Tetrahedron. Vol. 26, pp. 4419 to 4433 Pergamon Press 1970 Printed in Great Britain

THE GENERAL SYNTHETIC ROUTE TO AMINO ACID ESTERS OF NUCLEOTIDES AND NUCLEOSIDE-5'-TRIPHOSPHATES AND SOME PROPERTIES OF THESE COMPOUNDS

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(*Recieved in the UK* 20 *January* 1970; *Accepted for publication* 21 *April* 1970)

Abstract—A new common method of synthesis of 3'(2')-O-aminoacyl esters of nucleoside-5'-mono-and triphosphates has been elaborated. It involves the interaction between imidazolides of N-protected amino acids and nucleoside-5'-mono-and triphosphates with subsequent removal of the N-protecting group from the amino acid part. The scope of this method concerning the amino acids as well as nucleosides and nucleotides has been studied. The absence of racemization during the synthesis has been proved.

AMINO acid esters at 3'(or 2') hydroxyls of nucleosides and nucleotides in some reactions may be considered as the models of aminoacyl-tRNA*. Zachau and Karau[1,2] have demonstrated that amino acids are bound to their tRNA's by ester bonds at the 3'(2')-terminal hydroxyls of the latter. Later Waller *et al*[3] and Rychlik *et al.*[4] used aminoacyl-nucleosides and aminoacyl-dinucleoside phosphates as acceptors of peptide in their studies of the substrate specificity of the ribosome transferase center. Valuable information was obtained concerning the role of the nucleoside moiety, its heterocyclic nucleus, and the amino acid residue, etc. On the other hand, Monro *et al.*[5] applied N-acyl-aminoacyl-oligonucleotides to analogous studies to elucidate requirements to N-acyl-aminoacyl-oligonucleotides structure and to find out the role of different tRNA sites in ribosome-catalysed peptide synthesis.

The synthesis of 3'(2')-O-aminoacyl-nucleosides has been described[3,6,8] but nucleotide derivatives are more convenient models for some chemical and biochemical studies. For example, it appeared impossible to obtain tRNA aminoacyl derivatives using the DCC[6] and other methods that are used to prepare aminoacyl-nucleosides. On the other hand, it has been demonstrated that 3'(2')-O-L-phenylalanyl-pA is a better acceptor of peptide on ribosomes compared with 3'(2')-O-L-phenylalanyl-adenosine. Hence, elaboration of a general method for the synthesis of 3'(2')-O-aminoacyl-nucleotides was believed an important problem.

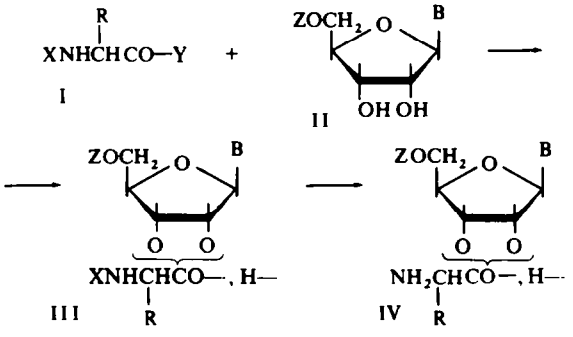
* Abbreviations: tRNA-transfer ribonucleic acid; pA-adenosine 5'-phosphate; pU-uridine 5'-phosphate; pG-guanosine 5'-phosphate; pC-cytidine 5'-phosphate; ppA-adenosine 5'-pyrophosphate; ppU-uridine 5'-pyrophosphate; ppG-guanosine 5'-pyrophosphate; ppC-cytidine 5'-pyrophosphate; pppA-adenosine 5'-triphosphate; pppU-uridine 5'-triphosphate; pppG-guanosine 5'-triphosphate; pppC-cytidine 5'-triphosphate; B-bases in nucleosides and nucleotides; dpT--thymidine 5'-phosphate; BOC--t-butyloxy-carbonyl; NPS--*o*-nitrophenylsulphenyl; MMOTr--monomethoxytrityl; Im--N-imidazolide; ONP *p*-nitrophenyl ester; OSU--N-hydroxysuccinimide ester; SPh--thiophenyl ester, OBu^t--t-butyl ester; DCC—dicyclohexyl-carbodiimide; CDI--N,N'-carbonyldiimidazole; DMF--dimethylformamide; DMS--dimethylsulphoxide; THF—tetrahydrofuran.

Two methods have been applied earlier to obtain amino acid esters of nucleotides. The method proposed by Wieland *et al.*[9] is based on the reaction between nucleotides (pA, pG, pC and pU) and amino acid thiophenyl ester hydrobromides (or hydrochlorides) and requires 15 min at 100–115° in DMS. By this method, esters of alanine, valine, leucine, phenylalanine have been obtained but the disadvantages are the small yield of aminoacyl-nucleotides (2–17%) and their contamination with a number of by-products.

In 1966 Žemlička and Chládek elaborated a new elegant method for the synthesis of amino acid esters of nucleotides, dinucleoside phosphates[10] and also of ppA and pppA.[11] The method is based on the condensation of N-carbobenzoxy-amino acid orthoesters with nucleotide components followed by hydrolysis of cyclic orthoester and removal of the N-protecting group. The yields are high; the method has been applied successfully to obtain the derivatives of pA, pU, pG and pC; two *cis*-hydroxyls are necessary. The two important limitations are: the rather drastic conditions of condensation and hydrolysis (80% acetic acid, 20°, 24 h, DMF with CF_3COOH) and also the method has been applied only to glycine derivatives.

In a series of papers we reported the imidazolide method of synthesis of 3′(2′)-aminoacyl-nucleosides, -nucleotides[12] and nucleoside di- and triphosphates.[13] At present, the conditions and scope of this method, its advantages and disadvantages are summarized.

The synthesis is based on the reaction of N-protected amino acid imidazolides (I, Y = Im) with nucleotides or nucleside-5′-di- or triphosphates (II) followed by removal of the protecting group from the aminoacyl residue (III, Scheme 1).



The condensation of the amino acid component (I) with the nucleotide (II) proceeds as a nucleophilic substitution. As the hydroxyl of the ribosyl moiety is a relatively weak nucleophile, the amino acid activation is extremely important. Also as the synthesis with nucleotides was performed in aqueous solution (see below), the amino acid activation had to be valid under these conditions. Finally, the relative lability of nucleotides and especially of nucleoside triphosphates limited the choice of the activation method to those providing rapid condensation at low temperature. With activated BOC-alanine and pA it was shown that the activation with *p*-nitrophenol, N-hydroxysuccinimide, symmetrical anhydride and thiophenyl ester are not valid in aqueous–organic medium at 20° for 3·5 hr. In the literature there was evidence that

imidazole produces a catalytic effect.[14] This prompted us to perform the same reactions in 1M imidazole buffer at pH 7·0, and a pronounced catalytic effect was found with the thiophenyl ester BOC-Ala-SPh [3′(2′)-O-BOC-Ala-(pA) was obtained in a yield of 12–15%]. At the same time, the thiophenyl ester of BOC-alanine did not react with pA in imidazole buffer at pH 5·0, where imidazole is completely protonized and thus cannot catalyze the reaction.

Another important problem in the synthesis was the choice of N-protection for the amino acid. Bearing in mind the specifity of synthesis and properties of compounds of the type III and IV, the protective group ought to meet the three following requirements: (i) the possibility of its removal under mild acidic conditions; (ii) the relatively good solubility of N-protected amino acid imidazolides in aqueous–organic media, containing more than 50% of water; and (iii) absence of any racemization of the N-protected amino acid residue during the synthesis.

Our attention was attracted to three protecting groups: the *o*-nitrophenylsulphenyl, the monomethoxytrityl and the t-butyloxycarbonyl groups. The best yields in the condensation of pU with N-protected phenylalanine (a hydrophobic amino acid difficult for work in aqueous solution) activated as imidazolide was obtained with the BOC-group (Table 1). On the other hand, the removal of BOC-protection of the NH_2

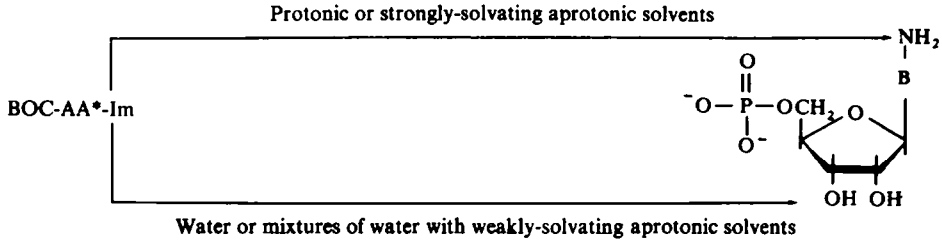
TABLE 1. YIELDS OF N-PROTECTED 3′(2′)-O-AMINOACYL-PU'S (III) IN THE REACTION OF N-PROTECTED AMINO ACID IMIDAZOLIDES (I) WITH PU

Nr	I	Yield of III, %
1	BOC–Phe–Im	27–30
2	NPS–Phe–Im	20–22
3	MMOTr–Phe–Im	8–10
4	MMOTr–Gly–Im	19–20

group of compounds of the type III is performed by treatment with dry trifluoroacetic acid (15 min, 0°) or with 10% hydrogen chloride in dry ether (15 min, 0°). In both the cases, no cleavage takes place of the ester bond in compounds IV with all the major ribonucleotides (pA, pU, pG and pC), but some apurinization takes place in the synthesis of 3′(2′)-O-aminoacyl-pA (IV) [adenine was isolated from the reaction mixture]. Moreover, treatment of 3′(2′)-O-BOC-aminoacylnucleotides (III) with ethereal hydrogen chloride does not result in complete removal of BOC-grouping because III is not soluble in ether.

The most difficult problem was to find reaction conditions preventing aminoacylation of the relatively nucleophilic amino groups of nucleotides pA, pC and pG or of nucleotide phosphate groups. Studies of the condensation in dry solvents (like formamide, DMF, DMS) with BOC-Ala-Im revealed that no action occurs with the nucleotide phosphate groups. In addition, no reaction takes place with the amino group of pA, but in pC the amino group is acylated first and quantitatively. At the same time, the condensation of BOC-Ala-Im with all the four nucleotides in water–DMF (5:1, v/v) proceeds at only the ribose residue hydroxyls. However, the reaction can be performed under these conditions but with amino acid imidazolides whose N-BOC-derivatives are soluble enough in water (other functional groupings if present

must also be protected). Using this method we prepared 3′(2′)-O-nucleotide esters (III) of BOC-glycine and BOC-alanine. To perform the reaction in homogeneous solution with non-polar protected amino acids, more organic solvent must be added to the reaction medium. Condensation at the interface of two immiscible solvents (benzene–water, chloroform–water) resulted in small yields and was complicated. For this reason, we studied a number of homogeneous organic–aqueous media (water–DMF, water–DMS, water–formamide, water–pyridine, water–acetonitrile, water–THF, water–acetone). With pC it was demonstrated, that if the content of strongly solvating aprotonic solvents (DMS, DMF, formamide or pyridine) is increased more than 30% the reaction of BOC-Ala-Im (and BOC-Phe-Im) is mainly at the amino group of the cytidine residue. On the other hand, if the content of weakly-solvating aprotonic solvents (acetonitrile, THF, acetone) is increased above 50%, no change in the direction of BOC-amino acid attack takes place with all four nucleotides at only ribose residue hydroxyls (Scheme 2). Finally, the medium providing the highest



yield of 3′(2′)-O-BOC-Phe-(pA) (and -pC), 3′(2′)-di-BOC-Lys-(pA) (and pC), 3′(2′)-O-BOC-Asp(OBu^t)-(pA) (and -pC), etc, proved to be mixture of acetonitrile and water, 1:1.

Having studied the effects described, the scope of the method with respect to nucleophilic and electrophilic components was investigated.

The imidazolide method proved especially advantageous when used in aqueous–organic media to obtain 3′(2′)-O-aminoacyl-nucleotides according to Scheme 1. It appeared applicable to all four major ribonucleotides—pA, pU, pG and pC. Studies with BOC-Ala-Im and the four nucleotides proved that it is most convenient to perform the reaction in water–DMF (5:1) for a 2–3 hr period at room temperature. 20% DMF is present in the reaction medium because BOC-Ala-Im was synthesized from BOC-alanine and CDI in dry DMF, and this reaction mixture was added without isolation to the aqueous solution of the nucleotide. The condensation can be performed at 4°, without increasing the time of reaction significantly (4 hr). As BOC-Ala-Im is simultaneously hydrolysed, an excess in respect to nucleotide (10:1 in moles) must be used.

As the reaction in water is extremely selective, only unchanged starting nucleotide, BOC-alanine and 3′(2′)-O-BOC-Ala-nucleotides were isolated from the reaction mixtures. It is possible to perform the reaction in dry medium (e.g., in DMS) with pA and pU, but not with pC, and to take a smaller excess of reagent (2:1); the yields are usually somewhat greater under these conditions, but sometimes by-products are formed whose structures have not been studied in detail. Table 2 shows the yields of

different 3′(2′)-O-BOC-aminoacyl-nucleotides calculated for amounts of nucleotide taken into reaction and for those entering the reaction. The condensation products were isolated by preparative paper chromatography, and in all the experiments we failed to elute quantiatively the nucleotide material from paper. Greater yields of III were not achieved due to these losses rather than due to side reactions.

TABLE 2. RESULTS OF THE SYNTHESIS OF O-BOC-AMINOACYL-NUCLEOTIDES

Starting compounds		Amount of II taken into reaction OU	Solvent	Obtained after reaction		Yield of III, %	
II' Z = —PO_3H_2, its form	I (I:II molar ratio 4:1(I))			of starting II	of III	for II taken into reaction	for II that entered reaction
pA(NH_4)	BOC–Ala–Im	352	water–DMF 5:1	178	145	41·5	84·0
pA(Na)	BOC–Ala–Im	600	DMF–DMS 1:5	380	214	36·0	98·3
pA(Na)	BOC–Ala–Im	239	DMF–water–acetonitrile 1:5:2	130	77	32	77
pA(Na)	BOC–Phe–Im	120	THF–acetonitrile–water 3:1:4	32	42·2	35·1	48·0
pA(Na)	BOC–Asp(OBu^t)–Im	430	DMF–acetonitrile–water 1:1:4	250	86	20·0	47·9
pA(Na)	BOC–Asp(OBu^t)–Im	218	DMF:DMS 1:3	28	157	72	82
pA(Na)	di-BOC–Lys–Im	172	DMS	—	44	25·6	—
pA(Na)	di-BOC–Lys–Im	6600	water–THF–acetonitrile 5:3:1	—	1950	29·5	—
pC(Na)	BOC–Ala–Im	270	water:DMF 5:1	185	69	25·6	80·5
pC(Na)	BOC–Phe–Im	150	DMF–acetonitrile–water 1:4:5	88·5	37·6	25·1	61·1
pC(Na)	di-BOC–Lys–Im	160	DMF–acetonitrile–water 3:4:8	—	16	10	—
pG(Na)	BOC–Ala–Im	324	water–DMF 5:1	126	154	47·5	77·2
pU(H)	BOC–Ala–Im	112	water–DMF 5:1	—	64	56·7	—
dpT(NH_4)	BOC–Ala–Im	288	water–DMF 5:1	32	253	87·9	98·8
dpA(NH_4)	di-BOC–Lys–Im	180	DMF–acetonitrile–water 3:4:8	140	0	0	0
dpA(NH_4)	di-BOC–Lys–Im	200	DMF–DMS 1:3	165	8	4	23

1 mg of nucleotides contains: 44·5 OU of pA, 28·0 OU of pU, 37·6 OU of pG and 30·8 OU of pC (at pH 7·0).

As seen in Table 2, the yields of 3′(2′)-O-BOC-aminoacyl-nucleotides (III) are different with different nucleotides. To compare directly the results, the syntheses of 3′(2′)-O-BOC-alanyl-nucleotides were performed under identical conditions (time, temperature, reagents ratio, dilution, post-treatment). The results are presented in Table 3. The yields of 3′(2′)-O-BOC-aminoacyl-nucleotides depending on the nucleotides decrease in the following sequence:

$$pU > pG \geqslant pA > pC$$

This sequence is in correlation with the basicity of heterocyclic nuclei and may be regarded as a measure of the heterocycle electron-acceptor effect. To find out whether

there is in fact a relationship between nucleus basicity and yield of aminoacyl-nucleotides, N^4-acetyl-cytidylic acid was synthesized (pC^{Ac^4}) where the presence of an electronegative group increases the bulk electron acceptor effect of the base. The condensation of pC^{Ac^4} with BOC-Ala-Im resulted in a 50% yield of 3′(2′)-O-BOC-Ala-pC^{Ac^4} compared with 26% for free pC; this is in accord with the assumption that the yield depends on the basicity of the nucleus NH_2 group.

TABLE 3. DEPENDENCE OF THE YIELDS OF 3′(2′)-O-BOC-ALA-NUCLEOTIDES (III) ON THE TYPE OF STARTING NUCLEOTIDE (III)

	Starting nucleotides				
	pU	pG	pA	pC	pC^{Ac^4}
Yield of III, %	56	48	42–46	26	50
pK of heterocyclic base in II[15]	—	2·30	3·70	4·24	

Removal of BOC-protection from the aminoacyl residue of 3′(2′)-O-BOC-aminoacyl-nucleotides was performed with dry trifluoroacetic acid. Table 4 contains the characteristics of the 3′(2′)-O-aminoacyl-nucleotides obtained.

With the two most difficult nucleotides pA and pC the generality of the imidazolide method has been demonstrated for different types of amino acids, such as alanine and phenylalanine,[16] as well as for polyfunctional amino acids—lysine[17] and aspartic acid. In the latter cases, imidazolides of N^{α}, N^{ε}-di-BOC-lysine and the β-t-butyl ester of N-BOC-aspartic acid were taken as starting compounds. Some complications were encountered in the condensation of di-BOC-Lys-Im with pA and pC in water–acetonitrile (1:1) giving rise along with III to a product of unknown structure; decrease of the reaction period to 1 hr reduced its amount to 5%. With 3′(2′)-O-di-BOC-lysyl-pA and -pC, both the BOC protecting groups were removed simultaneously by treatment with dry trifluoroacetic acid (15 min, 0°). However, the elimination of the butyl protective group of the β-carboxyl of 3′(2′)-O-BOC-Asp(OBu^t)-(pA) proceeds completely in 30% HBr in acetic acid. It should be mentioned that even under these drastic conditions (10 min, 20°) the ester in 3′(2′)-O-Asp-(pA) appeared stable, although the treatment resulted in considerable destruction of the glycosidic bond.

To study the contribution of the ε-amino group to the general properties of 3′(2′)-O-Lys-(pA), 3′(2′)-O-ε-aminocaproyl-(pA), [ε-Aca-(pA)], was synthesized as a model by condensation of BOC-ε-Aca-Im with pA under usual conditions followed by removal of BOC protection. The synthesis of 3′(2′)-O-BOC-ε-Aca-(pA) in DMS, unlike those of the α-amino acid esters, was complicated by the formation of large amounts of nucleotide-containing by-products.

The synthesis of 2′-O-ε-aminocaproyl-3′:5′-cyclophosphate has been also performed by condensation of BOC-ε-aminocaproic acid imidazolide with adenosine-3′:5′-cyclophosphate in dry medium (yield 26%) followed by removal of the protecting group. Compounds of this type are not described in the literature. This representative of the new group of nucleotide amino acid esters in some aspects is very similar to 3′(2′)-O-aminoacyl-nucleoside 5′-phosphates.

TABLE 4. CHROMATOGRAPHIC AND ELECTROPHORETIC CHARACTERISTICS OF 3'(2')-O-BOC-AMINOACYL-NUCLEOTIDES (III) AND OF 3'(2')-O-AMINOACYL-NUCLEOTIDES (IV)

Compounds	Paper chromatography in systems				Paper electrophoresis in System 1	
	1	2	3	4	E_f^{Gly}	E_f^{Hyn}
BOC–Ala–(pA)	0·26	0·48	0·33	0·32	0·47	0·22
Ala–(pA)	—	—	—	—	0·85	0·59
BOC–Phe–(pA)	0·85	—	—	—	0·12	0·06
Phe–(pA)	0·21	—	—	—	0·79	0·46
di-BOC–Lys–(pA)	0·80	—	—	—	0·09	0·03
Lys–(pA)	0·12	—	—	—	1·34	0·83
BOC–Asp(OBu^t)–(pA)	0·82	—	—	—	0·09	0·03
Asp(OBu^t)–(pA)	—	—	—	—	0·7	0·4
Asp–(pA)	—	—	—	—	0·61	0·30
Val–(pA)	0·55	—	—	—	0·53	0·32
di-BOC–Lys–(dpA)	0·75	—	—	—	0·09	0·03
Lys–(dpA)	—	—	—	—	1·57	1·03
BOC–Ala–(pC)	0·29	—	0·75	0·38	0·17	0·10
Ala–(pC)	—	—	—	—	0·88	0·56
BOC–Phe–(pC)	0·75	—	—	—	0·15	0·08
Phe–(pC)	—	—	—	—	0·87	0·48
di-BOC–Lys–(pC)	0·82	—	—	—	0·10	0·03
Lys–(pC)	—	—	—	—	1·37	0·85
BOC–Ala–(pG)	0·17	0·57	0·62	0·25	0·30	0·16
Ala–(pG)	—	—	—	—	0·70	0·45
BOC–Ala–(pU)	0·36	—	0·43	—	—	—
Ala–(pU)	—	—	—	—	0·73	0·40
BOC–Ala–(dpT)	0·35*	—	—	—	—	—
Ala–(dpT)	—	—	—	—	0·74	0·39

* System 5.

Although the imidazolide method can be applied to the synthesis of O-aminoacyl-nucleosides,[12] it does not exhibit any advantages over the known synthesis, e.g., those with DCC[6] or with symmetrical anhydrides.[7]

Further, we demonstrated that 3'(2')-O-aminoacyl-nucleoside-5'-pyro and triphosphates (III, Z = $P_2O_6H_3$ and $P_3O_9H_4$)[13] can be obtained by the imidazolide method. Little is known about compounds of this type. In 1959, the synthesis of 3'(2')-O-tryptophanyl-(pppA) and of 3'(2')-O-leucyl-(pppA)[18] was reported without any mention about the yields and properties of the compounds. In 1968 a paper[11] described the synthesis of 3'(2')-O-Gly-(ppA) and -(pppA), but no information was given concerning their chemical properties. The reactivity of compounds of this type are of interest because two macroergic bonds—pyrophosphate and ester—are present in their molecules.

The synthesis of 3'(2')-O-Ala-nucleoside-5'-di- and -tri-phosphates (IV, Z = $P_2O_6H_3$ or $P_3O_9H_4$) was performed according to Scheme 1 by condensation of BOC-Ala-Im (I) with nucleoside 5'-di- and triphosphates (II) followed by removal of protective groups. The conditions of the condensation, separation and purification, and the proof of structure are similar to those applied to the nucleotide derivatives.

Somewhat milder conditions were used for removal of protecting groups—1% ethereal hydrogen chloride during 15 min at 0°. The BOC protection was thus removed in a relatively high yield, while almost no disruption of ester bond was caused.

However, two compounds containing BOC-alanine and nucleoside-5'-di- or -triphosphate have been obtained in the condensations of BOC–Ala–Im with each pppA, pppU and ppG. Studies of the properties of these two BOC-Ala-nucleoside polyphosphates revealed, that in each the amino acid and the nucleoside derivative are present at a ratio 1 : 1 (hydroxylamine method[18]); the UV spectra are the same as those of the starting compounds (no aminoacylation at heterocyclic nuclei). Mild

TABLE 5. THE YIELDS, λ^{max} AND R_f OF 3'(2')-O-BOC-AMINOACYL-NUCLEOSIDE DI- AND TRIPHOSPHATES (III)

Starting nucleoside polyphosphates	Amount of II taken into reaction, OU	R_f of II in System Nr 1	R_f of III (two isomers)	Yield of III				λ_{max} nm
				Starting II eluted, OU	III eluted (two isomers) OU	% for II taken into reaction (two isomers)	% for II that entered reaction	
pppA	240	0·04	0·45	140	45	18·9	45·0	261
			0·65		10	4·2	10·0	
pppU	175·5	0·10	0·32	84·5	31·5	17·9	34·6	265
			0·41		49·5	28·1	54·5	
ppG	72·5	0·08	0·35	49·5	8	11·1	34·3	255
			0·21		5·2	7·2	30·9	
ppC	121	0·74*	0·92	62·5	21	17·4	35·9	272

* System 3.

alkaline hydrolysis transforms both the compounds to the starting amino acid and nucleoside polyphosphate. Finally, no *cis*-glycol group is present as revealed by periodate oxidation. The chromatographic and electrophoretic properties of the two compounds are very similar (Table 6). The above data suggest that the compounds are the 3'- and the 2'-isomer. This conclusion was confirmed by the similar stability of the compounds to ester bond hydrolysis (Table 7). Table 5 shows the yields of 3'(2')-O-BOC-Ala-nucleoside di- and -triphosphates (III) calculated for amounts of II taken and involved in reaction.

As mentioned above, there is no evidence concerning the stability of the ester bond in 3'(2')-O-aminoacylnucleoside 5'-di- and triphosphates of the type IV. We have compared 3'(2')-O-Ala-(pA), 3'(2')-O-Ala-(pppA) and 3'(2')-O-Ala-(pppU) at pH 2·5 and 7·2 (Table 9). These pH values were chosen because it was desired to obtain additional evidence in favour of the presence of ester rather than anhydridic bond in the compounds. With 3'(2')-O-aminoacyl-pA and an aminoacyl adenylates (P-O-C-bonding) it was demonstrated[18] that the former as well as the latter compounds are stable during 15 min at pH 2·5, whereas at pH 7·2 the former are relatively stable while the latter are completely hydrolysed. Paper chromatography revealed, that at both pH 2·5 and 7·2 no cleavage of the pyrophosphate bond occurred in 3'(2')-O-Ala-nucleoside 5'-triphosphates.

TABLE 6. THE R_f VALUES OF STARTING NUCLEOSIDE POLYPHOSPHATES (II), OF 3'(2')-O-BOC-L-ALANYL-NUCLEOSIDE DI- AND TRIPHOSPHATES (III) AND OF 3'(2')-O-L-ALANYLNUCLEOSIDE DI- AND TRIPHOSPHATES (IV)

Compound subjected to chromatography			R_f in system		
Designation	B	Z	1	3	2
I	Adenine	$P_3O_9H_4$	0·04	0·67	0·65
III*	Adenine	$P_3O_9H_4$	0·41	0·84	0·71
			0·47	0·76	0·67
IV*	Adenine	$P_3O_9H_4$	0·89	0·79	—
			0·87	0·71	—
I	Uracil	$P_3O_9H_4$	0·10	0·65	0·60
III*	Uracil	$P_3O_9H_4$	0·33	0·80	0·62
			0·37	0·72	0·58
IV*	Uracil	$P_3O_9H_4$	0·85	0·54	—
			0·82	0·65	—
I	Guanine	$P_2O_6H_3$	0·09	0·42	0·63
III*	Guanine	$P_2O_6H_3$	0·29	0·61	0·55
			0·35	0·50	0·57
IV*	Guanine	$P_2O_6H_3$	0·66	0·54	—
			0·65	0·56	—
I	Cytosine	$P_2O_6H_3$	0·17	0·79	—
III	Cytosine	$P_2O_6H_3$	0·21	0·89	—

* Two isomers.

TABLE 7. THE STABILITY OF 3'(2')-O-ALA-(PA), 3'(2')-O-ALA-(PPPA) and 3'(2')-O-ALA-(PPPU) TO HYDROLYSIS AS MEASURED BY HYDROXAMIC TEST (HYDROLYSIS DURING 15 MIN AT 37°)

Substance studied		% of unhydrolysed product	
		pH 2·5	pH 7·2
Ala–(pA)		96·0	89·5
Ala–(pppA)	Isomer 1	57·3	31·2
	Isomer 2	55·5	30·4
Ala–(pppU)	Isomer 1	69·5	33·2
	Isomer 2	71·2	35·6

As seen in Table 7, the stability of the ester bond in 3'(2')-O-aminoacyl-nucleoside 5'-triphosphates is considerably less than that of the corresponding 5'-monophosphates.

The imidazolide method can be used also to introduce fragments greater than amino acids into nucleotides. The possibility has been demonstrated in the synthesis of 3'(2')-O-peptidyl-nucleoside 5'-mono- and triphosphates [19–20] with two peptides – BOC-alanyl-alanine and BOC-leucyl-glycine.

It was demonstrated that not only N-protected, but also unprotected amino acid imidazolides can be introduced into condensation with nucleotides in aqueous and in dry organic media to give 3'(2')-O-aminoacyl-nucleoside 5'-phosphates in a single

stage. Trifluoroacetates or hydrochlorides of amino acids (valine, alanine, phenylalanine) react with carbonyldiimidazole in dry DMF and this solution subsequently added to nucleotides dissolved in dry dimethylsulphoxide or in aqueous buffer pH 7. The mixture is stirred during 5 hr at 20° followed by precipitation with organic solvents, paper electrophoresis and paper chromatography to give 3'(2')-O-aminoacyl-nucleotides. The yields of the compounds depend on the nature of nucleotides and solvents that are used as reaction media. Table 8 contains the yields of 3'(2')-O-L-valyl-nucleotides obtained by condensation of amino acid imidazolide trifluoroacetates with nucleotides.

TABLE 8. THE YIELDS OF 3'(2')-O-VALYL-NUCLEOTIDES (IV) THAT WERE OBTAINED WITHOUT PRELIMINARY PROTECTION OF AMINO ACID AMINO GROUP

Starting nucleotides	Yield of 3'(2')-O-valyl-nucleotide, %	
	In dry organic solvents	In water
pA	22–25	15–20
pU	10–12	4–5
pG	0	4–5
pC	Side reactions are the case	0

It should be mentioned that no side reactions occur with the nucleotide components pA, pU and pG in either dry medium and water [the yields of 3'(2')-O-valyl-nucleotides for amounts of nucleotides that enter into reactions are nearly quantitative; the only complication being apurinization with pA]. However, pC is converted in a high yield to a by-product of unknown structure and does not afford in dry medium any valine ester (IV). In aqueous medium, all pC is unchanged.Special studies revealed that no 3'(2')-O-poly-valyl-nucleotides was formed.

Similar results have been obtained with phenylalanine and alanine esters at nucleotide 3'- and 2'-hydroxy groups. This promising method is now under investigation to outline its possibilities and scope.

A very important problem has been whether any racemization of amino acid moieties occurs during either synthesis of imidazolides or condensation. To investigate this point, we employed the method based on circular dichroism of amino acid aldimine copper complexes elaborated earlier and applied to Schiff bases of amino acids and pyridoxal. Chelated coplexes of these bases with cupric ions exhibit abnormal optical rotatory dispersion—in the absorption band 370–390 nm. In the spectra of circular dichroism the ellipticity value depends on the individual properties of the aldimine chelated compound, whereas the sign of the ellipticity depends on the amino acid configuration.[21]

In the studies published,[21] the aldimine complexes of pyridoxal with α-amino acids were obtained at pH 8·5. We somewhat modified the method, particularly, because some minor racemization at this pH cannot be precluded. For the purpose, salicylic aldehyde was used instead of pyridoxal and the reaction was performed in dry ethanol to exclude any racemization. With pure Calbiochem preparations of L- and D-valine and of L- and D-phenylalanine the molar extinction coefficients were

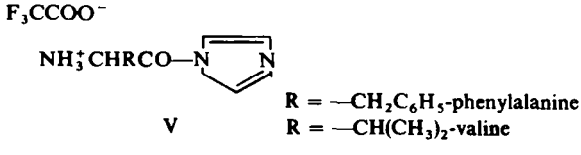
TABLE 9. THE CHARACTERISTICS OF ALDIMINDES AND ALDIMINE COPPER CHELATED COMPOUNDS OBTAINED FROM L- AND D-VALINE AND FROM L- AND D-PHENYLALANINE STANDARDS COMPARED WITH THOSE OF SIMILAR DERIVATIVES OBTAINED FROM AMINO ACIDS HYDROLYZED OFF THE N-IMIDAZOLIDES (V) AND THE O-ESTERS (IV) OF THE L-FORMS OF AMINO ACIDS

Amino acid	UV-spectra						Spectra of circular dichroism		
	Molecular extinctions of salicilidenimines at absorption maxima .10^{-3}			Molecular extinctions of copper compounds of aldimines at absorption maxima .10^{-3}			λ_{max} nm	$\Delta\varepsilon$	$[\theta]^{max}$
	322 nm	340 nm	397·5 nm	340 nm	365 nm	397·5 nm			
L-Valine standard	1·125	1·19	0·181	1·20	1·36	0·466	367	2·43	−8050
D-valine standard	1·125	1·20	0·182	1·22	1·38	0·469	368	2·47	+8120
L-valine from imidazolide (V)	1·110	1·14	0·181	1·21	1·35	0·466	366	2·50	−8230
L-valine from ester (IV)	1·128	1·14	0·172	1·20	1·38	0·466	368	2·49	−8200
L-phenylalanine standard	1·31	1·38	0·324	0·737	0·625	0·172	365	10·35	−34400
D-phenylalanine standard	1·34	1·40	0·330	0·728	0·624	0·171	364	10·50	+34600
L-phenylalanine from imidazolide (V)	1·34	1·37	0·326	0·725	0·627	0·172	365	10·20	−33600
L-phenylalanine from ester (IV)	1·28	1·47	0·335	0·697	0·641	0·173	363	10·30	−33900

$[\theta]_{max}$—molar ellipticity calculated according to formula: $[\theta] = 2{\cdot}303\ (4500/\pi)\cdot\Delta\varepsilon$ where $\Delta\varepsilon$ is the difference in the molar extinction coefficients in dextro- and levo-polarized light.

determined at absorption maxima in the UV spectra of the corresponding aldimines and of their chelated copper compounds, and also the ellipticity values (Table 9).

Absence of any amino acids racemization during their imidazolides condensation with nucleotides (particularly, pA) in water-DMF (5:1) was tested in two stages. (a) Imidazolides V were obtained from L-amino acid (L-valine and L-phenylalanine) trifluoroacetates by our usual procedure (experiment 7 in the Experimental) and hydrolyzed in water during a 6 hr period at 20°. The amino acids obtained were isolated and purified under conditions preventing racemization, and their aldimines were obtained. As seen in Table 9, no racemization occurs during the formation of N-protonized amino acid imidazolides. (b) 3′(2′)-O-phenylalanyl-pA (IV) and 3′(2′)-O-valyl-pA (IV) obtained from L-phenylalanine and L-valine imidazolides trifluoroacetates were subjected to hydrolysis with 2 N HCl (36 hr, 37°). The amino acids obtained were transformed into aldimines. As seen in Table 9, no racemization occurs during the synthesis of either phenylalanine or valine derivatives. Hence, racemization is not the case during the synthesis of 3′(2′)-O-aminoacyl-nucleoside 5′-phosphates from N-protonated amino acids by the imidazolide method in aqueous medium.



V

R = —$CH_2C_6H_5$-phenylalanine
R = —$CH(CH_3)_2$-valine

To find the pH values where 3'(2')-O-aminoacyl-nucleotides are most stable, the rates of the hydrolysis of the compounds at different pH-values have been measured (Fig 1). It was demonstrated that the ester bond in aminoacyl-nucleotides is hydrolyzed rapidly in alkaline and neutral media. The intervals of the greatest relative stability of aminoacyl-nucleotides are somewhat different for the derivatives of different amino acids (Fig 1). The two former compounds are rapidly hydrolysed at pH above 4·5–5·0, whereas the aspartyl ester of pA, Asp-(pA), having an additional carboxyl group, is stable up to pH 6·5.

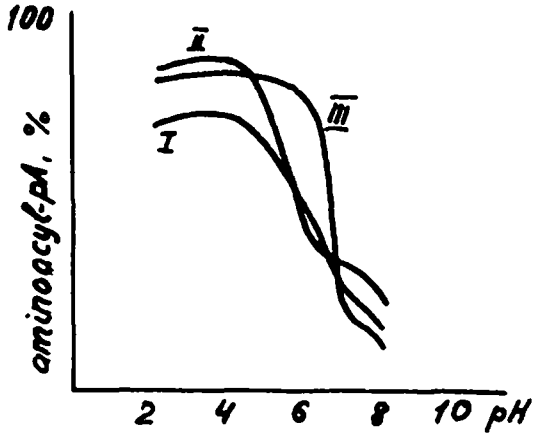


FIG 1. The stability of some O-aminoacyl-ncleotides at different pH values. I–Phe–(pA); II–Asp(OBu ^{t})–(pA); III–Asp–(pA).

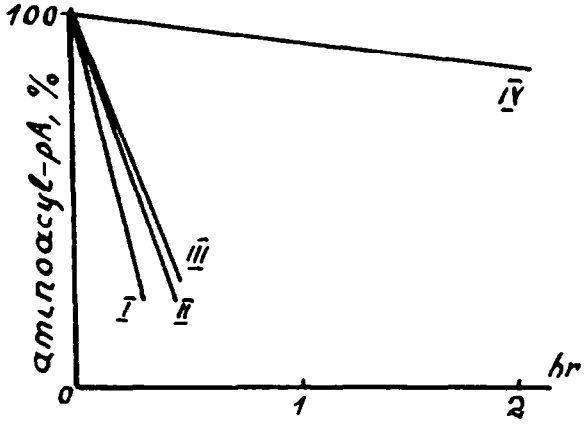


FIG 2. The comparative rates of hydrolysis of O-aminoacyl-(pA)'s at pH 7·8. I–Lys–(pA); II–Asp–(pA); III–Phe–(pA); IV–ε–Aca–(pA).

The high rate of the aminoacyl-nucleotides ester bond hydrolysis depends to a great extent on the presence of an α-amino group. As seen in Fig. 2, Phe-(pA), Lys-(pA) and Asp-(pA) at pH 7·8 are hydrolysed more rapidly ($\tau^{1/2}$ 19, 12 and 18 min, respec-

tively) than ε-Aca-pA ($\tau_{1/2}$ 360 min). The rates of the hydrolysis of Phe-pA and Lys-pA at this pH are close to those of the corresponding natural aminoacyl-tRNA's.[22–24] The hydrolysis of O-aminoacyl-nucleotides with protected amino acid residue amino groups proceeds much slower than that of the esters with free amino groups.

EXPERIMENTAL

Chromatography was performed on a Leningrad "fast" paper in solvent systems: n-butanol–water–acetic acid 5:3:2 (Nr 1); saturated aqueous ammonium sulphate–iso-propanol–water 79:2:17 (Nr 2); iso-butyric acid–water–acetic acid 100:50:1 (Nr 3); t-butanol–1N H_2SO_4 8:2 (Nr 4); iso-butyric acid–0·5 N ammonia 10:6, pH 3·6 (Nr 5).

Electrophoresis was performed on a Leningrad "slow" or Whatman 3MM paper in 6% AcOH (pH 2·5, system Nr 1) and in phosphate buffer, pH 7·2 (system Nr 2) at 22 V/cm during 1–1·5 hr. The E_j values were referred to glycine and histidine (when ions moved to cathode) or to pycric acid (when ions moved to anode).

UV spectrophotometry was performed with a spectrophotometer SF-4A (USSR) or EPS-3T (Japan). The spectra of circular dichroism were measured with a dichrograph of Roussel–Jouan (France).

1. *The synthesis of* 3′(2′)-O-*t-butyloxycarbonyl-*L-*aminoacylnucleotides* (III) *in water—DMF* (5:1) [3′(2′)-O-BOC-L-Ala-nucleotides (III) as example]

CDI (m.p. 109–113°; 71 mg) was added to a soln of 70·8 mg I (m.p. 72–73°) in 0·2 ml dry DMF and the mixture stirred for 5–10 min at 20–22°. The amide formed was poured into a soln of ca. 0·04 mmoles of II in 1 ml water. The mixture was stirred for 3·5 hr at 20–22° and applied onto sheet of Whatman 3 MM paper (58 × 47 cm). The chromatography was performed in System 1 during 10–12 hr. The UV absorbing bands were cut out and the substances eluted with water (30–50 ml) in an elution chamber. The aqueous solns were evaporated and the yields of the compounds determined spectrophotometrically, as OU (optical units) at λ_{max} characteristic of given nucleotide. The results for each of the nucleotides are presented in Table 2. The preparations of III were purified from substances extracted from paper by gel-filtration on Bio-Gel P2 in 0·5% AcOH (60 × 1 cm column for 5–10 mg of substance).

2. *The synthesis of* 3′(2′)-O-*t-butyoxycarbonyl-*L-*aminoacylnucleotides in water:acetonitrile:THF* (4:3:1) [3′(2′)-O-BOC-L-Phe-nucleotides (III) as example]

CDI (10 mg) was added to soln of 12 mg t-butyloxycarbonyl phenylalanine in 0·3 ml THF, and the mixture agitated for 5–10 min at 20–22°. The imidazolide obtained was added to a soln of 7 μmoles of Na-salt of II in a mixture of 0·4 ml water and 0·1 ml acetonitrile. The mixture was stirred for 3 hr, purified by extraction with 0·3 ml EtOAc and finally treated according to procedure 1. The results are presented in Table 2.

3. *The synthesis of* 3′(2′)-O-*t-butyloxycarbonyl-*L-*aminoacylnucleotides* (III) *in DMF–DMS* (1:3) [β-t-butyl ester of 3′(2′)-O-BOC-L-Asp-adenylic acid (III) as example]

CDI (32 mg) was added to a soln of 47 mg BOC-aspartic acid β-t-butyl ester in 0·1 ml DMF, and the imidazolide obtained was during 5–10 min poured into 0·3 ml DMS containing 5 mg (0·014 mmoles) sodium adenylate. The reaction mixture was left for 15–17 hr at 20–22° and III was isolated as in procedure 1. The results are presented in Table 2.

4. *The synthesis of* 3′(2′)-O-*t-butyloxycarbonylaminoacylnucleoside* 5′-*di- and triphosphates* (III, Z = $P_2O_6H_3$ *and* $P_3O_9H_4$). [3′(2′)-O-BOC-Ala-nucleoside 5′-di- and -triphosphates as example]

CDI (71 mg) was added to a soln of 70·8 mg N-t-butyl-oxycarbonyl-L-alanine in 0·2 ml dry DMF, the mixture agitated for 5–10 min at 20–22° and added to a soln of ca. 0·04 mmoles nucleoside di- or triphosphate (II) in 1 ml water. The mixture was stirred for 3·5 hr at 20–22° and applied on Whatman 3 MM paper (58 × 47 cm). The chromatography was performed during 10–12 hr in System 1. The UV absorbing bands were cut out and the substances eluted with water (30–40 ml) at 4°. The aqueous soln was evaporated. The yields of the compounds were measured spectrophotometrically as OU (optical units) at λ_{max} characteristic of given nucleoside di- or triphosphate. The results are presented in Table 5.

5. *Removal of t-butyloxycarbonyl protecting group from* 3'(2')-O-*t-butyloxycarbonyl-aminoacyl-nucleotides.*

(*a*) *With trifluoroacetic acid.* 5–20 mg samples of 3'(2')-O-BOC-aminoacyl-nucleotides were dissolved at 0° in 0·5 ml dry trifluoroacetic acid, the soln left at 0° for 15 min and rapidly evaporated *in vacuo* at a temp not exceeding 30°. The residue was dissolved in water, the soln neutralized, if necessary, with 1% ammonia and the substances purified by paper chromatography in System I. The chromatographic and electrophoretic characteristics of the compounds obtained are presented in Table 4.

(*b*) *With* 10% *ethereal hydrogen chloride.* 5–20 mg samples of 3'(2')-O-BOC-aminoacyl- nucleotides were treated with 5 ml 10% HCl in dry ether at 0°—the ppt was triturated for 5 min at 0°. The solvent was removed at a temp below 10°, the residue dissolved in water, the soln neutralized, if necessary, with 1% ammonia and the substances purified by paper chromatography in System 1. The results are presented in Table 4.

6. *The removal of t-butyloxycarbonyl protecting group from* 3'(2')-O-*t-butyloxycarbonyl-aminoacyl-nucleoside 5'-di- and triphosphates*

Samples of 3'(2')-O-BOC-aminoacyl-nucleoside 5'-di- or triphosphates (5–20 mg) were treated with 5 m 1% HCl in dry ether at 0°, the ppt was triturated for 5 min at 0°, the solvent decanted and the residue washed with ether (5 times with 5 ml) and dried *in vacuo*. Further purification was performed as in experiment 5. The chromatographic and electrophoretic characteristics of the compounds obtained are presented in Table 6.

7. *The synthesis of* 3'(2')-O-*aminoacylnucleotides* (IV) [3'(2')-O-L-valyl-nucleotides as example]

CDI (71 mg; 0·44 mmoles) was added to a soln of 22·4 mg (0·4 mmoles) L-valine trifluoroacetate in 0·1 ml DMF, and the mixture agitated for 5–10 min. 0·1 mmoles of nucleotide in 0·5 ml 0·1 M phosphate buffer pH 7·0 was added and the mixture stirred for 6 hr at 20–22°. 15 ml ice-cold acetone was added, the ppt was centrifuged and dissolved in 0·2 ml water. The substances were separated by paper electrophoresis (three sheets, each 40 cm wide). The 3'(2')-O-valyl-nucleotides obtained were eluted with water at 4° and the solns freeze-dried. The results are presented in Table 8.

8. *Synthesis of the copper chelated compounds of amino acid salicyl-aldimines* (N-salicylidene-L-valine as example).

Suspension of L-valine (167 mg, 1·43 mmoles) and salicylic aldehyde (116·7 mg, 0·956 mmoles) in 50 ml dry EtOH was stirred for 14 hr at 20–22°, excess of unreacted L-valine removed by filtration and the optical density determined at absorption maxima 322, 340 and 397·5 nm (the ratio of the optical densities at 322:397·5 nm was 6·2, and that at 340:397·5 nm was 6·50; this ratio in other experiments with valine was used to evaluate the purity of salicylidene valine). 3·8 ml of 50% aqueous EtOH containing $1{\cdot}428.10^{-2}$ mmoles cupric sulphate was added to 0·2 ml of the salicylidene valine soln obtained and after the determination of optical density at 340 and 365 nm (340:365 nm ratio 0·885) the circular dichroism spectra were measured (the results are presented in Table 9).

Compound V obtained as described in experiment 7 was left for 6 hr in 2 ml water, the soln evaporated and the residue in 0·2 ml 1N HCl applied to a column with Dowex 50 × 8 (H^+) 50–100 mesh (5 × 0·7 cm), the column washed with water to neutral pH and L-valine eluted with 60 ml 6% pyridine. The eluate was evaporated and the valine obtained reacted with salicylic aldehyde.

2 mg (4·5 μmoles) of 3'(2')-O-L-valyl-(pA) obtained as described in experiment 11 was hydrolysed in 2 ml 2 N HCl during 36 hr at 37°, the reaction products separated by electrophoresis on paper and further purified as described above. L-valine obtained was transformed to salicylidene valine.

9. *Determination of the rates of* O-*aminoacyl-nucleotides hydrolysis at different pH values*

OU samples of compounds (3–4) were dissolved in 0·02 ml water and 0·1 ml buffer (pH 2·8) was added. The moment of buffer addition was assumed as zero time. At time intervals the solns were cooled to 0° and applied on paper. The separation of hydrolysates was performed by chromatography in System 1 with Phe-(pA) and ε-Aca-(pA) or by electrophoresis with Lys-(pA) and Asp-(pA). The spots were cut out and eluted with 5 ml 5% NH_4OH and the concentrations determined spectrophotometrically. Correction for losses and degradation of substances during the separation was obtained from experiments with control samples; the little (ca. 5%) destruction of glycosidic bond was not taken into account in the calculations.

10. *The rates of the hydrolysis of O-aminoacylnucleotides at pH 7·8*

Phosphate buffer (pH 7·8; 0·1 ml; 0·1 M) was added to 3–4 OU sample of O-aminoacyl-(pA) in 0·02 ml water and incubated at 37° for 5, 10, 15, 20, 25 and 30 min. The further treatment was performed as described in experiment 9.

This paper was translated into English by M. A. Grachev (Novosibirsk, Institute of Organic Chemistry, Academy of Sciences of USSR).

REFERENCES

[1] H. G. Zachau and W. Karau, *Chem. Ber.* **93**, 1830 (1960); H. G. Zachau, *Ibid.*, **93**, 1822 (1960)

[2] J. Sonnenbichler, H. Feldmann and H. G. Zachau, *Z. Physiol. Chem.* **334**, 283 (1963); H. Feldmann and H. G. Zachau, *BBRC* **15**, 13 (1964)

[3] J.-P. Waller, T. Erdös, F. Lemoine, S. Guttmann and E. Sandrin, *BBA* **119**, 566 (1966)

[4] J. Rychlík, S. Chládek and J. Zemlička, *Ibid.* **138**, 640 (1967)

[5] R. E. Monro, J. Černá and K. A. Marcker, *Proc. Nat. Acad. Sci., USA* **61**, 1042 (1968); R. E. Monro, K. A. Marcker, *J. Mol. Biol.* **25**, 347 (1967)

[6] Z. A. Shabarova, N. I. Sokolova and M. A. Prokofyev, *Dokl. Akad. Nauk SSSR* **128**, 740 (1959): N. I. Sokolova, V. A. Bakanova, Z. A. Shabarova and M. A. Prokofyev, *Zh. Obshch. Khimii* **33**, 2480 (1963)

[7] D. H. Rammler and H. G. Khorana, *Federation Proc.* **19**, 349 (1960); *J. Am. Chem. Soc.* **85**, 1997 (1963)

[8] J. Žemlička and S. Chládek, *Tetrahedron Letters* No. 34, 3057 (1965), *Coll. Czechoslov. Chem. Commun.* **31**, 3775 (1966)

[9] Th. Wieland, H. Merz and G. Pfleiderer, *Chem. Ber.* **93**, 1816 (1960); Th. Wieland, F. Taenicke, H. Merz and M. Ossorio, *Liebigs Ann.* **613**, 95 (1958)

[10] S. Chládek, J. Zemlička and F. Šorm, *BBRC* **22**, 554 (1966); S. Chládek and J. Zemlička, *Coll. Czechoslov. Chem. Commun.* **32**, 1776 (1967); **33**, 232 (1968)

[11] J. Zemlička and S. Chládek, *Ibid.* **33**, 3293 (1968)

[12] B. P. Gottikh, A. A. Krayevsky, P. P. Purygin, T. L. Tsilevich, Z. B. Belova and L. N. Rudzite, *Izv. Akad. Nauk SSSR, ser. khim.* 2571 (1967); A. A. Krayevsky, P. P. Purygin, L. N. Rudzite, Z. S. Belova and B. P. Gottikh, *Ibid.* 378 (1968)

[13] P. P. Purygin, A. A. Krayevsky and B. P. Gottikh, *Ibid.* 1084 (1968)

[14] F. H. C. Stewart, *Chem. & Ind.* 1967 (1960), *Austral. J. Chem.* **21**, 1639, 2543 (1968)

[15] E. Chargaff and J. Davidson, *The Nucleic Acids*, IL, p. 491. Moscow (1957)

[16] N. B. Tarussova, A. A. Krayevsky, P. P. Purygin, T. L. Tsilevich and B. P. Gottikh, *Izv. Akad. Nauk SSSR, ser. khim.* in the press

[17] N. B. Tarussova, L. Kuznetsova, A. A. Krayevsky and B. P. Gottikh, *Ibid.* in the press

[18] S. Weiss, H. G. Zachau and F. Lipmann, *Arch. Biochem. Biophys.* **83**, 101 (1959)

[19] B. P. Gottikh, A. A. Krayevsky and P. P. Purygin, *Izv. Akad. Nauk SSSR, ser. khim.* in the press

[20] P. P. Purygin, A. A. Krayevsky and B. P. Gottikh, *Ibid.*, in the press

[21] Yu. M. Torchinsky, *Biokhimia* **31**, 1046 (1966)

[22] J. Rychlík, *Coll. Czechoslov. Chem. Commun.* **30**, 2259 (1965)

[23] Y. Lapidot, N. de Groot and J. Fry-Shafrir, *BBA* **145**, 292 (1967)

[24] C. F. Heredia and H. O. Halvorson, *Biochemistry* **5**, 947 (1966)