SYNTHESIS OF PHOSPHONYLMETHYL ANALOGUES OF DIRIBONUCLEOSIDE MONOPHOSPHATES CONTAINING MODIFIED INTERNUCLEOTIDE BOND

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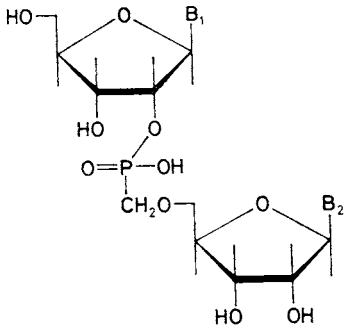
Received February 23rd, 1987

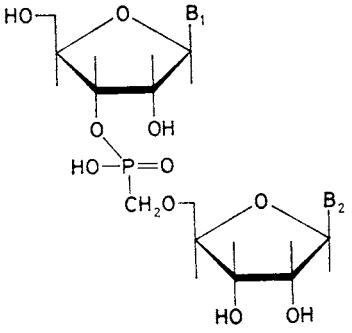
Two types of (2′—5′)- and (3′—5′)-isomers of analogues of diribonucleoside monophosphates derived from O-phosphonylmethyl derivatives of ribonucleosides, differing in the position of the methylene group in the internucleotide bond (type *A*, *B*, *C*, and *D*) have been synthesized. The compounds were prepared from methyl esters of O-phosphonylmethylribonucleosides *I* and *XVII* by a procedure analogous to the phosphotriester method of oligonucleotide synthesis. The phosphonate moiety was protected with the methyl group. After protection of the hydroxyl or amino groups, the compounds *I* or *XVII* were condensed with protected ribonucleosides *VIII*, *XI*, *XIV*, *XXIII* to afford the neutral diesters *IX*, *XII*, *XV*, *XXIV*, *XXVI*, and *XXVIII* which were isolated by short column chromatography on silica gel. Deprotection, ion-exchange chromatography, and semipreparative HPLC gave (2′—5′)- and (3′—5′)-isomers of both types of O-phosphonylmethyl analogues of diribonucleoside monophosphates (*X*, *XIII* and *XXV*, *XXVII*). All these compounds are resistant towards cleavage with ribonucleases A and T2 and with snake venom exonuclease. Under conditions of alkaline hydrolysis of RNA, the analogues of the type *A* and *B* are completely stable whereas compounds of the type *C* and *D* are degraded to form 2′- or 3′-O-phosphonylmethylribonucleosides and 3′-terminal ribonucleosides.

Within the framework of systematic investigation on isosteric isopolar nucleotide analogues that resist degradation with nucleolytic enzymes we prepared a novel type of analogues containing a methylene group inserted between the phosphorus atom and the oxygen atom of the nucleoside sugar moiety (for a review see refs[1,2]). These O-phosphonylmethyl derivatives resist, and some of them even inhibit, the action of dephosphorylating enzymes[3]. In order to ascertain whether monoesters of these analogues (*i.e.* analogues of phosphoric acid diesters) are resistant towards enzymes catalyzing hydrolysis of the phosphodiester bonds, we recently prepared methyl esters and cyclic esters of 2′- and 3′-O-phosphonylmethylribonucleosides and found that they are not cleaved with ribonucleases[4]. Ribonucleases (cyclizing ribonucleate: nucleotide 2′-transferases, EC 2.7.7) cleave the internucleotide bond by transfer reaction under formation of 2′,3′-cyclic nucleotide which in the next step undergoes stereospecific hydrolysis. The model analogues mentioned[4] exclude the action of ribonucleases in a hydrolytic reaction; it is, however, known that the 3′-terminal

nucleoside of the internucleotide bond has a considerable cooperative effect in complex formation with the enzyme as well as in the catalyzed transfer reaction[5]. Therefore, the resistance of the above-mentioned methyl esters of 3'-O-phosphonylmethyl-ribonucleosides[4] cannot be regarded as a definitive proof of internucleotide bond stability in the transfer reaction. The previously studied analogue UpU resisted degrading enzymes[3]; however, such resistance need not be general. To obtain a convincing evidence on stability of an analogous bond towards ribonucleases and other relevant enzymes, *i.e.* exonucleases of the type I and II (which degrade internucleotide bonds in RNA, DNA, and oligonucleotides), and also to be able to study conformational problems connected with character of an analogous internucleotide bond, we prepared systematically all the four possible types of diribonucleoside monophosphate analogues $(A-D)$, containing a phosphonylmethyl group etherically bonded



Type *A* (*X*)



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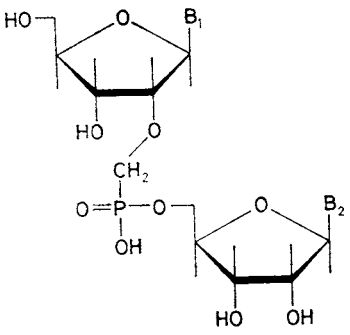
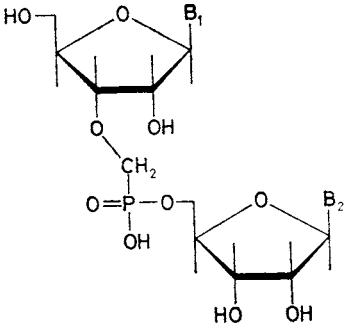


Type *C* (*XXV*)



Type *D* (*XXVII*)

to hydroxyl group of the nucleoside sugar moiety. The types *A* and *B* are analogues of $(2'-5')$- and $(3'-5')$-dinucleoside phosphates with an ether bond attached to

the 3′-terminal nucleoside whereas the types *C* and *D* contain an isomeric internucleotide bond with the phosphonylmethyl ether grouping bonded to the 2′- or 3′-hydroxyl of the 5′-terminal nucleoside. According to theoretical considerations, compounds of the type *D* might perhaps be degraded by ribonuclease-catalyzed transfer reaction or by ribonucleate (deoxyribonucleate): 3′-nucleotidohydrolase (EC 3.1.4.7, *e.g.* spleen phosphodiesterase); on the other hand, types *A* and *B* could possibly be cleaved by action of ribonucleate (deoxyribonucleate): 5′-nucleotidohydrolase (EC 3.1.4.9, *e.g.* venom exonuclease).

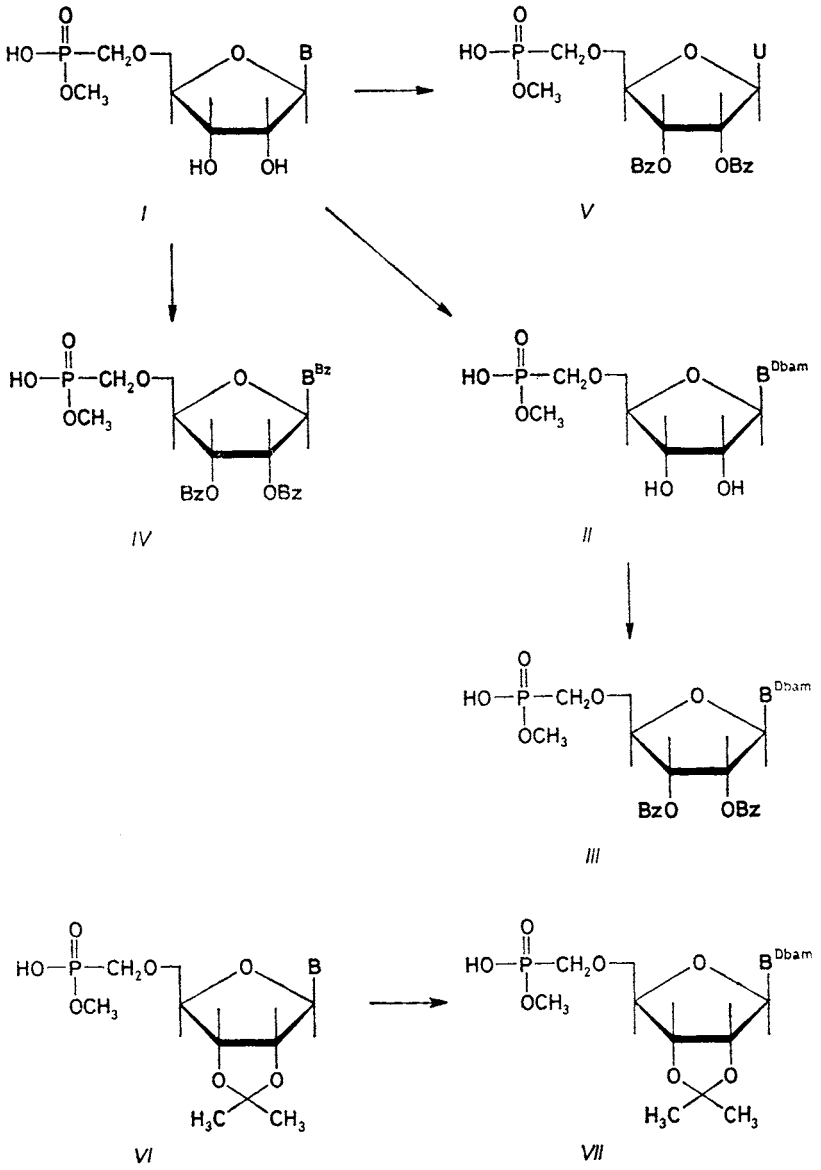
The aim of this work was to verify, in the four mentioned types of dinucleoside phosphate analogues with various bases B_1 and B_2, the stability of the analogous bonds towards selected enzyme types, not only in order to confirm the theoretical assumptions but also to find whether the resistance of the bond to such enzymes can prove the presence of a phosphonate bond in an oligo- (or poly) nucleotide chain. The preliminary results, together with the synthetic strategy, have been published in part already earlier[6].

Originally, we prepared a compound of the type *B* (a UpU analogue) using a procedure, analogous to the phosphodiester method[3] of dinucleoside phosphates synthesis. Since, however, this method is not very effective, we resorted to the phosphotriester method that makes use of nucleotide monoesters to give, after condensation, neutral phosphotriesters. Because the hitherto used methods, leading to O-phosphonylmethyl derivatives of nucleosides[4,7,8], exclude in principle a preparative analogy of the phosphite[9], phosphoramidite[10] or H-phosphonate[11,12] methods of internucleotide bond synthesis, the analogy with the phosphotriester method[13,14] represents the only alternative for the preparation of compounds *A*–*D*. Pairs of the (2′–5′)- and (3′–5′)-isomers (*A* and *B* or *C* and *D*) were prepared either separately by regiospecific syntheses or as mixtures with subsequent preparative or analytical separation of the corresponding pairs after deprotection.

The synthetic strategy utilizes the methyl esters of O-phosphonylmethylribonucleosides *I* and *XVII*, prepared previously in our Laboratory[4,7,8]. The methyl ester group in these compounds serves as a sufficiently stable but easily removable protecting group of the phosphonate internucleotide bond. Originally, methyl ester was introduced by Letsinger and coworkers[9] as a phosphate-protecting group in the phosphite approach to oligonucleotide synthesis. Later, this group was used also by other authors[15,16]. Introduction of suitable, particularly alkali-labile groups, protecting the hydroxy and amino groups, was advantageous only at the stage of preformed phosphonates *I* and *XVII*.

In the preparation of compounds of the type *A* and *B* derived from 5′-O-phosphonylmethylribonucleosides (compounds *X*, *XIII*, and *XVI*), the exocyclic amino groups in the starting compound *I* were protected with the benzoyl or dibutylaminomethylene groups[17]; the latter group is more stable than the generally used[18,19] dimethylaminomethylene group and, because of its considerable hydrophobic

character, it exhibits also suitable chromatographic properties. The 2',3'-*cis*-diol grouping in compounds *I* was protected with the benzoyl or isopropylidene groups (Scheme 1). Since removal of the acid-labile isopropylidene group requires such

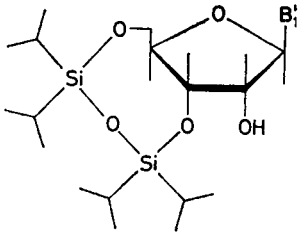


SCHEME 1

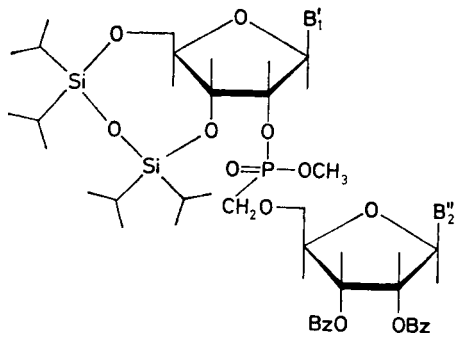
In formulae *I*, *VI*: *a*; B = U, *b*; B = C, *c*; B = A, *d*; B = G. In formulae *II*—*IV*, *VII*: *a*; B = C, *b*; B = A, *c*; B = G. (See footnote in Table I)

conditions which might induce isomerization of the formed internucleotide bond, this protection was used only in the preparation of mixed isomers *A* and *B* (*XVI*).

The thus-protected 5'-O-phosphonylmethyl derivatives *III*–*VII* were condensed with ribonucleosides containing one free hydroxyl functionality. For the preparation of compounds *A* (*X*) we used 3',5'-O-tetraisopropyldisiloxanyl derivatives *VIII*

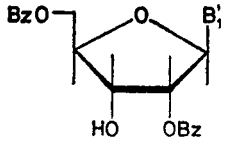


VIII

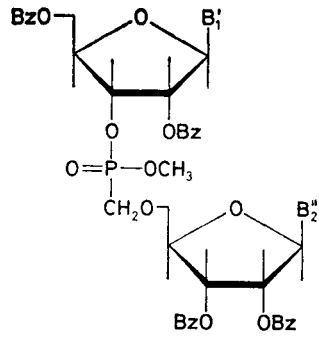


IX

Combination of bases B'_1, B''_2 in formulae *VIII*, *IX* and B_1, B_2 in formula *X* is given in Tables I and III



XI

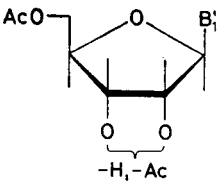


XII

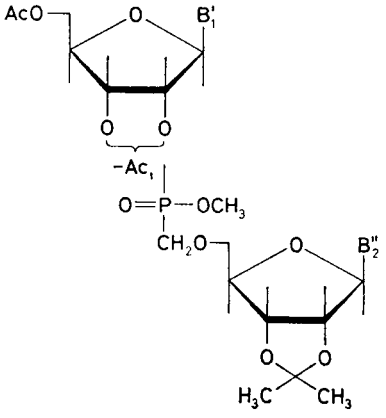
Combination of bases B'_1, B''_2 in formulae *XI*, *XII* and B_1, B_2 in formula *XIII* is given in Tables I and III

(see ref.[20]), compounds *B* (*XIII*) were synthetized by condensation with 2',5'-di-O--benzoylribonucleosides *XI* and the mixed isomers *A* and *B* were prepared using 2'(3'),5'-di-O-acetyl derivatives *XIV*, obtained by hydrolysis of 2',3'-O-ethoxyethylideneribonucleosides[21].

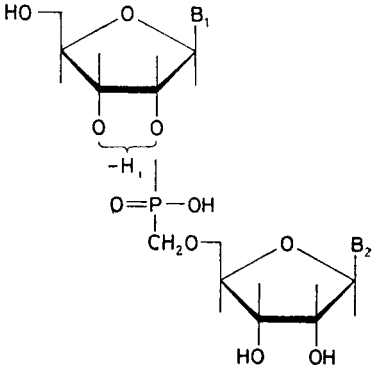
In analogues of the type *C* and *D* (compound *XVII*), derived from 2'- and 3'-O--phosphonylmethylribonucleosides, the hydroxyl and amino functionalities in the starting materials were protected with the same protecting groups as in compounds *I* (Scheme 2). In the case of *XVIIa*, we also used its di-O-tetrahydropyran-2-yl derivative *XIX* which was prepared by acid-catalyzed addition of dihydropyran. As



XIV



XV



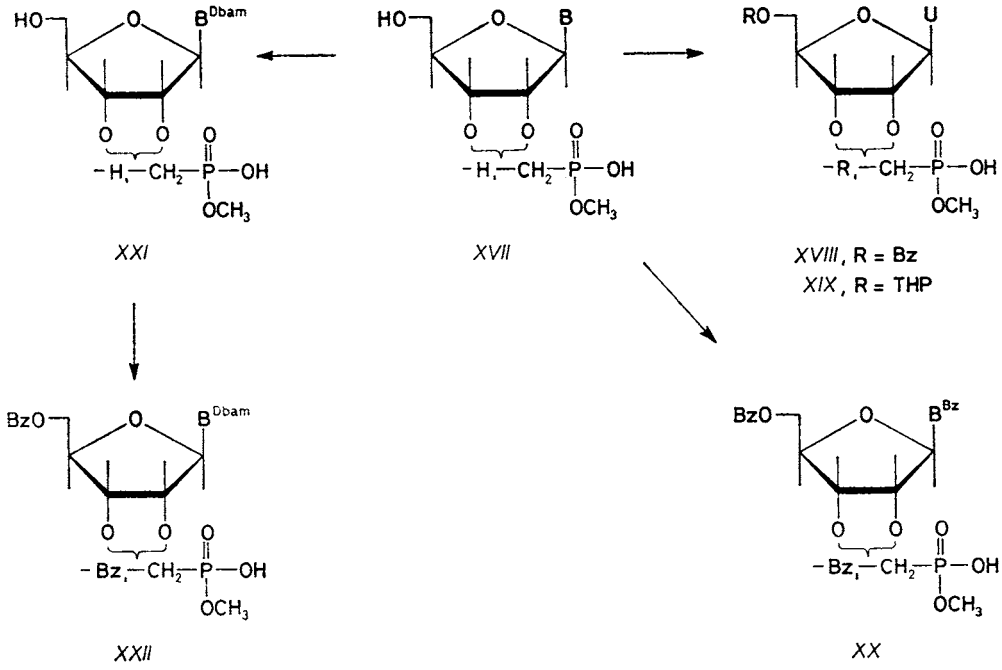
XVI

Combination of bases B'_1, B''_2 in formulae *XIV*, *XV* and B_1, B_2 in formula *XVI* is given in Tables I and III

nucleoside components for these condensations we used invariably 2',3'-O-isopropylideneribonucleosides *XXIII* with free 5'-hydroxyl group. The isopropylidene group (as well as other acid-labile groups) can be used for protection of the *cis*-diol grouping in compounds *C* or *D* because under conditions of its removal the acid--catalyzed intramolecular transesterification (isomerization) of the formed internucleotide bond is in principle excluded.

The hydroxyl groups (and exocyclic amino groups) in methyl esters of O-phosphonylmethylribonucleosides *I* and *XVII* were protected by two different methods.

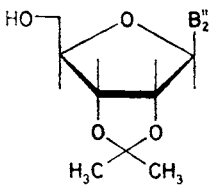
Treatment of lithium salts of unprotected compounds *Ib–d* and *XVIIb–e* with benzoyl cyanide[22] in dimethylformamide in the presence of triethylamine afforded he expected N,O-tribenzoyl derivatives *IVa–c* and *XXa–d* in relatively low yields;



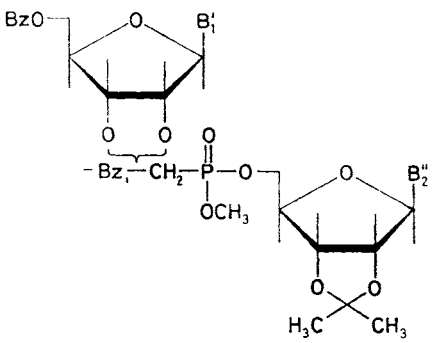
SCHEME 2

In formulae *XVII*: *a* (2′(3′)); B = U, *b* (2′(3′)); B = C, *c* (2′); B = A, *d* (3′); B = A, *e* (2′(3′)); B = G. In formulae *XX–XXII*: *a* (2′(3′)); B = C, *b* (2′); B = A, *c* (3′); B = A, *d* (2′(3′)); B = G. (See footnote in Table I)

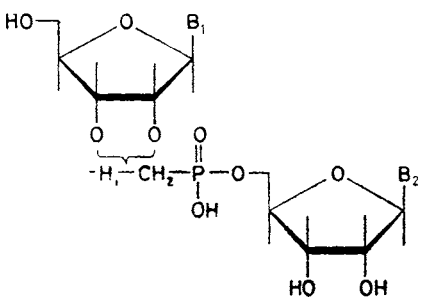
in addition to these compounds, chromatography of the reaction mixture on octadecyl silica gel afforded also 2′,3′- and 2′(3′),5′-di-O-benzoyl derivatives of compounds *Ib–d* and *XVIIb–e*, the total yield being over 90%. Only the uridine derivatives *Ia* and *XVIIa* afforded compounds *V* and *XVIII* in a quantitative yield. Under these benzoylation conditions, we detected neither the mixed anhydride in the case of compounds *I* nor the formation of six-membered 2′,3′-cyclic phosphonate (which would have arisen by an intramolecular cyclization of such reactive mixed anhydride with participation of the 2′- or 3′-hydroxyl group) in the case of derivatives *XVII*. Therefore, we tried another strategy consisting in subsequent protection of the amino and hydroxyl function in compounds *Ib–d* and *XVIIb–e*: In the first step, the N-dibutylaminomethylene group was selectively introduced by reaction



XXIII



XXIV, 2'-isomer
XXVI, 3'-isomer
XXVIII, 2'(3')-isomer



XXV, 2'-isomer
XXVII, 3'-isomer
XXIX, 2'(3')-isomer

Combination of bases B_1', B_2'' in formulae *XXIII*, *XXIV*, *XXVI*, *XXVIII* and B_1, B_2 in formulae *XXV*, *XXVII*, *XXIX* is given in Tables II and III

of lithium salts of *Ib–d* and *XXIa–d* with dibutylformamide dimethylacetal in dimethylformamide. Chromatography on octadecyl silica gel afforded the N-dibutylaminomethylene derivatives *IIa–c* and *XXIa–d* in good yields. Their subsequent benzoylation with benzoyl cyanide under the above-mentioned conditions gave compounds *IIIa–c* and *XXIIa–d* in quantitative yields. The dibutylaminomethylene group was also used for protection of the amino group in the isopropylidene derivatives *VIb–d*; compounds *VIIa–c* were again isolated by chromatography on octadecyl silica gel.

The diester condensation (Tables I and II) was performed invariably with 2,4,6-triisopropylbenzenesulfonyl chloride in acetonitrile in the presence of 1-methylimidazole[23]. As with nucleotides, the reaction was completed during several minutes. The protected neutral diesters *IX*, *XII*, *XV*, *XXIV*, *XXVI*, and *XXVIII* which were isolated from the mixture in 70–90% yields by short column chromatography on silica gel are listed in Tables I and II. In the deprotection the ester-bonded methyl

group was removed first, using aqueous pyridine[24]. The alkali-labile groups (acetyl, benzoyl, dibutylaminomethylene) were then removed by treatment with dioxane–aqueous ammonia and the acid-sensitive functionalities (isopropylidene, tetrahydropyranyl) were cleaved off with aqueous mineral acids (sulfuric or hydrochloric acid). The tetraisopropyldisiloxanyl group in compounds *IX* was removed by reaction with tetrabutylammonium fluoride in tetrahydrofuran. The free diribonucleoside monophosphate analogues *X*, *XIII*, *XVI*, *XXV*, *XXVII*, and *XXIX* were then isolated by ion exchange chromatography on DEAE-Sephadex A-25 in triethylammonium hydrogen carbonate, purified by reverse phase HPLC on octadecyl silica gel, and stored as lithium salts. All the prepared compounds were homogeneous according to HPLC, paper chromatography and electrophoresis, and their UV absorption spectra corresponded to the expected parameters for their dinucleoside phosphate counterparts (Table III).

The enzymatic assays were performed with pancreatic ribonuclease (compounds *B* and *D* with 5'-terminal uridine or cytidine), nonspecific ribonuclease T2 and snake

TABLE I

Protected analogues of dinucleoside phosphates with 5'-O-phosphonylmethylnucleotide bond

Compound	B_1' [a]	B_2'' [a]	Starting components	
			Nucleoside	Phosphonate
IXa	U	U	*VIIIa*	*V*
IXb	A^{Bz}	U	*VIIIc*	*V*
IXc	U	C^{Dbam}	*VIIIa*	*IIIa*
IXd	G^{Bz}	C^{Bz}	*VIIId*	*IVa*
IXe	U	A^{Dbam}	*VIIIa*	*IIIb*
IXf	A^{Bz}	A^{Bz}	*VIIIc*	*IVb*
IXg	C^{Bz}	G^{Dbam}	*VIIIb*	*IIIc*
XIIa	U	C^{Dbam}	*XIa*	*IIIa*
XIIb	U	A^{Dbam}	*XIa*	*IIIb*
XIIc	U	U	*XIa*	*V*
XIId	G^{Bz}	C^{Bz}	*XIb*	*IVa*
XVa	C^{Bz}	G^{Dbam}	*XIVa*	*VIIc*
XVb	A^{Bz}	U	*XIVb*	*VIa*
XVc	A^{Bz}	A^{Dbam}	*XIVb*	*VIIb*

[a] Abbreviations: A^{Bz} N^6-benzoyladenin-9-yl, A^{Dbam} N^6-dibutylaminomethyleneadenin-9-yl, C^{Bz} N^4-benzoylcytosin-1-yl, C^{Dbam} N^4-dibutylaminomethylenecytosin-1-yl, G^{Bz} N^2-benzoylguanin-9-yl, G^{Dbam} N^2-dibutylaminomethyleneguanin-9-yl, U uracil-1-yl, Bz benzoyl, THP tetrahydropyran-2-yl, TPSCl 2,4,6-triisopropylbenzenesulfonyl chloride.

venom exonuclease; the enzyme concentration used was sufficient for almost quantitative fission of natural diribonucleoside phosphates. Although in natural substrates both the mentioned ribonucleases cleave specifically the (3′–5′)-internucleotide bond, we checked under the given conditions also the behaviour of (2′–5′)-isomers *A* and *C* to exclude the possibility that the presence of O—CH_2—P grouping makes the conformation of (2′–5′)-analogues more similar to that in the natural substrate, and thus possibly more susceptible to the transfer reaction. Since snake venom exonuclease degrades both the (2′–5′)- and (3′–5′)-type of natural internucleotide bond, we studied its activity towards all the four analogue types. Even upon prolonged enzymatic treatment, we registered no observable degradation of the studied analogues with any of the enzymes used. In accord with our assumptions, the modified internucleotide bonds are resistant to both transfer and hydrolytic reactions catalyzed by the mentioned typical nucleolytic enzymes. In this work we did not investigate whether the diribonucleoside phosphate analogues interact with, or even inhibit, the mentioned enzymes, or whether they are degraded by the large excess of the enzyme protein. Under such conditions, the problem of bond fission is transferred from the category of enzyme catalysis to the field of chemical attack by a protein as a nucleophilic reagent.

In addition to the enzymatic assays, compounds *X*, *XIII*, *XXV*, and *XXVII* (types *A* – *D*) were subjected to chemical hydrolysis under standard conditions used for treatment of RNA (0·33 mol l^{-1} aqueous potassium hydroxide at 37°C, 16 h).

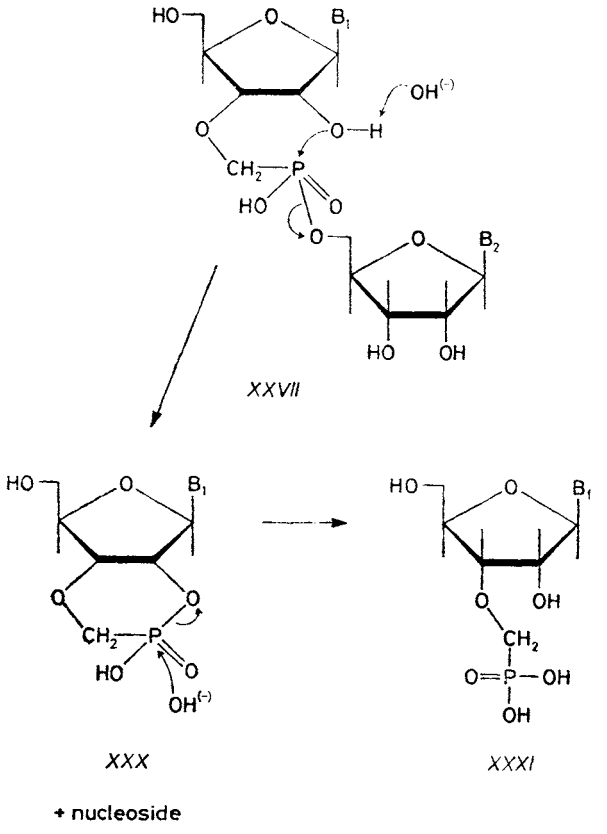
TABLE II

Protected analogues of dinucleoside phosphates with 2′- or 3′-O-phosphonylmethylnucleotide bond

Compound	B_1' [a]	B_2'' [a]	Starting components	
			Phosphonate	Nucleoside
XXIVa	A^{Dbam}	U	*XXIIb*	*XXIIIa*
XXIVb	A^{Bz}	A^{Bz}	*XXb*	*XXIIIc*
XXVIa	A^{Dbam}	U	*XXIIc*	*XXIIIa*
XXVIb	A^{Bz}	A^{Bz}	*XXc*	*XXIIIc*
XXVIIIa	U	U	*XVIII*	*XXIIIa*
XXVIIIb	C^{Dbam}	C^{Bz}	*XXIIa*	*XXIIIb*
XXVIIIc	G^{Dbam}	C^{Bz}	*XXIId*	*XXIIIb*
XXVIIId	U	A^{Bz}	*XVIII*	*XXIIIc*

[a] See footnote in Table I.

The internucleotide bond in oligoribonucleotides is cleaved by alkali similarly as in enzymatic hydrolyses with ribonucleases, *i.e.* following a two-step mechanism. The first step consists in formation of the 2',3'-cyclic nucleotide and the 3'-terminal nucleotide (with RNA) or nucleoside (with dinucleoside phosphates); in the subsequent alkaline hydrolysis the 2',3'-cyclic nucleotide is nonstereospecifically opened to give mixture of ribonucleoside 2'- and 3'-phosphate. As expected, compounds



SCHEME 3

of the type *A*, *B* are completely resistant to alkaline hydrolysis; on the contrary, compounds *C* and *D* are hydrolyzed to 2'- or 3'-O-phosphonylmethylribonucleosides *XXXI* and the corresponding nucleosides (Scheme 3). Alkaline cleavage of the phosphonate internucleotide bond in compounds of the type *C* and *D* probably proceeds by the same mechanism as that of the natural nucleotide bond; the only difference is that in the first step, instead of a five- a six-membered ring ester (*XXX*)

TABLE III

O-Phosphonylmethyl analogues of dinucleoside monophosphates *X*, *XIII* and *XXV*, *XXVII*

Comp.	B_1	B_2	k[a]		E_{Up}[b]	R_F[c]	UV-spectrum; λ_{max}, nm					
							acid[d]		neutral[e]		alkaline[f]	
			X	*XIII*			*X*	*XIII*	*X*	*XIII*	*X*	*XIII*
a	A	U	2·76	6·02[g]	0·32	0·31	260	263	262	263	263	263
b	U	A	1·67	3·64[g]	0·33	0·32	260	260	260	263	262	262
c	A	A	1·44	3·08[h]	0·27	0·35	260	260	261	260	261	260
d	U	U	0·99	1·58[g]	0·37	0·28	263	264	263	265	263	265
e	C	G	1·09	2·26[i]	0·31	0·19	279	280	259	257	269	268
f	G	C	1·42	3·95[i]	0·34	0·21	278	280	258	258	270	272
g	U	C	0·90	1·83[j]	0·36	0·30	270	271	267	267	267	268
			XV	*XXVII*			*XV*	*XXVII*	*XV*	*XXVII*	*XV*	*XXVII*
a	A	U	2·54	5·28[g]	0·34	0·31	261	262	262	263	263	263
b	U	A	1·61	3·51[g]	0·36	0·30	261	260	261	263	262	263
c	A	A	1·52	4·61[h]	0·30	0·37	261	260	262	260	261	262
d	U	U	0·82	0·97[g]	0·38	0·22	264	264	264	264	263	263
e	G	C	1·44	2·46[j]	0·36	0·22	279	281	257	258	269	271
f	C	C	0·82	1·36[j]	0·36	0·31	281	281	273	274	271	273

[a] Capacity factor (HPLC), $k = (t_R - t_0)$, t_R retention time, t_0 hold-up time; [b] electrophoretic mobility in S2 related to uridine 3′-phosphate; [c] chromatographic mobility in S1; [d] UV spectra measured in 0·01 mol l^{-1} aqueous hydrochloric acid; [e] in water; [f] in 0·01 mol l^{-1} aqueous sodium hydroxide; [g] HPLC in S11; [h] in S12; [i] in S9; [j] in S10.

TABLE IV

Alkaline hydrolysis of 2′- or 3′-O-phosphonylmethyl analogues of dinucleoside phosphates *XXV* and *XXVII*

Compound	B_1	B_2	Splitting, %		Products		
			XXV	*XXVII*	*XXV*	*XXVII*	Nucleoside
a	A	U	37	68	2′	3′	Urd
b	U	A	53	68	2′	3′	Ado
c	A	A	27	57	2′	3′	Ado
d	U	U	62	75	2′	3′	Urd

is formed in which only the ester bond is hydrolyzed to give O-phosphonylmethyl-ribonucleoside as the sole product. Under the mentioned conditions of alkaline hydrolysis, natural dinucleoside monophosphates (UpU, UpA, ApU, and ApA) are cleaved to the extent of >95%. However, the extent of cleavage of compounds *C* and *D* was markedly lower than that measured for natural dinucleoside phosphates (Table IV). Since the six-membered cyclic ester *XXX* is easily hydrolyzed, this difference can be interpreted in terms of a slower formation of this intermediate.

On the basis of this study, we can assume that an internucleotide bond of O--phosphonylmethylnucleosides, if formed by any chemical or biosynthetic reaction, would resist enzymatic degradations; oligonucleotides or polymers containing such bonds would thus be stable under *in vivo* conditions. It is also evident that the resistance of such analogous bond to the mentioned enzymes (and probably also other nucleolytic enzymes) can be utilized for the detection of such linkage in the presence of natural internucleotide bonds. The modified bonds in the chain can also be distinguished by chemical hydrolysis: only bonds to 2'- or 3'-O-phosphonylmethyl-nucleosides are cleaved in an alkaline medium whereas ester bonds derived from the isomeric 5'-ethers are entirely stable.

Chemical synthesis of dinucleoside phosphate analogues by the triester method in a homogeneous medium has proven the general applicability of this method of internucleotide bond synthesis. Clearly, it can be utilized also in constructing longer blocks in which these bonds exist together with the natural linkages. With an appropriate combination of protecting groups, this method can undoubtedly be adapted also to the solid-phase synthesis.

EXPERIMENTAL

Unless stated otherwise, the solutions were evaporated at 40°C/2 kPa and the compounds were dried at 13 Pa over phosphorus pentoxide. Ultraviolet spectra were measured in aqueous solutions on a Specord UV-VIS spectrophotometer (Carl Zeiss, Jena, G.D.R.). Paper chromatography was performed on a Whatman No. 1 paper in the system S1 (2-propanol–conc. aqueous ammonia–water, 7 : 1 : 2), paper electrophoresis on a Whatman No. 3MM paper in S2 (0·05 mol . l^{-1} aqueous triethylammonium hydrogen carbonate, pH 7·5) at 20 V/cm for 1 h. Thin-layer chromatography (TLC) was carried out on UV-254 Silufol plates (Kavalier, Czechoslovakia) in S3 (chloroform) and S4—S7 systems (5, 10, 15, and 20%, respectively, of methanol in chloroform) and the short column chromatography on Silpearl 30—60 μm (Kavalier, Czechoslovakia) in the system S3—S7. Reverse phase medium-pressure chromatography was done on a column of octadecyl silica gel (25 × 250, 500 mm; 30—60 μm, carbon content 18%; prepared by Service Laboratories of this Institute), elution with a linear gradient (0—50%) of ethanol in water, flow rate 5 ml min^{-1}, continuous detection with a Uvicord apparatus (LKB, Uppsala, Sweden). Ion exchange chromatography was performed on DEAE-Sephadex A-25 (HCO_3^- form), elution with linear gradient (0—0·2 mol l^{-1}) of triethylammonium hydrogen carbonate in water at pH 7·5. Compounds were deionized on columns of Dowex 50 (H^+ form) by washing with water and 50% aqueous methanol to UV-absorption drop of the eluate; the compounds were then eluted by 5% aqueous ammonia. Reverse phase HPLC was carried out on a stainless steel column

(4 × 250 mm) packed with Separon SGX C18 (7 μm) Laboratorní přístroje, Prague) in the systems S8 (0·05 mol l^{-1} aqueous triethylammonium hydrogen carbonate, pH 7·5), S9–S12 (3, 4, 5, and 8%, respectively, of acetonitrile in S8), flow rate 1 ml min^{-1}. Semipreparative reverse phase HPLC was performed on a stainless steel column (12·7 × 500 mm) packed with the same adsorbent as the analytical column, elution with a gradient of methanol in 0·05 mol l^{-1} aqueous triethylammonium acetate, pH 7·5, flow rate 4 ml min^{-1}.

Compounds and reagents: Ribonucleosides were Serva or Calbiochem (U.S.A.) products, benzoyl cyanide, triisopropylbenzenesulfonyl chloride, and 1-methylimidazole were purchased from Merck (F.R.G.). Di-n-butylformamide dimethyl acetal was prepared according to ref.[17], tetraisopropyldisiloxanyl bromide according to ref.[20]. Dimethylformamide and acetonitrile were distilled from P_2O_5, pyridine from potassium hydroxide and calcium hydride. Dioxane was mixed with 50% (w/w) aqueous sodium hydroxide solution (100 ml *per* litre of dioxane), dried over solid sodium hydroxide, refluxed over sodium and stabilized with di-tert-butyl-*p*-cresol (250 mg/l). All solvents were stored over molecular sieves (3 Å). Pancreatic ribonuclease was a Lachema (Czechoslovakia) product, ribonuclease T2 was isolated from a mixture with ribonuclease T1 by isoelectric focusation, snake venom exonuclease (*Crotalus terr. terr.*) was purchased from Boehringer (F.R.G.).

Preparation of Protected Ribonucleosides

Compounds VIIIa–d were obtained by reaction of 1,3-dibromotetraisopropyldisiloxane with uridine or N-benzoyl derivatives of cytidine, adenosine, and guanosine in a dimethylformamide–pyridine mixture in the presence of imidazole[20]. The compounds were purified by chromatography on silica gel in S3.

2,'5'-*Di*-O-*benzoyluridine* (XIa) was prepared according to ref.[25].

2',5'-O,N^2-*Tribenzoylguanosine* (XIb): Sodium methoxide in methanol (2 mol l^{-1}; 20 ml) was added at 0°C to a stirred solution of 2',3',5',N^2-tetrabenzoylguanosine[26] (10 mmol) in dioxane–methanol (1 : 1, 400 ml). After stirring for 4 min, Dowex 50X8 (pyridinium form, 60 ml) was added and after further 2 min the suspension was filtered, the Dowex washed with dioxane–methanol (1 : 1, 3 × 50 ml) and the combined filtrates were taken down. Chromatography on silica gel (100 g) in the systems S4–S8 afforded 5',N^2-dibenzoylguanosine (4·89 mmol; 49%). This product was dissolved in dimethylformamide (6 ml), triethyl orthobenzoate (5·5 ml) was added and the mixture was acidified with hydrogen chloride in dimethylformamide (6 ml; 3·4 mol l^{-1}). After stirring at room temperature for 3 h, triethyl orthobenzoate (2 ml) and hydrogen chloride in dimethylformamide (2 ml) were added and the homogeneous mixture was set aside at room temperature for 16 h. The solution was cooled to −20°C, made alkaline with triethylamine (moist pH indicator paper) and the solvent was evaporated at 40°C *in vacuo*. The residue was dissolved in chloroform and the compound was purified by chromatography on silica gel (50 g) in the system S3 with triethylamine (0·1 vol. %). Fractions, containing the product (2',3'-O-ethoxybenzylidene-5',N^2-dibenzoylguanosine), were combined, the solvent was evaporated and the residue was dissolved in 80% aqueous acetic acid. The solution was set aside at room temperature for 2 h, the mixture was taken down and the residue was codistilled with dioxane (5 × 20 ml) and dried over sodium hydroxide at 13 Pa. Methanol (50 ml) was added, the mixture was taken to the boil and cooled (repeated three times). The heterogeneous mixture was allowed to stand at 4°C for 16 h, the crystals were filtered, washed with a small amount of ice-cold methanol and dried over phosphorus pentoxide (13 Pa). Yield, 1·82 mmol (37%) of 2',5',N^2-tribenzoylguanosine (*XIb*). ^{1}H NMR spectrum (hexadeuteriodimethyl sulfoxide):

6·34 d, 1 H (H-1′, $J(1', 2')$ = 4·2); 5·94 dd, 1 H (H-2′, $J(2', 3')$ = 5·5); 5·00 t, 1 H (H-3′, $J(3', 4')$ = = 6·0); 4·4 m, 1 H (H-4′); 4·7 m, 2 H (H-5′); 8·29 s, 1 H (H-8′); 11·8 br, 1 H (NH).

Compounds XIVa,b were prepared by acetylation of 2′,3′-O-ethoxyethylidene derivative of N^4-benzoylcytidine and N^6-benzoyladenosine[21]. After acid hydrolysis of the ethoxyethylidene group, the 2(3′),5′-di-O-acetyl derivatives *XIVa,b* were isolated by chromatography on silica gel in systems S3—S5.

Compounds XXIIIb,c were prepared by benzoylation of 2′,3′-O-isopropylidene derivatives of cytidine and adenosine by the method described for 2′-deoxynucleosides[27].

Methyl Esters of O-Phosphonylmethyl-N-dibutylaminomethyleneribonucleosides *IIa—c*, *VIIa—c*, and *XXIa—d*

A solution of lithium salt of the compound (*Ib—d*, *VIb—d* ot *XVIIb—e*; 1 mmol) in a mixture of dimethylformamide (10 ml) and dibutylformamide dimethylacetal (1·5 ml) was stirred at room temperature for 20 h. After addition of another portion of the reagent (0·75 ml) in dimethylformamide (5 ml), the mixture was again stirred for 20 h at room temperature. The homogeneous solution was taken down at 40°C *in vacuo*, the residue was dissolved in 50% aqueous pyridine (20 ml) and solid carbon dioxide (3 g) was added. After 30 min at room temperature, the solvents were evaporated and the residue codistilled with pyridine (2 × 10 ml) and dioxane (4 × 10 ml). The product was purified by medium-pressure chromatography on C18 silica gel, the product fractions were combined, evaporated and the compound was converted to the lithium salt on a Dowex 50X8 column (lithium form) in 70% aqueous ethanol at 4°C. After evaporation *in vacuo*, the residue was codistilled with ethanol (3 × 10 ml), dioxane (3 × 10 ml) and dried over phosphorus pentoxide at 13 Pa. Yields: *IIa* (85%), *IIb* (70%), *IIc* (60%), *VIIa* (91%), *VIIb* (74%), *VIIc* (62%), *XXIa* (90%), *XXIb* (88%), *XXIc* (85%), *XXId* (60%). UV spectra in methanol; λ_{max}, nm: 322 (*IIa*, *VIIa*, *XXIa*); 314 (*IIb*, *VIIb*, *XXIb,c*); 309 (*IIc*, *VIIc*, *XXId*).

Benzoyl Derivatives of O-Phosphonylmethylnucleoside Methyl Esters *IIIa—c*, *IVa—c*, *V*, *XVIII*, *XXa—d*, and *XXIIa—d*

Triethylamine and benzoyl cyanide (2·5 molar equivalent each for *Ia*, *IIa—c*, *XVIIa*, and *XXIa—d*; 3·5 equivalents for *Ib—d* and *XVIIb—e*) were added in succession to a solution of lithium salt of the phosphonate (*Ia—d*, *IIa—c*, *XVIIa—e*, *XXIa—d*; 0·2—1 mmol) in dimethylformamide (10 ml/mmol) and the mixture was stirred at room temperature for 16 h. In the case of *Ib—d* and *XVIIb—e*, another portion (half of the above-specified amounts) of dimethylformamide, triethylamine and benzoyl cyanide was added and the solution was stirred for further 16 h at room temperature. The homogeneous solution was taken down at 40°C *in vacuo*, the residue was dissolved in ethanol (10 ml) and the solution was added dropwise under stirring into ether (150 ml). The precipitate was filtered, washed with ether and the obtained compounds *IIIa—c*, *V*, *XVIII*, and *XXIIa—d* were dried *in vacuo*. The benzoyl derivatives *IVa—c*, *XXa—d* were purified by medium-pressure chromatography on C18 silica gel and the pertinent fractions were concentrated at 30°C *in vacuo* (to 20 ml) with addition of dioxane. Dowex 50X8 (lithium form; 5 ml), suspended in aqueous dioxane (1 : 1, 20 ml) was added and the mixture was stirred for 5 min at 4°C. The suspension was filtered, the resin was washed with aqueous dioxane and the solution of the lithium salt was taken down at 30°C *in vacuo* with addition of dioxane. Finally, the residue was codistilled with dioxane (4 × 10 ml) and the product was dried *in vacuo*. Yields: *IIa—c* (100%), *IVa* (45%), *IVb* (32%), *IVc* (36%), *V* (100%), *XVIII* (100%), *XXa* (31%), *XXb* (38%), *XXc* (35%), *XXd* (30%), and *XXIIa—d* (100%).

Condensation of Protected O-Phosphonylmethylnucleosides with Protected Nucleosides (Tables I and II)

Dowex 50X8 (pyridinium form; 5 ml/mmol) was added at 0°C to a solution of lithium salt of the protected compound *I* (*III*—*VII*) or *XVII* (*XVIII*, *XX*, *XXII*) in 70% aqueous pyridine (20 ml/mmol) and the mixture was stirred in an ice-bath for 10 min. The resin was filtered, washed with 70% aqueous pyridine and the combined filtrates were evaporated at 30°C *in vacuo* with addition of pyridine. The residue was dried by codistillation with pyridine (4 × 10 ml) and mixed with pyridine (10 ml). The dried protected nucleoside (1·2 molar equivalent) was added, the solvent was evaporated and the residue was codistilled with pyridine (2 × 10 ml) and finally with dioxane (3 × 10 ml). A solution of 2,4,6-triisopropylbenzenesulfonyl chloride (2 equivalents) and 1-methylimidazole (4 equivalents) in acetonitrile (10 ml/mmol) was added and the mixture was shaken at room temperature (monitoring by TLC on silica gel in S4, S5). Water (0·1 ml/mmol) was added and the solution was concentrated *in vacuo*. The oily residue was dissolved in chloroform, the product was isolated by chromatography on silica gel in S3—S5 and the pertinent fractions were combined and taken down at 30°C *in vacuo*. The protected diesters were obtained in 70—90% yields.

Deprotection of Neutral Intermediates *IX*, *XII*, *XV*, *XXIV*, *XXVI*, and *XXVIII*

A solution of the protected intermediate (1 mmol) in 60% aqueous pyridine (30 ml) was allowed to stand at 40°C for 24 h and then the solvent was evaporated *in vacuo* to dryness. The mixture was further processed as follows:

A) In the case of compounds *XII*, the residue was allowed to stand in a mixture of dioxane and conc. aqueous ammonia (1 : 2; 50 ml/mmol) at 40°C for 15 h (*XIIa—c*) or 40 h (*XIId*) and the mixture was taken down *in vacuo*.

B) The mixture was processed as described under *A*) under the following reaction conditions: compounds *XV* 40 h at 40°C, *XXIVa* and *XXVIIIa* 15 h at 40°C, *XXIVb*, *XXVIb* and *XXVIIIb—d* 40 h at 40°C; the residue was set aside with 0·025 mol l^{-1} sulfuric acid (50 ml/mmol) at room temperature for 16 h and the mixture was deionized on Dowex 50 (*vide supra*). In the preparation of *XXIXa*, the mixture was directly neutralized with ammonia and taken down.

C) The reaction mixture from compounds *IX* was first processed according to *A*), the residue was codistilled with dioxane (3 × 20 ml) and set aside at room temperature for 30 min with 0·1 mol l^{-1} tetrabutylammonium fluoride in tetrahydrofuran (30 ml/mmol *IX*). Water was added (1 ml/mmol *IX*) and the solution was evaporated *in vacuo*.

The crude products obtained under *A*)—*C*) were purified by chromatography on DEAE-Sephadex A-25 (HCO_3^- form) and finally by semipreparative reverse phase HPLC in triethylammonium acetate. In the case of compounds *XVI* and *XXIX* the (2′—5′)- and 3′—5′)-isomers were isolated. The eluates were stirred for 5 min at 4°C with Dowex 50X8 (pyridinium form; 2 ml *per* 10 ml of effluent), the suspension was filtered, the Dowex was washed with 50% aqueous pyridine, the combined filtrates were evaporated at 30°C *in vacuo* and the residue was codistilled first with 50% aqueous pyridine (4 × 20 ml) and finally with dioxane (4 × 20 ml). The product was converted into its lithium salt on a column of Dowex 50X8 (lithium form). Compounds *X*, *XIII*, *XVI*, *XXV*, *XXVII*, and *XXIX* were isolated in 50—70% yields; their characterisation is given in Table III.

Enzymatic Assays

The incubation mixture contained 400 nmol of the compound in 100 µl of 0·05 mol l^{-1} sodium citrate buffer (pH 7·5 for RNase A, pH 6·3 for RNase T2) or tris-acetate buffer pH 8·3 (snake

venom exonuclease). The amount of the enzyme was chosen so as to cleave 80—90% of the same amount of natural dinucleoside phosphates UpA (RNase A) and ApU (RNase T2 and exonuclease) during 30 min at 37°C. The following compounds were tested with RNase A: *Xa—d*, *XIIIa—d*, *XXVa—d*, *XXVIIa—d*; with RNase T2: *Xa—g*, *XIIIa—g*, *XXVa—f*, *XXVIIa—f*; and with exonuclease: *Xa—g*, *XIIIa—g*, *XXVa—f*, *XXVIIa—f*. After incubation for 30 min at 37°C, 5 µl of the mixture was analyzed by HPLC. Under these conditions, none of the tested compounds was cleaved with the three mentioned enzymes (within the limit of accuracy 0·5—1%).

Alkaline Hydrolysis

A solution of the compound (400 nmol) in 0·33 mol l^{-1} aqueous potassium hydroxide (100 µl) was incubated for 16 h at 37°C. Under these conditions, the natural dinucleotides Upa, ApU, and ApA were more than 95% cleaved. The mixture (5 µl) was analyzed by HPLC and the results are given in Table IV.

The authors thank Dr I. Votruba for the enzymatic assays and Miss R. Kusá, Miss I. Doležilová and Mrs. H. Miklová for the excellent technical assistance.

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Translated by M. Tichý.