the natural tRNA$_y^{Ala}$.

The total synthesis of the biologically active tRNA$_y^{Ala}$ was successfully achieved in China in November 1981 after thirteen and half years of collaboration of six laboratories.

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