Tetrahedron. Vol. 34, pp. 853 to 855. Pergamon Press 1978. Printed in Great Britain

PREFERENTIAL FORMATION OF (2'-5')-LINKED INTERNUCLEOTIDE BONDS IN NON-ENZYMATIC REACTIONS

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(*Received in USA* 10 *August* 1977; *Received in UK for publication* 31 *October* 1977)

Abstract—The reactions of ImpU with MepU, MepC, MepA and MepG in aqueous solution yield mixtures of isomeric dinucleotides in which the (2'-5')-linked compound is 6–9 times more abundant than the (3'-5')-linked compound. The ratio of isomers obtained from ImpA and MepA under these conditions is similar, but increases to 18 when a poly(U) template is included in the reaction mixture.

The template-directed reactions of activated mononucleotides yield a mixture of isomeric oligonucleotides in which (2'-5') linkages predominate.[1,2] The reaction of ImpA† with A on a poly(U) template, for example, gives ApA which is 95% (2'-5')-linked. The preponderance of (2'-5')-over (3'-5')-linked isomers must be due in part to the greater nucleophilic reactivity of the 2'-OH group. However, orientation of the reactant nucleotides on the template strands could change the ratio of (2'-5')- to (3'-5')-linked products in either direction from the value that would be anticipated from the intrinsic reactivities of the 2'- and 3'-OH groups. We have now studied a series of model reactions in order to determine the ratio of the intrinsic reactivities, and illustrate the use of such information by analyzing the influence of the template on the isomer ratio obtained from A and ImpA.

RESULTS AND DISCUSSION

The importance of nucleosides in biochemistry has led to a number of studies of the relative reactivities of the 2'-, 3'- and 5'-OH groups towards alkylating, acylating, sulfonylating and phosphorylating reagents. The few studies that address the relative reactivities of the 2'- and 3'-OH groups establish that the 2'-OH group is the more reactive, but the quantitative interpretation of the data is often complicated because isolated yields reflect kinetic and thermodynamic factors to different extents in different studies. This work has recently been reviewed in detail.[3]

We have determined the relative reactivities of the 2'- and 3'-OH groups of the 5' methylesters of the natural nucleotides with ImpU. The former compounds were chosen as 5'-protected acceptors because they simulate the terminal nucleotide group of an oligonucleotide; ImpU was chosen as donor to minimize stacking interactions. Our results (Table 2) show that the 2'-OH group is substantially (6–9 times) more reactive than the 3'-OH group in each of the natural nucleotides.

†Abbreviations: Im, imidazole; MeIm, 1-methylimidazole; CDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; A, adenosine; C, cytidine; G, guanosine; U, uridine; pN (N = A, C, G, or U), nucleoside 5'-phosphate; ImpA, adenosine 5'-phosphorimidazolide; ImpU, uridine 5'-phosphorimidazolide; MepN(N = A, C, G or U), nucleoside 5'-(phosphoric acid-methylester); MepNpX, 5'-methylester of a dinucleotide pNpX, with N = A, C, G or U and X = A or U; poly(U), polyuridylic acid.

The origin of this substantial difference in reactivity is not obvious. Presumably, the inductive effect of the heterocyclic N-atom decreases the pK of the 2'-OH to some extent, and hence makes the 2'-anion more abundant than the 3'-anion.[4] Our data, therefore, suggest that the nucleophilicity of the 2'-anion relative to the 3'-anion is not decreased to the same extent. However, the reactivities of the two OH groups might also be affected by the conformation of the ribose ring and by steric effects.

The use of information of this kind in evaluating the importance of orientation on a template is illustrated by the data in Table 3. The reaction of ImpA with MepA in the absence of poly(U) again yields the (2'-5')- and (3'-5')-linked isomers in a ratio of about 6 : 1. However, in the presence of a poly(U) template, the ratio is about 18 : 1. Hence, orientation on the template enhances the reactivity of the 2'-OH group by a further factor of 3, relative to the reactivity of the 3'-OH group.

EXPERIMENTAL

Materials.

Imidazole (Matheson, Coleman & Bell) was recrystallized from benzene, 1-methylimidazole (Aldrich) was redistilled before use. CDI was purchased from Story Chemical Corp., triphenylphosphine and 2,2'-dipyridyl disulfide from Aldrich, the nucleoside 5'-phosphates from Sigma, the [^{14}C]-labelled nucleotides from Schwarz-Mann. Ribonuclease T_1 and Ribonuclease T_2 were supplied by Worthington, pancreatic Ribonuclease by Boehringer.

Poly(U) was prepared as described in the literature.[5] The methylesters of all four nucleotides[6,7] and the imidazolides of pA and pU[8] were prepared by modifications of published procedures (see below).

Chromatography and electrophoresis

Paper chromatography was performed on Whatman 3MM paper by the descending technique. The following systems were used:

System I, isopropanol, triethylamine and water (7 : 1 : 2)

System II, isopropanol, concentrated ammonia and water (7 : 1 : 2)

System III, 95% ethanol, 1M ammonium acetate made up to 2×10^{-3} M in EDTA and brought to pH 5.0 with glacial acetic acid (7 : 3)

System IV, saturated ammonium sulfate, 0.1 M sodium acetate (pH 6.5) and isopropanol (71 : 19 : 2)

Electrophoresis was carried out at 3500 V on Whatman 3MM paper using varsol as coolant.

System V, the buffer was 0.03 M KH_2PO_4 titrated to pH 7.1 with KOH.

The chromatographic and electrophoretic mobilities of various compounds are listed in Table 1.

Table 1. Chromatographic and electrophoretic mobilities

Compounds	I[a]	II[a]	III[a]	IV[a]	V[b]
A	1.00	1.00	1.00	1.00	0.00
U	0.88	0.89	1.13	4.47	0.04
pA	0.12	0.13	0.56	2.39	1.00
pC	0.08	0.11	0.51	4.78	1.08
pG	0.05	0.06	0.35	3.56	1.02
pU	0.06	0.13	0.63	5.17	1.15
ImpA	0.98	0.90			0.55
ImpU	0.93	0.79			0.65
MepA	0.72	0.79	0.83	1.51	0.61
MepC	0.62	0.63	0.85	4.12	0.69
MepG	0.41	0.45	0.74	2.93	0.62
MepU	0.75	0.65	1.04	3.90	0.68
$MepA_3^2pA$	0.13		0.46	1.08	0.76
$MepA_2^3pA$	0.10		0.46	0.49	0.76
$MepA_3^2pU$	0.09		0.45	2.66	0.91
$MepA_2^3pU$	0.09		0.45	1.54	0.84
$MepC_3^2pU$	0.07		0.46	3.69	0.97
$MepC_2^3pU$	0.07		0.46	3.69	0.97
$MepG_3^2pU$	0.06		0.36	4.17	0.95
$MepG_2^3pU$	0.06		0.36	2.34	0.95[c]
$MepU_3^2pU$	0.06		0.55	3.35	1.01
$MepU_2^3pU$	0.06		0.55	3.35	1.01
MepAp					1.48
MepCp					1.53
MepGp					1.44
MepUp					1.58

[a] The chromatographic mobilities are given relative to A

[b] The electrophoretic mobilities are given taking $R_{adenosine} = 0.00$ and $R_{adenylic\ acid} = 1.00$

[c] Lit. cit. 7

Table 2. Reaction of MepN-[^{14}C] (0.05M) with ImpU (0.05M) in presence of $MgCl_2$ (0.12M), NaCl (0.20M) and 1-Methylimidazole (0.20M, pH 8.0) after 25 days at 0°

	MepApU	MepCpU	MepGpU	MepUpU
Yield of dinucleotide (%)	9.3	4.8	9.3	5.9
3'-Isomer (%)	11.5	14.1	10.4	14.7
2'-Isomer (%)	88.5	85.9	89.6	85.3

Table 3. Reaction of MepA-[8-^{14}C] (0.05M) and ImpA (0.05M) in presence of $MgCl_2$, NaCl (0.20M) and 1-Methylimidazole-HCl (0.20M, pH 8.0), after 7 days at 0°. The reaction was performed in presence or absence of a template. The yields (%) are based on MepA.

poly(U)	MepApA (%)	MepApApA (%)
-	10.6	-
	2') 85.4%	
	3') 14.6%	
+	36.0	14.0
	2') 94.9%	
	3') 5.1%	

All experiments utilized radioactive MepN's. Quantitative estimates of the product yields were made by running the chromatograms or electropherograms through a Baird Atomic RSC 363 scanner with integrator. When necessary, spots were eluted from paper that had been run in one system, and rerun in a second system to achieve further separation. Yields are expressed as the percentage of the total radioactivity on the paper, after allowing for the background.

Preparations

ImpU. The anhydrous triethylammonium salt of pU (0.45 mmol) was dissolved in an anhydrous mixture containing triethylamine (0.90 ml), trioctylamine (0.90 ml) and dimethylformamide (30 ml). Triphenylphosphine (5.73 mmol) and 2,2'-dipyridyldisulfide (5.73 mmol) were added, and the mixture was then kept at room temperature for 1 h. Chromatographic analysis in System I (Polygram CEL 300 UV_{254} from Brinkmann) showed that the reaction was virtually complete.

The ImpU was isolated as sodium salt by dropwise addition of the mixture to a vigorously stirred anhydrous solution containing $NaClO_4$ (9 mmol) triethylamine (60 ml), acetone (900 ml) and ether (450 ml). The white precipitate was collected by centrifugation and washed carefully, first with acetone and then with ether. The yield of isolated material was usually better than 90%. Chromatography of an aliquot in System I showed it to be better than 98% pure, a trace of pU being the only UV-absorbing contaminant. The product was kept *in vacuo* over P_2O_5 and solid NaOH. ImpA was obtained in 95% yield using essentially the same procedure. Both imidazolides cochromatograph with authentic material synthesized by another established method.[9,10]

MepA. The pyridinium salt of pA (1 mmol) was dissolved in a mixture containing water (5 ml), pyridine (1 ml), dimethylformamide (10 ml) and MeOH (20 ml), with warming. After addition of CDI (3 mmol), the mixture was shaken for 16 h at room temp. Electrophoresis of an aliquot showed that MepA had formed in ca 80% yield. The mixture was diluted with water (200 ml) and applied on a column (1.6 × 30 cm) of Sephadex A25 QAE bicarbonate (Pharmacia). The column was eluted by a linear gradient of triethylammonium bicarbonate (0.005M-0.30M; 2 × 600 ml). MepA eluted at a conc. of 0.10–0.13M bicarbonate. The fractions containing the methylester were pooled and evaporated *in vacuo* in presence of pyridine. Finally, the carbonate-free residue was dissolved in water and lyophilized giving the methylester of pA (as triethylammonium salt) in about 65% yield. The product was chromatographically and electrophoretically pure. The methylesters of pC and pG were prepared by the same procedure. However, in the case of pU, water was omitted from the mixture and, to prevent adduct formation between pU and CDI, the reaction time was reduced to 1 h. The esters of pC, pG and pU were isolated in 61, 32 and 75% yield, respectively. The electrophoretic and chromatographic characteristics and the UV spectra of these compounds are consistent with published data.[6,7]

[8-^{14}C]-*MepA.* [8-^{14}C]-Adenosine 5'-phosphate (pyridinium salt; 10 μmole; 2 mCi/mmol) was dissolved in a mixture of water (50 μl), pyridine (20 μl) and dimethylformamide (50 μl). Then MeOH (200 μl) and CDI (10 mg) were added. After stirring the mixture for 20 h at room temperature, an aliquot was checked by electrophoresis. MepA had formed in 88.5% yield. The mixture was subjected to paper chromatography in System I. The major band was eluted with water. MepA was obtained by evaporation of the eluate. Radioactively-labelled pU, pC and pG were obtained by very similar procedures.

Reactions

ImpU and various MepN's. Four solns (pH 8.0) each containing 0.05M ImpU, 0.12M $MgCl_2$, 0.20M NaCl, 0.20M MeIm and a [^{14}C]-labelled MepN (0.2 mCi/mmol) were prepared. Soln (a) contained 0.05M MepA, soln (b) 0.05M MepC, soln (c) 0.05M MepG and soln (d) 0.05M MepU. After addition of toluene the solns were kept at 0° for 25 days.

Analysis of the MepNpX compounds. Small aliquots of each mixture were chromatographed in System I from time to time. After 25 days, ImpU had virtually disappeared from each of the mixtures. Aliquots of each solution were then analyzed in

Systems II and V. The newly appearing radioactive spots had mobilities similar to those anticipated for the appropriate MepNpX's. In order to have more material for a detailed analysis, the bulk of the reaction mixtures was then chromatographed on paper in System II. The MepNpX-containing bands were cut out and eluted with water. Aliquots of each sample were checked in Systems II, III, IV and V and found to be chromatographically and electrophoretically pure.

Each eluate was subjected to enzymatic degradation,[11] MepCpU and MepUpU with pancreatic RNase, MepGpU with T_1-RNase and MepApU with T_2-RNase. Subsequent electrophoresis showed in each case two radioactive spots, one which corresponded to undegraded material and another which migrated like authentic MepAp marker.[12] The electrophoretic mobility of compounds of the type MepNp is almost independent of the nature of the nucleoside N. The proportions of (2'-5')- and (3'-5')-linked isomers (Table 2) were obtained from the counts measured in the MepNpU and MepNp peaks, respectively.

In the case of MepApU and MepGpU chromatography in System IV resolved the (2'-5')- and (3'-5')-linked isomers (Table 1). This gave an independent measure of the isomer ratio which agreed closely with that obtained by enzymatic degradation.

MepA and ImpA. Two solns (a, b) each containing 0.05M [8-^{14}C]-adenosine 5'-(phosphoric acid methylester), 0.2 mCi/mmole, 0.01 ImpA, 0.20M NaCl, 0.20M MeIm were prepared. In addition, soln (a) contained 0.12M $MgCl_2$, while soln (b) contained 0.30M $MgCl_2$ and 0.20M poly(U). After adjusting the pH of the solns to 8.0, toluene was added to keep the solns sterile. The mixtures were kept for 7 days at 0°, and then analyzed by chromatography in Systems I, III and IV and by high voltage electrophoresis in System V (Table 3).

The MepApA containing spots from System I were eluted with water. A portion of the eluate chromatographed in System IV showed two radioactive spots which corresponded to the two isomers MepA^2pA and MepA^3pA. Another portion digested by T_2-RNase and then analyzed in System V gave two radioactive spots corresponding to undigested MepA^2pA and MepAp, the latter compound representing the 3'-isomer. The isomeric ratio obtained by the two independent methods again agreed closely.

Acknowledgements—This work was supported by National Institutes of Health Grant No. GM 13435. We wish to thank A. R. Hill and J. E. Tomaschke for technical assistance.

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