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Methods of Chemical Synthesis of Aminoacid and Peptide Esters of Nucleosides, Nucleotides, and Oligonucleotides†

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The review gives a systematic account of the literature data for and the methods of synthesis of aminoacid esters of nucleosides, nucleotides, and oligonucleotides. The principle of the selection of protecting groups, the methods of activation of aminoacids, and the reaction conditions necessary for the synthesis of specific compounds are discussed.
A separate section is devoted to the discussion of methods for the preparation of peptide derivatives, in particular peptidyl-tRNA on the basis of natural aminoacyl-tRNA derivatives.
The bibliography includes 93 references.

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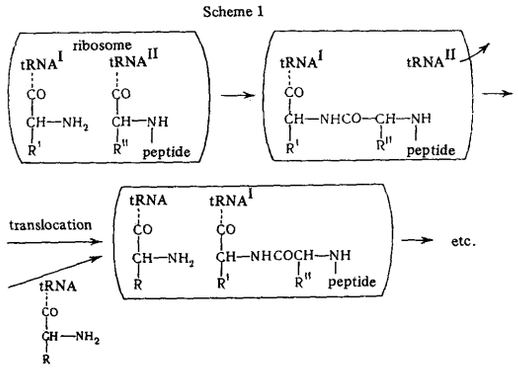
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I. INTRODUCTION

Aminoacyl-tRNA and peptidyl-tRNA are always involved in the synthesis of each peptide bond during the matrix biosynthesis of protein on ribosomes. The peptide bond is formed as a result of electrophilic attack by the C-end of the peptide residue of peptidyl-tRNA on the amino-group of aminoacyl-tRNA (Scheme 1). At the present time this process can be achieved *in vitro* both in the form of a continuous cycle in which ribosomes act upon an endogenic or synthetic RNA-matrix, with formation of a polypeptide chain containing at least several peptide bonds[1,2], or stepwise with control of each succeeding stage in ribosomal activity[3].

Apart from the natural participants in the biosynthesis of a polypeptide on a ribosome (aminoacyl-tRNA and peptidyl-tRNA), aminoacid esters of nucleosides[4], nucleotides[5], and oligonucleotides[6] (as acceptors of the peptide, i.e. as analogues of aminoacyl-tRNA) and peptidyl-(or acylaminoacyl)oligonucleotides[7] (as donors of the peptides, i.e. analogues of peptidyl-tRNA) are employed successfully in single model steps involved in the formation of the peptide bond.

Scheme 1



The use of such compounds helped demonstrate the location of the peptidyl transferase centre of the bacterial ribosome on the 50 S-subunit[7] and also yielded some information about the substrate specificity of this centre[5-7,8].

Bearing in mind that the ribosomal synthesis of peptides and individual proteins in *in vitro* systems is widely used to investigate the functioning of the ribosome, it is of interest to develop methods for the chemical synthesis of aminoacyl- and peptidyl-tRNA.

Such methods would enable investigators to acquire all kinds of "Hybrid" variants in which the aminoacid of peptide[9] would be joined to tRNA which is non-specific in relation to them.

Despite the considerable advances achieved in the chemistry of natural products in the synthesis of amino-acid nucleotide derivatives and related compounds, the search for new methods of synthesis remains an urgent task. Derivatives of this kind will find application in the study of ribosomes and possibly of many enzymes involved in nucleotide synthesis. We believe that development of new methods of synthesis is a [necessary] stage on the path to non-enzymatic synthesis of aminoacyl-tRNA.

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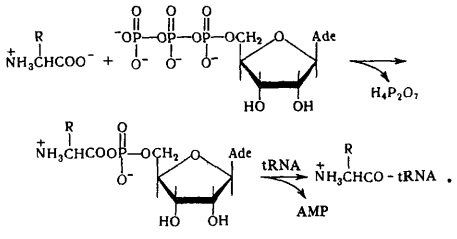
†List of abbreviations employed: tRNA = transfer ribonucleic acid, ATP = adenosine 5′-triphosphate, AMP or pA = adenosine 5′-monophosphate, pG = guanosine 5′-monophosphate, pC = cytidine 5′-monophosphate, pU = uridine 5′-monophosphate, pI = inosine 5′-monophosphate, A = adenosine, G = guanosine, C = cytidine, U = uridine, Ade = adenine, Gua = guanine, Cyt = cytosine; Ura = uracil, DMF = dimethylformamide, DMSO = dimethyl sulphoxide, THF = tetrahydrofuran, DCC = *NN′*-dicyclohexylcarbodi-imide, Z = benzyloxycarbonyl, BOC = t-butyloxycarbonyl, NPS = *o*-nitrophenylsulphenyl, Trit = trityl (triphenylmethyl), Mtrit = monomethoxytrityl, Ac = acetyl, F = formyl, and Im = imidazolyl. Residues of activating esters: OSu = hydroxysuccinimide, ONp = *p*-nitrophenyl.

The aminoacids and nucleic acid components have been named in accordance with the nomenclature adopted by IUPAC.

II. SYNTHESIS OF 3′(2′)-*O*-AMINOACYLNUCLEOSIDES

In protein-synthesising systems, aminoacyl-tRNA is formed from the aminoacid, tRNA, and ATP (Scheme 2) on special enzymes: aminoacyl-tRNA synthetases[2]. In the first stage of this enzymatic reaction, ATP is hydrolysed to AMP and inorganic pyrophosphate with coupled formation of aminoacyladenylate in which the aminoacid residue exhibits electrophilic properties. Next, this aminoacid residue is transferred to the 3′-hydroxyl of tRNA, which results in the synthesis of aminoacyl-tRNA with an ester linkage between the aminoacid residue and tRNA. The chemical nature of this linkage was established[10] in 1960 and it was shown to be a high-energy bond.

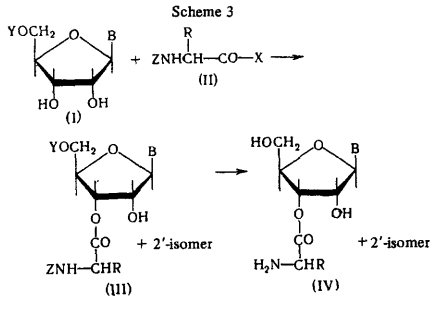
Scheme 2



In the development of approaches to the synthesis of aminoacylnucleosides and their more complex analogues, investigators have employed the principle of the biological process. In all cases the aminoacid residue was activated initially and then allowed to interact with the nucleoside component. Various methods of activation available in the chemistry of peptide synthesis were employed for this purpose. In terms of types of compounds and intermediate activated aminoacids, they can be classified as based on anhydrides, amides, and esters. The reactivities of the aminoacids or peptides activated in this way are different and moreover depend on reaction conditions to different extents. One must also bear in mind the possibility of the acylation of the amino-groups of the bases.

Numerous studies have been made on the synthesis of 3′(2′)-*O*-aminoacylnucleosides(IV).[11-18] The principle of the synthesis, which is common to all these investigations, consists in the reaction of 5′-*O*-protected nucleosides (I) and aminoacids (II) protected at the amino-groups and activated at the carboxyl (Scheme 3).

Scheme 3



where Y = triphenylmethyl or monomethoxytriphenylmethyl and Z = benzyloxycarbonyl, t-butyloxycarbonyl, or *O*-nitrophenylsulphenyl.

Since 5′-*O*-trityl or 5′-*O*-monomethoxytrityl-nucleosides(I) are insoluble in water, all the syntheses were performed in organic solvents such as pyridine, DMF, dioxan, and THF. It was shown that selective synthesis of aminoacid esters of nucleosides under these conditions without side reactions involving *N*-acylation of the bases is possible for adenosine, uridine, and guanosine. In the case of cytidine, the first step is the aminoacylation of the amino-group of the heterocyclic base, which necessitates a preliminary protection of this group.

Among the known methods for the activation of carboxy-groups of aminoacids for the synthesis of 3′(2′)-*O*-aminoacylnucleosides (IV), condensation with the aid of DCC,[4-7] symmetrical anhydrides of *N*-protected amino-acids[11,12], and imidazolides of *N*-protected[17] and *N*-protonated amino-acids[18] have been successfully employed. In the most fundamental study on the preparation of esters of all four main nucleosides (adenosine, guanosine, cytidine, and uridine) the synthesis was carried out by the method of symmetrical anhydrides[12]. As a result of all the stages in the synthesis, the yields of the phenylalanine esters of these nucleosides were respectively 36, 5.3, 19.3, and 33.6%.

3′-*O*-Aminoacid esters of 2′-deoxynucleosides (deoxyadenosine[12] and deoxythymidine[15]) were synthesised by a procedure similar to that used for the aminoacid esters of nucleosides, involving condensation with the aid of DCC or symmetrical anhydrides. The yields of these esters were close to those of ribonucleoside esters.

Among the *N*-protecting groups for aminoacids employed in the syntheses of 3′(2′)-aminoacylnucleosides, the benzyloxycarbonyl (Z),[11-16] t-butyloxycarbonyl (BOC),[4,19] and *o*-nitrophenylsulphenyl (NPS)[17,19] protecting groups have been used. It is not always a simple matter to make rigorous recommendations concerning the choice of a particular group when a new synthesis is planned. Each laboratory where aminoacylnucleosides were synthesised used a new set of protecting groups. However, we believe that the Z-protecting group, which can be eliminated by catalytic reduction without loss of purines, is most suitable for the preparative synthesis of aminoacyladenosine and aminoacylguanosine. Moreover, the comparatively low solubility of 5′-*O*-trityladenosine in the majority of the usual solvents, apart from pyridine and DMF, hinders the employment of the NCS group. For the aminoacid esters of uridine and cytosine, a wide-scale employment of BOC and NPS protecting groups is possible, their elimination being achieved by mild acid treatment—either with anhydrous trifluoroacetic acid or with ethereal hydrogen chloride—and does not lead to the cleavage of the ester linkage[19]. In one of the proposed methods of synthesis of 3′(2′)-*O*-aminoacyladenosines, the imidazolides of *N*-protonated aminoacids were employed[18]. This method does not require a preliminary introduction and subsequent elimination of a protecting group and is therefore suitable for microsyntheses, for example of radioactive esters. However, owing to the difficulty of dissolving the imidazolides of aminoacid trifluoroacetates, this procedure is fairly inconvenient for preparative syntheses.

Having described various methods for the synthesis of *O*-aminoacyl derivatives of nucleosides, a further important question must be discussed, namely their structures.

When nucleosides and nucleotides are aminoacylated, one may expect the formation of a mixture of the 2′- and 3′-isomers. The questions of the preferential aminoacylation of one of the hydroxy-groups of ribose and the migration of the aminoacyl residue between the 2′- and 3′-hydroxyls are not only of independent chemical interest but are also closely related to the determination of

the true position of the aminoacyl residue in aminoacyl-tRNA. It is important to know this in order to understand the mechanism of the formation of aminoacyl-tRNA on aminoacyl-tRNA synthetases [11] and the mechanism of the synthesis of the peptide bond on ribosomes [20].

In order to establish the location of the aminoacyl residue, a number of studies have been made both on synthetic aminoacyl derivatives of nucleosides and nucleotides [4,11,12,21-28] and on aminoacyladenosine isolated from aminoacyl-tRNA. [29,30] The conclusions arrived at proved to be extremely contradictory but considered together they demonstrate that, even if the synthetic methods for the preparation of *O*-aminoacyl-nucleosides and -nucleotides lead to a mixture of isomers, the 3′-isomer greatly predominates as the final product, although in some cases the aminoacylation reaction yielded both the pure 3′-isomer (Z-phenylalanyladenosine) [12] and the almost pure 2′-isomer (*O*-BOC-L-leucyladenosine) [4].

Apart from positive data on the aminoacylation of nucleosides under specified conditions (organic solvents, room temperature, several hours), negative results are also important. For example, it has been shown that the *p*-nitrophenyl, *N*-hydroxysuccinimide and 5-chloro-8-hydroxyquinolinyl esters of *N*-protected aminoacids do not react with the nucleoside hydroxyls, while *p*-nitrophenyl and *N*-hydroxysuccinimide esters react slowly with the amino-group of cytosine [31,32].

Concluding the discussion of the methods of aminoacylation of nucleosides, the following two conclusions may be reached:

1. For reaction with the hydroxyls of the riboside residue, the activation of the *N*-protected aminoacid must be fairly powerful; the imidazolides and anhydrides of aminoacids are capable of reacting with the hydroxyls (the synthesis using DCC probably also involves intermediate anhydrides).

2. Apart from the ribose hydroxyls, in anhydrous solvents only the NH_2 group of the cytosine residue reacts with the anhydrides, amides, (imidazolides), and esters of *N*-protected aminoacids. (Here and henceforth only the four main heterocyclic bases—adenine, guanine, uracil, and cytosine—are considered as part of the composition of nucleosides or nucleotides).

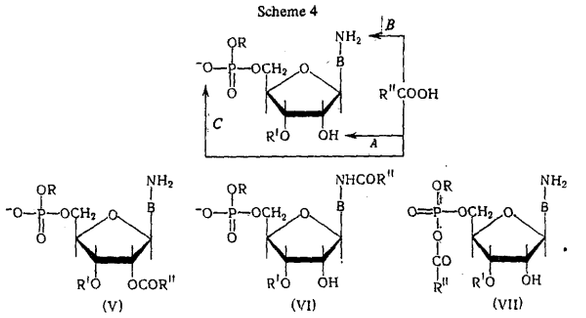
Like the synthesis of 3′(2′)-*O*-aminoacylnucleosides, the method using DCC makes it possible to introduce *N*-protected peptides into 5′-*O*-tritylnucleosides with formation of 3′(2′)-*O*-peptidylnucleosides [15]. Unfortunately, in this case, owing to the intrinsic features of the chosen scheme, appreciable racemisation of the *C*-terminal aminoacid of the peptide is bound to occur, and its extent remains unknown.

III. SYNTHESIS OF 3′(2′)-*O*-AMINOACID ESTERS, NUCLEOTIDES, NUCLEOSIDE 5′-DIPHOSPHATES AND 5′-TRIPHOSPHATES, AND OLIGONUCLEOTIDES

The difficulty of selective aminoacylation of ribose hydroxyls greatly increases on passing from nucleosides to nucleotides. Here the hydroxyls of the phosphate residue are added to the existing groups with nucleophilic properties—the ribose hydroxyls and the amino-groups as the bases. As a result, there is a possibility of the formation of three types of compounds on aminoacylation—esters (V), amides (VI), and mixed anhydrides (VII) (Scheme 4).

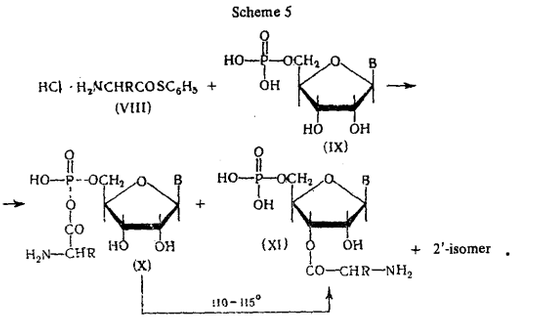
Indeed, the first attempts to extend the method of symmetrical or mixed anhydrides in anhydrous solvents or to carry out a condensation with the aid of DCC under the same conditions led exclusively to mixed anhydrides of type (VII). This showed that it is necessary to find conditions suitable for the aminoacylation of nucleotides in which the reactivity of the phosphate group is negligible.

Scheme 4



In 1958–1960 the first method was developed for the aminoacylation of nucleotides, consisting of the reaction of the thiophenol esters of aminoacid hydrochlorides (or hydrobromides) (VIII) with the nucleotides (IX) at 110 to 115°C in DMSO (Scheme 5). [33,34]

Scheme 5



The authors of the method showed that, when the reaction is carried out at 40°C, a mixture of the anhydride (X) and the ester (XI) is formed; when the temperature is raised to 110–115°C, the anhydride (X) rearranges to the ester (XI).

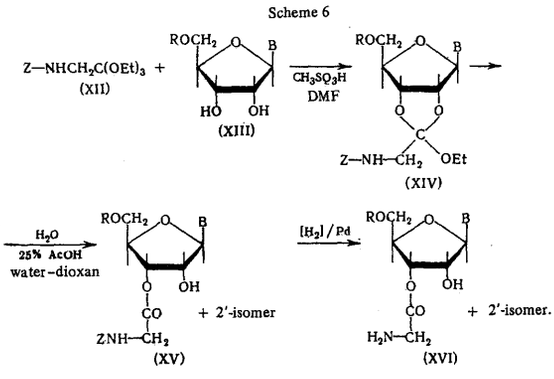
Aminoacid derivatives of (XI) were obtained by the thiophenol ester method in yields of 12–18% for pA esters, 9–10% for pU and pG esters, and 2–3% for 3′(2′)-*O*-aminoacyl-pC. In terms of aminoacids, the synthesis has been extended to valine, leucine, alanine, and methionine. It has been claimed that 3′(2′)-*O*-leucyladenosine 5′-triphosphate has also been obtained by this procedure [35], but the yield of this ester did not exceed 2% and convincing evidence for the structure of the compound obtained was not adduced.

The syntheses of 3′(2′)-*O*-aminoacylnucleotides by the thiophenol ester method, which are the first examples of the synthesis of such compounds, were in addition very important as models for the determination of the type of linkage between the aminoacids and tRNA. [10] It was found that the properties of the ester linkage between the aminoacid and the nucleotide in these compounds are very similar to the properties of the bond between the aminoacid and tRNA, which proves that the latter is also an ester linkage.

However, for preparative purposes the thiophenol ester method suffers from one major disadvantage in addition to low yields. Under the condensation conditions, the nucleotide is appreciably broken down, as a result of which at the end of the reaction the reaction medium contains a mixture of compounds from which the required substance is difficult to isolate. For example, when one of the syntheses by the thiophenol ester method was repeated in our laboratory, at least eight side products in appreciable amounts and several minor admixtures were obtained in addition to the 3′(2′)-*O*-aminoacyl nucleotides, but its yield was the same as that quoted in the literature.

From 1965, a series of studies were published in which a general method was proposed for the synthesis of glycine esters of nucleosides[22], nucleotides[36], and dinucleoside phosphates[37,38]. It is based on a selective reaction of the Z-glycine orthoester (XII) with the *cis*-hydroxy-groups of the nucleoside, nucleotide, or oligonucleotide (XIII). The resulting cyclic orthoester (XIV) hydrolyses to the corresponding Z-glycylnucleoside or its derivative (XV), from which the Z-protecting group is then removed, yielding 3′(2′)-*O*-glycylnucleoside (XVI) or its derivative (Scheme 6).

Scheme 6



(R = H, PO_3H_2, $P_3H_4O_9$, or nucleoside 3′-phosphate)

In the method described the transesterification involves exclusively the *cis*-glycol group: single hydroxyls do not react with the orthoester (XII). The yields of products are fairly high at all stages (60–80%). However, the method has two significant limitations. Firstly, hitherto it has been applicable exclusively to the synthesis of nucleoside or nucleotide esters of glycine, since the orthoesters of other L-α-aminoacids have not been described. Secondly, the conditions governing the interaction of the orthoester (XII) with the nucleoside (or the nucleotide) (XIII) and also the subsequent hydrolysis of the orthoester (XIV) are fairly severe (transesterification in DMF for 1–3 days in the presence of large amounts of methanesulphonic acid, hydrolysis in 25% acetic acid in aqueous dioxan over a period of several hours). Probably these disadvantages precluded the employment of the method for the synthesis of glycyl-tRNA.

Here mention should be made of yet another application of this method, in which the orthoester of benzyloxycarbonylglycine and $N_{(6)}$-dimethylaminomethyleneadenosine 5′-phosphate was used for condensation with $N_{(4)}$-dimethylaminomethylene-5′-*O*-tritylcytidine with the aid of DCC, which resulted in the formation of the corresponding orthoester of the nucleoside diphosphate; after hydrolysis and hydrogenolysis, it yielded CpA-Gly.[39] CpI-Gly was obtained by a similar procedure. This synthesis shows that the orthoester of the aminoacid can be used successfully to protect the *cis*-glycol group in the synthesis of oligonucleotides and thus gives rise to the possibility, in principle, of synthesising the glycine esters of oligonucleotides.

The first report of a series of studies in which a general method was proposed for the synthesis of aminoacid esters of pA, pU, pG, and pC using aminoacid imidazolides, was published in 1967. In the development of this method, the authors began with the following two conditions. Firstly, either water or a water–organic solvent mixture with water predominating was chosen as the condensation medium. The use of water made it possible to reduce sharply the nucleophilicity of the phosphate hydroxyls owing to their dissociation: $pK_a^I = 0.7–1$ and $pK_a^{II} = 5.9$ to 6.0 for pA, pU, pG, and pC. Moreover, it was found that, under these conditions, the reactivity of the amino-group of cytidylic acid, which does not react at all with activated aminoacids, is significantly reduced.

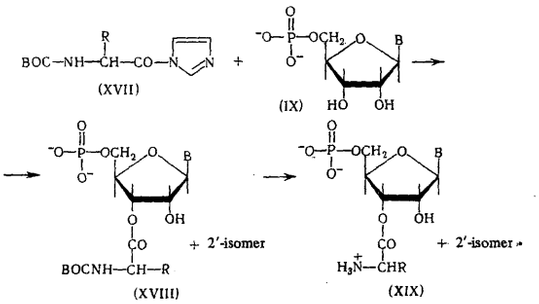
These characteristics of the behaviour of phosphates and amino-groups were known earlier. For example, whereas in acetylation with acetic anhydride in anhydrous solvents the *N*-acetyl derivative of cytidylic acid is always synthesised and the mixed anhydride of the nucleotide and acetic acid is formed[40,41], in an aqueous medium nucleotides or oligonucleotides are acetylated by acetic anhydride[42,43] exclusively at the ribose hydroxyls.

However, the hydroxyls of the riboside residues in nucleotides are also less nucleophilic in an aqueous medium than in anhydrous organic solvents. For example, in organic solvents the symmetrical anhydrides of *N*-protected aminoacids react vigorously with the hydroxyls of the riboside residues of nucleosides[11,12], while in an aqueous medium the same anhydrides altogether fail to react with nucleotides[19,44]. Furthermore, DCC has been widely used as the condensing agent for the synthesis of the ester linkages between *N*-protected aminoacids and nucleosides[45], while a water-soluble carbodi-imide {*N*′-cyclohexyl-*N*″-[β-(*N*-methylmorphilinium)ethyl]-carbodi-imide} is incapable of forming an ester linkage with the similar hydroxyls of tRNA.[46] Therefore successful condensation in water requires the choice of a powerful activating agent, which would make possible reaction with weak nucleophilic groups.

Imidazole was chosen as such activating agent. The imidazolides of *N*-protected aminoacids were used earlier to form the amide linkage in the synthesis of peptides[47,48] and also the ester linkage in depsipeptides[48]. The imidazolides of *N*-protected aminoacids were obtained by the reaction of *N*-protected aminoacids with *NN*′-carbonyldiimidazole by a standard method[47,48]. The condensation of BOC-alanylimidazolide with all the principal components of ribonucleic acids in a 5 : 1 water–DMF mixture at 20°C over a period of 3.5 h yielded 3′(2′)-*O*-BOC-alanylnucleotides in acceptable yields, ranging from 25 to 60% with respect to the initial nucleotide[19,49] (Scheme 7). In all cases the amides or anhydrides of type (VI) and (VII) (see p. 824) were completely absent from the reaction products. Virtually the only products were 3′(2′)-*O*-BOC-alanylnucleotides (XVIII, R = CH_3). Apart from them, the initial nucleotides (IX) and aminoacids were isolated from the reaction mixture. The yields of the esters with respect to the nucleotide which had reacted were 80–98%.

The BOC protecting group in (XVIII) was then removed by treatment with anhydrous trifluoroacetic acid (15 min at 0°C), which gave high yields of 3′(2′)-*O*-alanylnucleosides (XIX, R = CH_3). In the case of 3′(2′)-*O*-alanyladenosine 5′-phosphate, partial loss of the purine (sometimes to the extent of 15%) was observed.

Scheme 7

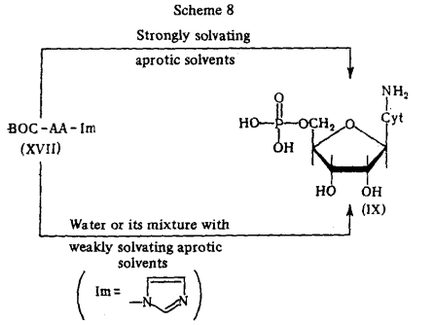


However, it was found that in this version the method is satisfactory only for alanine and glycine. The imidazolides of other BOC-aminoacids cannot be dissolved to a sufficient extent in a medium with 80% of water. Other readily removed *N*-protecting groups (for example Z, Trit, Mtrit, and NPS) only reduce further the solubility of aminoacid imidazolides. Therefore subsequently a large number of various solvents in 1 : 1 mixtures with water were investigated and it was found that the *O*-acylation of all nucleosides can be achieved in 1 : 1 mixtures of water with weakly solvating solvents such as acetonitrile, THF, and acetone. If the amount of strongly solvating aprotic solvent (DMF, DMSO, formamide, and pyridine) reached 30% of the total volume of the reaction medium, the reaction also involved the amino-group of pC (Scheme 8). [50] Thus esters involving the 3′(2′)-hydroxy groups of nucleotides with phenylalanine, lysine, aspartic acid, valine, proline, asparagine, etc. were obtained in such mixtures (the water–acetonitrile mixture was best) [19, 50, 51, 52].

One should note that 3′(2′)-*O*-aminoacylnucleotides of adenylic and uridylic acids are formed in high yields in anhydrous solvents, for example in a 5 : 1 DMSO–DMF mixture. However, this involves also the formation of a certain amount of impurities by side reactions, including 2′,3′-di-*O*-aminoacylnucleotides [19].

The imidazolide method also makes it possible to introduce into nucleotides fragments larger than aminoacids. The applicability of the method to all four nucleotides has been demonstrated for two peptides—BOC-Leu-Gly-Trp-OH and BOC-Ala-Ala-OH. [53] The BOC-protecting groups were removed by treatment with trifluoroacetic acid.

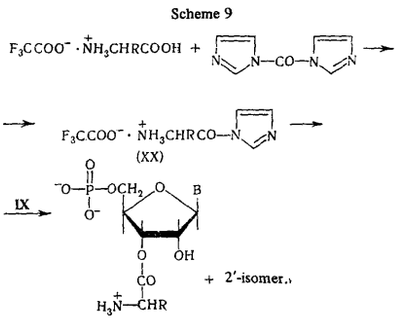
Scheme 8



It is noteworthy that the racemisation of the *C*-terminal aminoacid is not ruled out in this procedure.

The next version of the imidazolide method [19, 54, 55] involved the use of the imidazolides of *N*-protonated aminoacids (XX) in which the amino-group was protected by protonation. Aminoacid trifluoroacetates (or hydrochlorides) were converted into aminoacid trifluoroacetate imidazolides, which were then condensed with nucleotides in an aqueous medium at pH 7. Here the synthesis of 3′(2′)-*O*-aminoacylnucleotides (XIX) takes place virtually in only one stage (Scheme 9).

Scheme 9



It was found that the nucleotide (IX) reacts in different ways with the imidazolides of the *N*-protonated aminoacids (XX). Under the same conditions (for example with the same molar ratio of the reactants, dilution, time, and temperature), the imidazolides of valine and alanine trifluoroacetates reacted best with pA (yields 15–20%), less satisfactorily with pU and pG (yield 4–5%), and altogether failed to react with pC. In the last case all the pC was recovered unchanged from the reaction. (It is noteworthy that in the reaction with pA, pU, and pG the substances isolated contained, apart from the reaction products, mainly the initial nucleotides and only small amounts of side products.)

If the differences between the reactivities of nucleotides are represented by an inequality, the following formulation is possible:

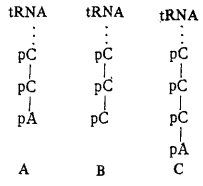
$$pA \gg pU = pG \qquad pC = 0.$$

A similar sequence was observed in the synthesis with thiophenol esters, where *N*-protonated aminoacids were also employed [33, 34]:

$$pA > pU = pG > pC.$$

These data are of interest from the standpoint of the aminoacylation of tRNA *in vivo*. Regardless of the type of tRNA or its origin [2], the end of tRNA carrying the aminoacid (the acceptor end) is known to contain always the same polynucleotide fragment —pCpCpA (A). Among cellular enzymes, there is a special enzyme which builds this fragment into tRNA, namely ATP(CTP)-tRNA nucleotidyltransferase [56]. In its usual form (A), tRNA is capable of being aminoacylated on the corresponding type of aminoacyl-tRNA-synthetase, forming aminoacyl-tRNA. However, if the terminal pA nucleotide is removed with the aid of ATP(CTP)-tRNA nucleotidyltransferase and pC is introduced instead, then the resulting molecule (B) is no longer capable of being aminoacylated. On the other hand, if one obtains with the aid of the same enzyme under

anomalous conditions a type of molecule, modified in relation to natural tRNA, in which one superfluous pC residue is present



(C), then the latter are capable of being aminoacylated on aminoacyl-tRNA synthetase [57] although they are larger than (A). This emphasises the role of the pA residue in enzymatic aminoacylation.

One must stress that the preferential chemical aminoacylation of pA has been demonstrated in both methods where the aminoacid is introduced into the synthesis with the amino-group protected by protonation, i.e. as under the natural conditions on the enzyme. On the basis of these data, one may suppose that the structure of pA makes it most reactive in relation to aminoacylation when two pC residues before the terminal pA in tRNA rule out the erroneous aminoacylation of the incomplete tRNA molecule.

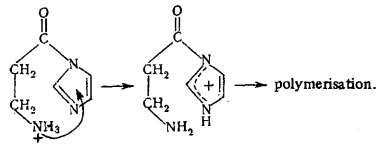
A study of the scope and limits of the applicability of the imidazolides of *N*-protonated aminoacids showed that anhydrous solvents may be used to synthesise *O*-aminoacyl-pA and *O*-aminoacyl-pU. In this case the yield increased to 30–35% for aminoacyl-pA and to 10% for aminoacyl-pU. However, in anhydrous solvents (DMSO, DMF, hexamethylphosphoramide), a number of impurities are formed together with aminoacylnucleotides; when pC is aminoacylated under these conditions, a mixture of side products is formed exclusively in a high yield [19].

Since it was not known from the available literature data whether the optical purity is retained in the synthesis of activated forms of *N*-protonated aminoacids and in their subsequent reaction, the stereospecificity of the synthesis of 3′(2′)-*O*-aminoacylnucleotides obtained with the aid of the imidazolides of *N*-protonated aminoacids was investigated. pA-Phe and pA-Val, obtained from pA, L-Phe, and L-Val, were hydrolysed with hydrochloric acid; next the aminoacids were purified and converted by reaction with salicylaldehyde into *N*-salicylidene derivatives. In the form of their copper complexes, such Schiff bases were investigated by circular dichroism (CD). The CD spectra of the copper complexes of the Schiff bases of salicylaldehyde and α-aminoacids show an anomalous rotation at 360–370 nm, the molecular ellipticity depending on the structure of the aminoacids and its sign on the configuration of the asymmetric carbon atom [58]. It was shown by this method that, within the limits of its sensitivity (up to 3%), the aminoacid is not racemised.

Further development of the aminoacylation method with the aid of the imidazolides of *N*-protonated aminoacids showed that the protection of the amino-groups by protonation is possible solely for α-aminoacids. If the amino-group is separated from the carboxy-groups by more than one carbon atom [for example in $NH_3(CH_2)_n COOH$, $n > 1$ (2–10)], the formation of imidazolides involves a very rapid polymerisation:

$$\overset{+}{N}H_3(CH_2)_n\text{—CO—Im} \rightarrow H\text{—}[NH(CH_2)_n CO]_m\text{—OH}.$$

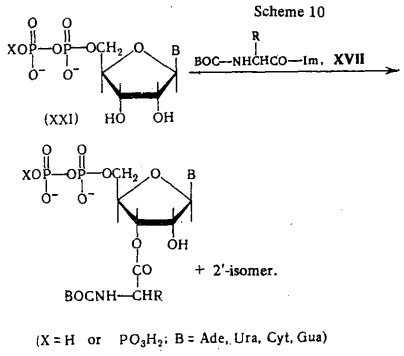
From the standpoint of the formal p*K* values for amino-groups, such a process appears unlikely. Thus, whereas the p*K* for the NH_2 group of glycine (the imidazolide of which is comparatively stable) is 9.6, for $n = 2$ and above the p*K* for the NH_2 group increases to 10 and above; therefore in this case the protection of the amino-group should be unreliable. A tentative explanation of this phenomenon involves a probable intramolecular proton transfer from the $\overset{+}{N}H_3$ group of the aminoacid imidazolide to N(3) of the imidazole ring. For α-aminoacids, such transfer is impossible due to steric hindrance, while for aminoacids with $n > 1$ it is theoretically (according to Stuart models) quite feasible. For example,



With α-aminoacids, the polymerisation reaction also occurs but its rate is very low. Probably here it involves an intermolecular mechanism, the dipeptide imidazolides formed undergoing a rapid further polymerisation, since their α-amino group is separated from the imidazole ring by more than four atoms. In an aqueous medium, the formation of oligopeptidylnucleotides takes place to a very small degree. In absolute solvents, dipeptidylnucleotides are formed in amounts up to 10%, their yield depending on the nature of the initial carbonyldi-imidazole.

A report of the use of the imidazolides of *N*-protected aminoacids for the synthesis of 3′(2′)-*O*-aminoacylnucleoside 5′-diphosphates and 5′-triphosphates (XXI) [59] under conditions resembling those in the aminoacylation of nucleotides (Scheme 10) was published in 1968.

Scheme 10

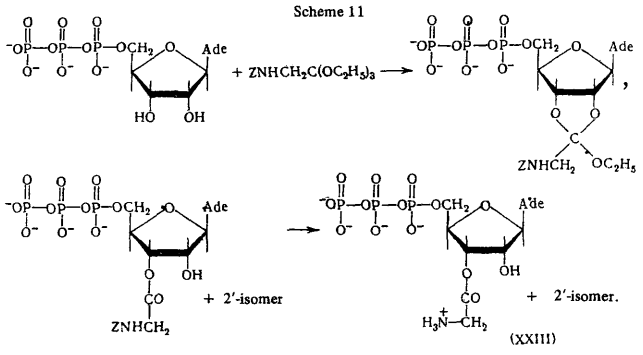


(X = H or PO_3H_2; B = Ade, Ura, Cyt, Gua)

3′(2′)-*O*-Peptidylnucleoside 5′-triphosphates were obtained similarly [60].

The applicability of the orthoester method in the synthesis of 3′(2′)-*O*-glycyl ATP (XXIII) was demonstrated in the same year [61] (Scheme 11).

Scheme 11



Investigators encountered the greatest difficulty in attempts to carry out a chemical aminoacylation of tRNA. tRNA is known to comprise 70–80 nucleoside units bound by (3′-5′)-phosphodiesters linkages. In such polymeric molecules (molecular weight about 25 000) there are on average 80 intermediate 2′-hydroxyls of the riboside residues, one 2′,3′-*cis*-diol group (to which the amino-acid in fact adds in enzymatic aminoacylation), a phosphomonoester group at the 5′- end of tRNA, and about 60 amino-groups of the bases. Under certain conditions, all these groups are capable of reacting with the activated aminoacid. Therefore it is not fortuitous that none of the methods, which are not distinguished by a high selectivity of the reaction with respect to the hydroxyls or by the mildness of the conditions of synthesis, yielded aminoacylated tRNA. The first and so far the only hope of developing a method for chemical aminoacylation arose in connection with the imidazolide method. A report concerning the interaction of the imidazolide of formylphenylalanine and BOC-glycine with a preparation of total tRNA from bakers' yeast was published in 1967.[62] The possibility, in principle, of the aminoacylation of tRNA in a mainly aqueous medium at 20°C over a period of several hours was demonstrated. Under the reaction conditions, compounds with ester type linkages and not anhydride or amide linkages are formed. Stable and high yields of aminoacyl-tRNA were achieved when the reaction was carried out in 1 M imidazole buffer. The BOC protecting group was removed from compound (XXIV) by treatment with anhydrous trifluoroacetic acid (Scheme 12).[63]

Scheme 12

$$\underset{\text{(XVII)}}{\text{BOC—NHCHCO—Im}} \xrightarrow{\text{tRNA}} \underset{\text{(XXIV)}}{\text{BOC—NHCHCO-tRNA}} \xrightarrow{F_3CCOOH} \underset{\text{(XXV)}}{\overset{+}{N}H_3CHCO\text{-tRNA}},$$

where R = H (glycine) or $C_6H_5CH_2$ (phenylalanine).

In the process of elimination of the protecting group from the molecule, the aminoacyl-tRNA lost their adaptor function, i.e. the capacity to combine specifically with information RNA on ribosomes and thus participate in the biosynthesis of protein. Therefore it was necessary to eliminate this severe stage of the synthesis. This was achieved by employing the imidazolides of *N*-protonated aminoacids, which react with tRNA forming aminoacyl-tRNA in one stage (Scheme 13).[64]

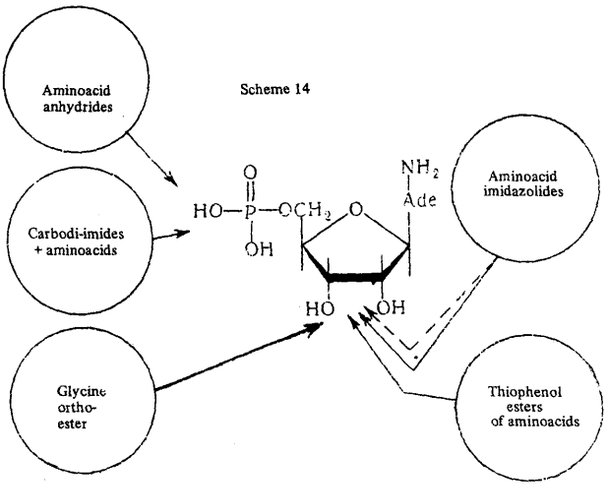
Scheme 13

$$F_3CCOO^- \cdot \overset{+}{N}H_3CHRCO—Im + tRNA \rightarrow \underset{\text{(XXV)}}{\overset{+}{N}H_3CHRCO\text{-tRNA}}.$$

This reaction took place under very mild conditions—in 1 M imidazole buffer in water at pH 7 and 4°C over a period of several hours. The aminoacyl-tRNA separations obtained in this way are being studied at present.

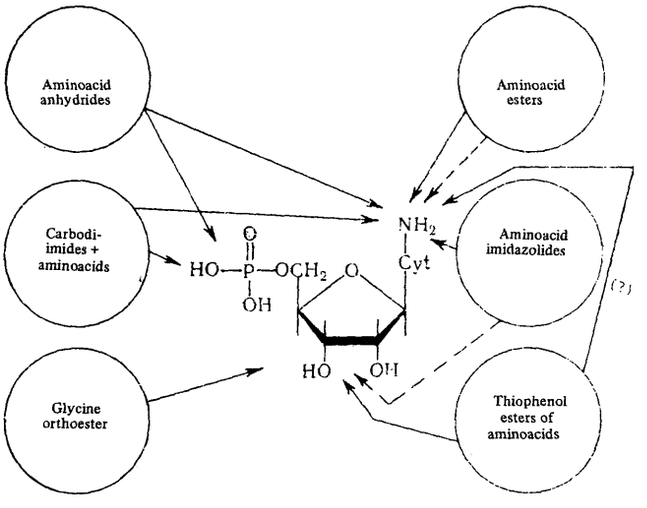
In conclusion, we shall attempt to summarise the data on the mode of the aminoacylation reaction as a function of the conditions and the reactivity of the activated aminoacid (Schemes 14 and 15). The nucleotides in the examples are adenylic acid (pA), the esters of which are widely employed in biochemistry, and cytidylic acid (pC) which has the most reactive amino-group. The properties of *p*-nitrophenyl and *N*-hydroxysuccinimide esters of *N*-protected aminoacids, which do not react either with the phosphate groups or with the ribose hydroxyls, should be considered separately. As regards their interaction with the amino-group of pC, the available data are contradictory: on the one hand, it is asserted that in water–organic solvent media such a reaction does not occur[65]; on the other hand, other investigators[32] did observe the reaction. Whatever the actual state of affairs, the unreacted nucleotides are isolated from the reaction mixture when the nucleotides are treated in a water–organic solvent medium at 20°C. These data served as a foundation for a large series of investigations which led to the discovery of methods for the selective acylation of the amino-group in aminoacylnucleotides and also aminoacyloligonucleotides (including aminoacyl-tRNA) (Section IV).

Scheme 14



The reactions designated by continuous arrows are carried out in organic solvents and those designated by broken arrows are carried out in solvents where water predominates.

Scheme 15



The reaction designated by continuous arrows are carried out in organic solvents and those designated by broken arrows are carried out in a medium where water predominates.

IV. SYNTHESIS OF ACYLAMINOACYL AND PEPTIDYL DERIVATIVES OF NUCLEOSIDES, NUCLEOTIDES, OLIGONUCLEOTIDES, AND tRNA

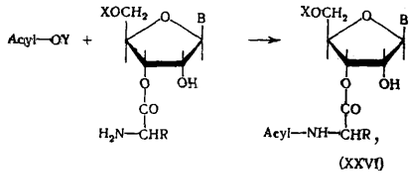
The last section of the review deals with the selective aminoacylation of aminoacyl derivatives of nucleosides, nucleotides, oligonucleotides, and tRNA. Above it was already stated that a rational approach to the search for aminoacylating agents of this type consists of the following steps: (a) the choice of activated forms of aminoacids the reactivity of which is insufficient for the interaction with ribose hydroxyls but permits condensation via the amino-group of the aminoacid residue of the aminoacylnucleoside and (b) the choice of conditions ruling out the reaction via the amino groups of the heterocyclic bases and via the phosphate groups.

Among the activated forms of carboxylic acids (including *N*-protected aminoacids), attention has been concentrated on activated esters. Activated esters such as the *p*-nitrophenyl and *N*-hydroxysuccinimide esters are most widely employed in peptide synthesis. These esters were in fact investigated in the first place as possible activating agents for aminoacylnucleosides and aminoacyl-tRNA.

Activated carboxylic acid esters (acetates, benzoates, etc.) were first allowed to interact with pC in anhydrous solvents, which gave high yields of $N_{(4)}$-acetyl-[66] or $N_{(4)}$-benzoyl-cytidine[67]. (As mentioned above, the amino-groups of adenine and guanine resist acylation by more reactive acylating agents such as anhydrides). However, it was shown in 1967[65] that in a mixture of water and an organic solvent the *N*-hydroxysuccinimide esters of acetic, formic, and other acids do not react with pC. On the basis of these data, a rational synthesis of acylaminoacyl-tRNA and peptidyl-tRNA was developed and is described below.

In 1967–1970 two reports were published[31,32] where it was shown that acyloxysuccinimides and acyl-*p*-nitrophenols can react in a water-organic solvent medium with the amino-group of pC at 24°C, and the yields of *N*-acylcytidylic acid can reach more than 35–45% in 24 h. In order to avoid this process, the authors suggested milder acylating agents, namely 5-chloro-8-hydroxyquinoline esters[32], which do not react with pC in a water–organic acid medium but do acylate the amino-group of the aminoacid residue. The acylation (aminoacylation) of the aminoacid esters of tRNA components or tRNA itself is shown in a general form in Scheme 16.

Scheme 16



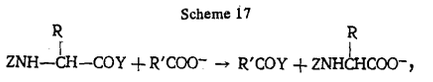
(XXVI)

where Y is the alcoholic component of the activated ester and X = H (nucleosides) or H_2O_3P (nucleotides or oligonucleotides).

A-(AcGly), A-(Val-Gly), A-(Lys-Gly), CpA-(AcGly), and CpA-(Lys-Gly) have been synthesised by activation with N-hydroxysuccinimide according to Scheme 16.[68,69]

Aminoacylation of aminoacylnucleosides and their analogues should not be carried out in acetate or formate buffer solutions. On the basis of the final results, one may suppose that in the presence of acetates of formates in organic solvents or in water there is an exchange of the activating group between the acylamino-acid and acetate or formate (Scheme 17).[70,71]

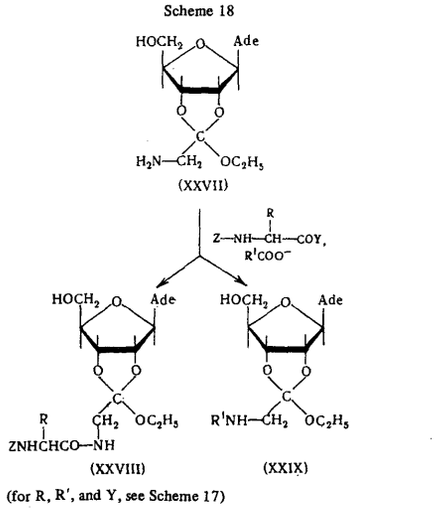
Scheme 17



where R = $CH(CH_3)_2$ or $(CH_2)_4NHZ$, Y = OSu or ONp, and R′ = H or CH_3.

The overall reaction, between the glycyladenosine orthoester (2′,3′-*O*-aminomethylethoxymethyleneadenosine) (XXVII) (Scheme 18) and the *p*-nitrophenyl or *N*-hydroxysuccinimide esters of benzyloxycarbonylvaline or $N_{(\alpha)}N_{(\epsilon)}$-dibenzyloxycarbonyl-lysine in the presence of acetate or formate in both anhydrous DMF or DMSO and in their mixtures with water results in the formation of a mixture of 2′,3′-*O*-(*N*-acyl)aminomethylethoxymethylene-adenosines. Apart from the main products, 2′,3′-*O*-(*N*-benzyloxycarbonylvalyl)- and 2′,3′-*O*-(*N*-dibenzyloxy-carbonyl-lysyl)-aminomethylethoxymethyleneadenosines (XXVIII), 2′,3′-*O*-(*N*-acetyl)- and 2′,3′-*O*-(*N*-formyl)-aminomethylethoxymethyleneadenosines (XXIX) were isolated from the reaction mixtures, their yield in a mixture of DMF and 2 M aqueous acetate buffers amounting to 39–42%.

Scheme 18



(for R, R′, and Y, see Scheme 17)

However, few examples of the synthesis of peptidyl-nucleosides or oligonucleotides have been described so far. The acylation of aminoacyl-tRNA has been investigated to an incomparably greater degree. Acylaminoacyl-tRNA is of particular interest for the investigation of the mechanism of the biosynthesis of protein on ribosomes. Acylaminoacyl-tRNA initiates the formation of the polypeptide chain in ribosomes (for example, FMet-tRNA in the protein synthesising system of *Escherichia coli*[72], AcPhe-tRNA in a model system with ribosomes and polyuridylic acid as the matrix[73], and in other cases).

FMet-tRNA was synthesised for the first time in 1964 by the reaction of *p*-nitrophenyl formate with Met-tRNA.[74] Acetic anhydride has been employed similarly[73] for the acylation of aminoacyl-tRNA, but this reaction does not

follow a single pathway: there is partial acylation of certain other parts of tRNA.[75,76] A convenient general method was developed in 1967–1970 using *N*-hydroxysuccinimide esters[75,76]. The condensation of *N*-hydroxysuccinimide esters of carboxylic acids with aminoacyl-tRNA is carried out in a mixture of water and organic solvents at 20°C and pH 5–6.

Among the numerous organic solvents employed, DMSO proved to be the most convenient. The yields of a number of acylaminoacyl-tRNA (FPhe-tRNA, AcPhe-tRNA, capryloyl-Phe-tRNA, lauroyl-Phe-tRNA, palmitoyl-Phe-tRNA, AcVal-tRNA, and AcMet-tRNA) were nearly qualitative and the yield of $N_{(\alpha)}N_{(\epsilon)}$-diAcLys-tRNA was about 90%. The acylaminoacyl-tRNA obtained did not lose their adaptor properties, i.e. in the presence of the corresponding codons they are capable of binding to ribosomes[76] and of initiating polypeptide synthesis[77,78].

The synthesis of oligopeptidyl-tRNA was first achieved using a water-soluble carbodi-imide[43,73]. Above it was already stated that this condensing agent in an aqueous medium is incapable of forming ester linkages between aminoacids and the hydroxyls of the ribose residues of tRNA; the amino-groups of the heterocyclic bases and the phosphate residues also fail to react under these conditions. This made it possible to develop with the aid of *N'*-cyclohexyl-*N''*-[β-(*N*-methylmorpholinium)ethyl] carbodi-imide (CME-carbodi-imide) the first method for the synthesis of formylpeptidyl-tRNA. Gly-Val-tRNA[46] and FGly-Gly-Val-tRNA[79] were obtained in this way. However, this method has not found wide application, possibly partly owing to the difficulty of removing the formyl protecting group without disrupting the structure of formylpeptidyl-tRNA.

A method employing *N*-hydroxysuccinimide esters, which nowadays makes it possible to synthesise derivatives up to deca- and trideca-peptidyl-tRNA, was developed more thoroughly. The synthesis of Gly-Phe-tRNA was achieved for the first time by the condensation of the *N*-hydroxysuccinimide ester of Mtrit-glycine (XXX) with Phe-tRNA to form Mtrit-Gly-Phe-tRNA (XXXI) followed by elimination of the protecting group (Scheme 19).

Scheme 19

$$\underset{\text{(XXX)}}{\text{MtritGlyOSu}} + \text{Phe-tRNA} \rightarrow \underset{\text{(XXXI)}}{\text{MtritGly-Phe-tRNA}} \rightarrow \underset{\text{(XXXII)}}{\text{Gly-Phe-tRNA}} .$$

The choice of the protecting group in (XXX) and of the conditions for the condensation of (XXX) with tRNA is not trivial, since the protecting group must be removed subsequently from compound (XXXI) under very mild conditions. Otherwise the labile peptidyl-tRNA bond might be easily broken. t-Butyloxycarbonyl (BOC), trityl (Trit), and monomethoxytrityl (Mtrit) *N*-protecting groups were tested for this purpose, but the attempts to eliminate the BOC and Trit protecting groups under mild conditions were unsuccessful. The Mtrit group was removed from (XXXI) with 5% dichloroacetic acid at 4°C in 5 min; the Gly-Phe-tRNA (XXXII) obtained as a result of such treatment possessed the adaptor function and was capable of functioning as a peptide donor on ribosomes[78].

Tests of this method of synthesis on a large group of dipeptidyl-tRNA showed[80] that the condensation of *N*-hydroxysuccinimide esters of Mtrit-aminoacids with aminoacyl-tRNA takes place just as satisfactorily at pH 5–8 in a 4:1 DMSO–aqueous buffer mixture. Among the organic solvents tested (DMSO, DMF, ethanol, THF, and ethyl acetate), DMSO was found to be most convenient. In this solvent the reaction takes place under the most homogeneous conditions and as a result has the highest yield.

The structure of the aminoacid in compound (XXX) (Scheme 19) also affected the yield of (XXXI). Whereas for the *N*-hydroxysuccinimide esters of Mtrit-glycine and Mtrit-alanine the yields were nearly quantitative[80–84], for the *N*-hydroxysuccinimide esters of Mtrit-phenylalanine and Mtrit-valine the yields did not exceed 55–85%.[80] This decrease of the yield can be accounted for by the steric hindrance of the aminoacid component to the synthesis of compound (XXX), since both the *N*-protecting group and the side group of the aminoacid are in this case fairly bulky. In order to overcome this difficulty, the NPS *N*-protecting group, which is readily removed from NPS-peptidyl-tRNA with a 0.4 M solution of sodium thiosulphate in water at 30°C in 2 h, was employed. Val-Gly-Phe-tRNA[85] and many other derivatives[86,87] were obtained in this way in nearly quantitative yields.

Peptidyl-tRNA with larger peptide residues were synthesised according to a scheme which is similar in principle but involves both stepwise and block procedures in the formation of the peptide chain[82]. The synthesis of penta-alanyl-tRNA (XXXV, Scheme 20) may be quoted as an example.

Scheme 20

$$\underset{\text{(XXXIII)}}{\text{Ala-tRNA}} \xrightarrow{\text{MtritAla-AlaOSu}} \text{Mtrit-(Ala)}_2\text{-tRNA} \rightarrow$$

$$\underset{\text{(XXXIV)}}{\text{(Ala)}_3\text{-tRNA}} \xrightarrow{\text{MtritAla-AlaOSu}} \text{Mtrit-(Ala)}_5\text{-tRNA} \rightarrow \underset{\text{(XXXV)}}{\text{(Ala)}_5\text{-tRNA}} .$$

According to this scheme, the pentapeptide (XXXV) is formed from 1 + 2 + 2 aminoacid units. The yield of (XXXIV) relative to (XXXIII) employed in the reaction was 93% and the yield of (XXXV) relative to (XXXIV) was 80%. Several tri- and tetra-peptides containing valine, phenylalanine, alanine, leucine, and methionine as aminoacids introduced by an enzymatic procedure and glycine or alanine introduced synthetically were obtained by a similar method[83]. $(Gly)_n$-Phe-tRNA, $(Gly)_n$-Val-tRNA, $(Ala)_n$-Phe-tRNA and $(Ala)_n$-Ala-tRNA, where $n = 4–12$, were also synthesised in 4–5 stages[84]. In the synthesis of such relatively large peptidyl-tRNA, the condensations were carried out at 30°C over a period of 12 h.

The use of the NPS-protecting group permitted the extension of this method to hydroxyl-containing aminoacids (serine, tyrosine, threonine[86], and phenylalanine[87]). In the case of these aminoacids, the condensation of NPS-peptidyl-OSu with aminoacyl-tRNA was carried out at 30°C in 2 h in a mixture of an aqueous buffer at pH 8.1 and DMSO in proportions of 1:(1–4) by volume; there was then no need to protect the hydroxyls of serine, tyrosine, and threonine. The yields of the peptidyl-tRNA were nearly quantitative.

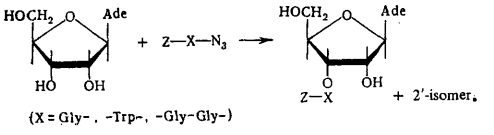
Activation of the formation of *N*-hydroxysuccinimide esters is now beginning to find application in the synthesis of special variants of acylaminoacyl-tRNA and peptidyl-tRNA for the solution of specific biochemical problems. For example, phenoxyacetyl-Phe-tRNA (forming part of the overall tRNA) was synthesised from the *N*-hydroxysuccinimide ester of phenoxyacetic acid and Phe-tRNA (forming part of the overall tRNA). This made it possible to develop a method for the isolation of the individual phenylalanyl-tRNA[88]. A similar method has been

developed also for Val-tRNA[89]. A series of acylamino-acyl-tRNA ($tRNA^{Phe}$, $tRNA^{Val}$), with a stable radical attached to the amino-group of the aminoacyl-tRNA, were obtained by the formation of the *N*-hydroxysuccinimido-ester of 2,2,5,5-tetramethylpyrroline-3-carboxylic acid N-oxide containing the stable radical[90,91]. The tertiary structures of the above compounds were investigated with the aid of ESR spectra.

A number of peptidyl-tRNA have been employed as substrates for the enzyme peptidyl-tRNA hydrolase[92], which hydrolyses selectively aminoacyl-tRNA.

In conclusion we must point out that the possibilities of the synthesis of the aminoacid esters of nucleosides, nucleotides, and polynucleotides have still been comparatively little investigated. This is illustrated by a recent study in which 3′(2′)-*O*-benzyloxycarbonylglycyl-, 3′(2′)-*O*-benzyloxycarbonyltryptophanyl- and 3′(2′)-*O*-benzyloxycarbonyldiglycyl-adenosines were obtained[93] (Scheme 21).

Scheme 21



(X = Gly-, -Trp-, -Gly-Gly-)

The synthesis was achieved by the condensation of benzyloxycarbonylaminoacid and benzyloxycarbonyldipeptide azides with adenosine in 3 : 1 aqueous dioxan at pH 9 and 8°C. This method is interesting from several points of view. Firstly, it does not require the protection of the 5′-hydroxyl. Secondly, it will probably make it possible to prevent the racemisation of the *C*-terminal aminoacid in peptides, provided that it can be extended to optically active peptides. Thirdly, the unusual behaviour of the azides of *N*-protected aminoacids and peptides in a medium containing mainly water at a high pH has been demonstrated in this case. It is known from experimental peptide chemistry that the use of aminoacid and peptide azides is possible solely for reactions involving the amino-group, since the hydroxyls of serine, threonine, and tyrosine do not react with azides under these conditions.

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