Oligomerization of activated derivatives of 3'-amino-3'-deoxyguanosine on poly(C) and poly(dC) templates

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ABSTRACT

3'-amino-3'-deoxyuridine reacts with the nucleoside 5'-phosphorimidazolides in aqueous solution to give dinucleoside phosphoramidates. The reactions are one to two orders of magnitude faster than the corresponding reactions of uridine.

In the presence of poly(C) or poly(dC) it is known that guanosine-5'-phosphorimidazolidine does not condense efficiently or regiospecifically. However, the introduction of a methyl group at the 2-position of the imidazole ring leads to efficient synthesis of long 3'-5'-linked oligomers. The corresponding imidazole derivatives of 3'-amino-3'-deoxyguanosine-5'-phosphate both condense on these templates to give virtually identical families of products. Our results suggest that the intrinsically greater nucleophilicity of the amine groups will permit a much wider range of efficient template-directed syntheses with 3'-amino-3'-deoxynucleoside derivatives than with the corresponding derivatives of the parent nucleosides.

INTRODUCTION

The use of oligonucleotides to facilitate the non-enzymatic synthesis of complementary oligomers has been studied extensively (1). Reactions of this kind obey the Watson-Crick pairing rules in the sense that a given base facilitates the incorporation of the complementary nucleotide into product oligomers and has little or no effect on the incorporation of the other three nucleotides. However, the reactions are usually relatively inefficient and lead to non-regiospecific products in which 2'-5' linkages often predominate (1, 2). Only recently has a system been described which is efficient, regiospecific and, to some extent, applicable to templates containing two or more

I

bases (2, 3, 4).

In order to obtain an efficient and regiospecific condensation giving 3'-5'-linked products, it was necessary to use a special set of activated substrates: the derivatives I of 2-methylimidazole. Closely related compounds, for example derivatives of unsubstituted imidazole or 4-methylimidazole, undergo much less efficient and non-regiospecific reaction (2). We believe that the hydroxyl groups of nucleosides are insufficiently reactive to support rapid oligomerization in aqueous solution except when the hydroxyl group is constrained to a nearly optimal position relative to the neighbouring activated phosphate. Synthesis of the 3'-5'-linked oligomers requires further that the 3'-OH group is correctly oriented for reaction, while the 2'-OH group is held away from the activated phosphate. These conditions are not readily satisfied, so it is not surprising that only one of a substantial number of the activated nucleotides that we tested gave a high yield of 3'-5'-linked oligomers.

The requirement for a precise orientation of the reacting groups can be relaxed if the 3'-OH group is replaced by a more nucleophilic substituent. This may lead to better yields and greater specificity of reaction even for non-optimal orientations. In the template-directed reactions of activated derivatives of A, the substitution of the 2'-NH₂ group for the 2'-OH group does indeed lead to 2'-5'-linked oligomeric phosphoramidates in high yield (5). It is interesting, therefore, to study the reactions of 3'-amino-3'-deoxyguanylic acid derivatives on poly(C) and poly(dC) templates, first for a system in which the reaction of the corresponding hydroxyl compound is efficient and regiospecific and then for another in which the reaction of the hydroxyl compound is inefficient and non-regiospecific.

MATERIALS (6)

Unless otherwise specified, materials were reagent or analytical grade and used without further purification. Phosphorus oxychloride (MCB-Reagents) was freshly distilled. Trimethyl phosphate was distilled under reduced pressure and kept under a nitrogen atmosphere. Dimethylformamide, triethylamine and acetonitrile were distilled from CaH₂ and stored over 4A molecular sieves. 1,2-dichloroethane was distilled from P₂O₅ and stored over 4A molecular sieves. Trimethylsilyltrifluoromethanesulphonate was a product of Fluka AG (Switzerland). [8-¹⁴C]guanine HCl, specific activity 54.4mCi/mmol, was purchased from ICN-Radiochemicals. UpU, UpA, and G2'pG were commercial pro-

ducts. Sodium salts of ImpN and 2-MeImpN were prepared as previously described (7).

The following enzymes were used for structural determinations: Pancreatic ribonuclease and Ribonuclease T₁ from *aspergillus oryzae* were products of Boehringer (FRG), Ribonuclease T₂ was obtained from Calbiochem-Behring, Phosphodiesterase II from calf spleen, 5'-nucleotidase from *crotalus adamanteus* venom and Dinucleotide nucleotidohydrolase type III from *crotalus atrox* venom (lot 110F-9765) were purchased from Sigma.

The following compounds were synthesized according to the known procedures (8, 9, 10):

3-azido-1,2-di-O-acetyl-5-O-(4-methoxybenzoyl)-3-deoxy-β-D-ribofuranose (1): mp. 74.5–75°C (MeOH) 23° [α]_D²⁰ +11.44° (c 0.064, CHCl₃).

NMR (CDCl₃): 7.97, 7.25 (2d, J=8 Hz, 4H arom.), 6.17 (s, H-1), 5.38 (d, J₂₋₃ 4.7 Hz, H-2), 4.65 and 4.43 (2dd, J_{5ab-4} 3.6 Hz, J_{5a-b} 12.2 Hz, H-5ab), 4.36 (m, J₄₋₃ 8.6 Hz, J_{4-5ab} 3.6 Hz, H-4), 4.23 (dd, J₃₋₂ 4.7 Hz, J₃₋₄ 8.6 Hz, H-3), 2.42 (s, H₃C-arom), 2.20 and 1.94 (2s, 2 H₃C-CO).

3'-azido-3'-deoxyuridine (2): UV(0.05M phosphate pH 7.0): λ_{max}265(ε10x-10³) λ_{min}232, A_{230/260} 0.25, A_{280/260} 0.38.

NMR (DMSO-d₆): 7.88 (d, J₆₋₅ 7.9 Hz, H-6), 6.18 (d, J_{HO-CH} 5.3 Hz, HO-2'), 5.75 (d, J_{1'-2'} 5.5 Hz, H-1'), 5.66 (d, J₅₋₆ 7.9 Hz, H-5), 5.28 (bs, HO-5'), 4.40 (dd, J_{2'-1'} 5.5 Hz, J_{2'-3'} 0 Hz, H-2'), 4.05 (dd, J_{3'-4'} 5.0 Hz, J_{3'-2'} 0 Hz, H-3'), 3.88 (dt, J_{4'-3'} 5.0 Hz, J_{4'-5'} 3.0 Hz, H-4'), 3.62 and 3.55 (2dd, J_{5'-4'} 3 Hz, J_{5'a-5'b} 12 Hz, 2H-5'a,b).

3'-azido-3'-deoxyguanosine (3): UV(0.05 phosphate pH 7.0): λ_{max}256(ε13.3x10³), 268 (sh), λ_{min}226, A_{230/260} 0.32, A_{280/260} 0.65.

NMR (DMSO-d₆): 7.93 (s, H-8), 6.50 (brs-NH₂), 6.19 (d, J_{HO-CH} 5.5 Hz, HO-2'), 5.68 (d, J_{1'-2'} 6 Hz, H-1'), 5.20 (t, J_{HO-CH} 5.3 Hz, HO-5'), 4.78 (dd, J_{2'-1'} 5.7 Hz, J_{2'-3'} 0 Hz, H-2'), 4.23 (dd, J_{3'-4'} 3.8 Hz, J_{3'-2'} 0 Hz, H-3'), 3.88 (dt, J_{4'-3'} 3.8 Hz, J_{4'-5'} 3.9 Hz, H-4'), 3.60 and 3.53 (2dd, J_{5'-4'} 3.9 Hz, J_{5'a-5'b} 12 Hz, 2H-5'a,b).

3'-amino-3'-deoxyuridine (4) and 3'-amino-3'-deoxyguanosine (5) were prepared by reduction from 2 and 3 according to a published procedure (9).

3'-azido-3'-deoxy[8-¹⁴C]guanosine was synthesized by modification of the procedure used for preparation of unlabelled 3'-azido-3'-deoxyguanosine (10).

N²-palmitoyl[8-¹⁴C]guanine (6) (11)

Unlabelled guanine HCl (1.23mmole) was dissolved in 1M HCl (2ml) and mixed with [8-¹⁴C]guanine HCl (0.876mCi) in 0.01M HCl (10ml). The solution

was made alkaline (pH 9.2) with 25% NH_4OH (final volume 22ml) and kept at 0°C for 45 minutes. The precipitate was separated by centrifugation, washed with H_2O (4x20ml) until it gave a negative test for Cl^- ion, and then washed again with acetone and ether. Drying at 108°C over P_2O_5 in vacuo gave 152mg of [8- ^{14}C]guanine, specific activity 0.789mCi/mmole. This material was suspended in dry pyridine (6ml), and 0.9ml freshly distilled palmitoyl chloride was added. The mixture was stirred and refluxed with protection from atmospheric moisture for 2 hours. Pyridine was removed by repeated co-evaporation with toluene. The residue was refluxed in ethanol (35ml) for 1 hour, then the solid was separated by centrifugation and washed with warm ethanol (2x20ml). Drying at 108°C over P_2O_5 in vacuo (16 hours) gave 326mg (84%) of chromatographically homogeneous product (TLC, S_3 Rf. 0.57).

3'-azido-3'-deoxy[8- ^{14}C]guanosine (7)

0.84mmole 6, 5.6ml hexamethyldisilazane (freshly distilled under N_2) and 0.8ml trimethylsilylchloride were mixed and heated under reflux with protection from atmospheric moisture for 5 hours. After co-evaporation with anhydrous xylene (3x5ml) the residue was dissolved in DCE, then 1.26mmole of 1 in acetonitrile (10ml), and 0.3ml TMSTF were added. The mixture was refluxed for 2 hours with protection from atmospheric moisture, then another portion of 0.15ml TMSTF was added and refluxing was continued for a further 7.5 hours. The reaction mixture was diluted with DCE (45ml), cooled on ice and extracted with cold saturated NaHCO_3 (2x30ml) and water (30ml). Drying over anhydrous Na_2SO_4 and evaporation gave 0.742g of a mixture which was separated by flash column chromatography on silica gel (4.5x7cm) eluting with benzene-ethylacetate (25-55%). The yield of isolated fully protected nucleoside was 0.234g (39.5%). TLC (silica gel) Rf. 0.30 in system S_4 .

0.23mmole of the radioactive product was mixed with an equal amount of unlabelled material and deprotected using 6M NH_3 in methanol (10ml) at $+7^\circ\text{C}$ for 60 hours. Solvents were removed and the residue was dissolved by warming in ethanol-methanol- H_2O (3:4:3;100ml) and mixed vigorously with hexane-ether (2:1;40ml). The aqueous layer was separated, diluted with ethanol-methanol- H_2O (3:4:3;50ml) and applied on a column (2.8x7cm) of AG1x2 (200-400 mesh) ion exchanger in the $[\text{OH}^-]$ form. The column was eluted first with ethanol-methanol- H_2O (3:4:3;350ml), and then with 0.2M TEAB. Fractions containing chromatographically homogeneous 7 were evaporated, co-evaporated with aqueous ethanol (5x30ml) and dried over P_2O_5 in vacuo. Yield 109mg (77%) of 7, specific activity 0.411mCi/mmole.

3'-azido-3'-deoxy[8-¹⁴C]guanosine 5'-phosphate, triethylammonium salt (8)

0.35mmole of **7** was evaporated with anhydrous pyridine (5x2ml), and dried overnight *in vacuo* (P₂O₅). Trimethyl phosphate (2.5ml) was added and, after stirring for 1 hour at room temperature, POCl₃ (0.15ml) was added at +4°C with stirring. After 20 hours at +4°C an ice-cooled 0.2M TEAB (50ml) solution was added to the reaction mixture and stirring was continued for 1 hour at 0°C. Solvents were removed by evaporation and the residue was co-evaporated with aqueous ethanol (2x10ml). The residue was dissolved in H₂O (50ml), the solution was adjusted to pH 8.0 with TEA, and the mixture was then applied on a DEAE-Sephadex A-25 column (2.8x23cm) in the [HCO₃⁻] form. The column was washed first with 0.02M TEAB (500ml), and was eluted with a linear gradient of TEAB (0.02M-0.4M, 3.5l). Fractions containing **8** were pooled, evaporated, co-evaporated with ethanol (5x5ml) and, after dissolving in water (2ml), freeze dried. The yield was 56%. This product was 95% radiochemically pure as judged by paper chromatography in system S₂ and was completely hydrolyzed (15 minutes/37°C) to the parent nucleoside **7** by 5'-nucleotidase in 0.1M glycine-NaOH buffer (pH 8.6) in the presence of 0.01M MgCl₂ (12). Consecutive preparations of unlabelled **8** from **7** gave 65-70% yield.

MS-FAB (-VE) 387 [M-H]⁻, (+VE) 490 [MH+TEA]⁺, 389 [MH-TEA]⁺, 239 [M-Gua], 152 [GuaH]⁺.

3'-azido-3'-deoxy[8-¹⁴C]guanosine-5'-phosphoro(2-methyl)imidazolidate, sodium salt (9)

0.098mM of **8** was dried by repeated co-evaporation with DMF (4x2ml) and placed in a dry box. The residue was dissolved in DMF (3.5ml) and the following reagents were added with stirring: 0.49mM 2-methylimidazole, 0.70mM TEA, 0.245mM Ph₃P and 0.245mM 2,2'-dipyridyldisulphide. After 3 hours at room temperature a 60mM solution of NaI in anhydrous acetone (4ml) was added and stirring was continued for an additional 15 minutes. The product **9** was precipitated with anhydrous ether (40ml) and separated by centrifugation. The fine precipitate was washed with anhydrous ether (2x15ml), anhydrous acetone (3x15ml) and finally with ether. Drying *in vacuo* over P₂O₅ gave 47mg (97%) of the sodium salt of **9**.

3'-amino-deoxy[8-¹⁴C]guanosine-5'-phosphoro(2-methyl)imidazolidate, sodium salt (10)

In a dry box, 0.096mM of **9** was dissolved in a mixture of anhydrous MeOH (12ml) and anhydrous TEA (2.25ml). Ph₃P (0.479mM) was added and the whole mixture was stirred for 4 hours at room temperature. Then anhydrous tributylamine (50μl) was added and the reaction mixture was evaporated into about 2ml

volume. The product was precipitated by gradual addition of anhydrous ether (80ml), then separated by centrifugation, and washed with ether (3x20ml). Drying over P_2O_5 in vacuo gave 44mg (98%) of **10**. This product was more than 96% radiochemically pure as judged by paper chromatography in system S_3 .

3'-amino-3'-deoxy[8- ^{14}C]guanosine-5'-phosphorimidazolidate, sodium salt (**11**) was prepared 97% pure following the same two step procedure described above.

3'-amino-3'-deoxyguanosine-5'-phosphate (**12**)

0.073mM of unlabelled **8** was dissolved in a mixture of 28% NH_4OH (4ml) and pyridine (3ml). After addition of a 6-fold excess of Ph_3P , the mixture was stirred at room temperature for 18 hours. Solvents were removed by evaporation, and the residue was partitioned between H_2O (50ml) and ether (20ml). The aqueous layer was extracted with ether (2x10ml), filtered through Millipore HA(0.42 μ m) and concentrated by evaporation to about 5ml. This solution was applied on a BioGel P-2 (200-400 mesh) column (1.5x15cm). The column was eluted with 0.02M TEAB. The appropriate fractions were pooled, evaporated and co-evaporated with aqueous ethanol. The yield was 75%.

MS-FAB: (-VE) 361 $[M-H]^-$.

5'-phosphoryl-(3'-amino-3'-deoxyquanylyl)-(3'→5')-3'-azido-3'-deoxyguanosine (**13**), and 5'-phosphoryl-(3'-amino-3'-deoxyquanylyl)-(3'→5')-3'-amino-3'-deoxyguanosine (**14**)

Equimolar amounts of unlabelled **9** (0.031mM) and **12** were dissolved in 0.15M 1-methylimidazole buffer pH 7.5 (1ml). The mixture was incubated at 50°C for 4 days, then diluted with H_2O (10ml), and applied on a DEAE-Sephadex A-25 column (1.5x24cm) in the $[HCO_3^-]$ form. The column was eluted with a linear gradient of TEAB (0.05-0.5M, 2l) at a flow rate of 0.8ml/minute. Dinucleoside phosphates **13** and **14** were eluted at buffer concentrations of 0.30-0.33M and 0.37-0.40M respectively, then isolated after the usual work-up procedure. The total yield of **13** and **14** calculated on the amount of **9** was 40%.

MS-FAB (+VE) 733 $[MH]^+$ for **13**; (-VE) 732 $[M-H]^-$, 755 $[M-H+Na]^-$ for **13**, and 706 $[M]^-$ for **14**.

Compound **13** is quantitatively converted into **14** by reduction with Ph_3P-NH_4OH /Pyridine as described above.

METHODS

UV measurements were made on a Beckman Mod.35 spectrophotometer. The molar extinction coefficient for phosphorylated derivatives of G_{N_3} or G_{NH_2} was assumed to be the same as that determined for the parent ribonucleoside. Unless otherwise indicated estimated yields are based on UV measurements. Proton

Table I. Chromatographic and Electrophoretic Mobilities

	PC in S ₂ *	TLC in S ₂ **	PE in S ₅ ***
Up	1.00	0.43	1.00
pG	0.59	0.27	-
U _{N3}	1.89	0.76	0.00
U _{NH2}	1.44	0.58	-0.08
G _{N3}	1.63	0.78	0.05
G _{NH2}	1.06	0.44	-0.03
pG _{N3}	0.89	0.41	0.78
pG _{NH2}	0.54	0.28	0.57
2-MeImpG _{N3}	1.82****	0.78	0.32
2-MeImpG _{NH2}	1.74****	0.60	0.29
ImpG _{N3}	1.46****	0.69	-
ImpG _{NH2}	1.20****	0.53	0.32
pG (N) pG _{N3}	0.45	-	0.68
pG (N) pG _{NH2}	0.28	-	0.51

* The chromatographic mobilities are relative to Up = 1.00.

** On Cellulose F.

*** The electrophoretic mobilities are given taking R_U = 0.00 and R_{Up} = 1.00.

**** In system S₃.

NMR spectra were recorded on a 360MHz Fourier transform spectrometer equipped with an Oxford Instruments magnet and a Nicolet 1180E computer. Chemical shifts are reported in parts per million and are referenced relative to TMS. MS measurements were performed on a VG ZAB-HF high resolution mass spectrometer (England) with an FAB source operated at 8KV. Samples were introduced on H₂O-glycerol or thioglycerol matrices.

Thin layer chromatography was performed on Cellulose F or Kieselgel 60F₂₅₄ precoated plates (DC-Fertigplatten-Merck, FRG). Paper chromatography was performed on Whatman 3MM sheets by the descending technique (development time 22-24 hours). Paper electrophoresis was performed on Whatman 3MM paper at 53V/cm using Varsol (Exxon) as coolant.

The following solvents systems and buffers were used: S₁, i-PrOH-28%-NH₄OH-H₂O (7:1:2); S₂, n-PrOH-28%-NH₄OH-H₂O (55:10:35); S₃, n-PrOH-TEA-H₂O (55:10:35); S₄, CHCl₃-MeOH (96:4); S₅, 0.03M KH₂PO₄ pH 7.1.

The chromatographic and electrophoretic mobilities are summarized in Table I.

When radioactive components were employed, yields were estimated by scanning strips from the paper chromatograms with a Baird Atomic 363 scanner with integrator or by cutting out the radioactive zones and, after immersion in Liquifluor (20ml) from New England Nuclear (concentration 42.25ml/l of toluene solution), counted in a Beckman scintillation counter. The HPLC analysis, and semi-preparative separations were performed on RPC-5 columns as described elsewhere (3).

Reaction of 2-MeImp[8-¹⁴C]N with 3'-amino-3'-deoxyuridine and uridine

Reaction mixtures each containing 0.1M of **4** and 0.01M of 2-MeImp[¹⁴C]N (0.5μCi, where N = U, A, G) in 50μl of 0.1M 2-MeIm.HCl buffer pH 7.45 were prepared and kept at room temperature for 15 days. Small aliquots (5μl) of each mixture were analyzed from time to time by paper chromatography in system S₂, and the distribution of radioactivity was measured. UV absorbing radioactive spots which had mobilities similar to those of authentic samples of UpU, UpA and UpG could be detected. In a parallel set of reactions employing uridine, virtually no significant formation of dinucleoside phosphates could be detected.

After 15 days radioactive spots corresponding to U(N)pU, U(N)pA and U(N)pG were isolated, and subjected (about 2 OD₂₅₄ of each) to enzymatic degradation using 0.2 units of ribonuclease T₂ or phosphodiesterase II, in 7mM sodium citrate (pH 6.1) or 0.2 units of pancreatic ribonuclease in 5mM TRIS.HCl (pH 7.6). Incubation at 37°C for 1 hour showed that dinucleoside phosphoramidates were completely degraded by ribonuclease T₂ and pancreatic ribonuclease to the corresponding ribonucleoside and a phosphorylated derivative of **4** which behaves like uridine 2',3' cyclic phosphoramidate (13). We did not analyze it further. Dinucleoside phosphoramidates were resistant to the action of phosphodiesterase II as previously reported (14).

Template-directed Reaction

Template-directed oligomerization reactions were carried out in polyethylene Eppendorf tubes (1ml) at 0°C. When gel-formation or precipitation occurred, the initial mixture was divided into separate tubes before the reaction began. After appropriate time periods the samples were diluted with 0.1M EDTA and analyzed. Unless otherwise indicated the composition of the template-directed oligomerization reaction mixture was as follows: 0.6M NaCl, 0.2M MgCl₂, 0.05M poly(C) or poly(dC) template, 0.4M EPPS.Na (pH 7.9) buffer and 0.1M 2-MeImpN or ImpN. In the experiments monitored by radioactivity,

0.4–0.5 $\mu\text{Ci}/20\mu\text{l}$ of ^{14}C was introduced into each tube.

Oligomers were isolated from reaction mixtures by collecting the HPLC eluate corresponding to the appropriate peak in the elution profile. After desalting on a BioGelP-2 column, the corresponding fractions were subjected to enzymatic degradation followed by HPLC analysis. Coinjection of independently prepared 14 with the original reaction mixture allowed us to identify the dimer.

Enzymatic Analysis

Isolated fractions corresponding to the dimer, tetramer and hexamer were digested by ribonuclease T₁ (0.1M TRIS.HCl (pH 7.6), 0.05M MgCl₂, 0.5 U enzyme, and about 0.1 OD of a substrate for 3–6 hours at 37°C). In parallel experiments corresponding oligoriboguanylates were digested under identical conditions. The nature of the side products represented by the smaller peaks in the elution profiles was investigated using dinucleotide nucleotidohydrolase (EC 3.6.1.9). Reaction conditions: 0.05M TRIS.HCl (7.4), 0.025M MgCl₂, 0.05 units of the enzyme per 0.1 OD of substrate. The incubation time at 37°C was dependent on the length of the oligomers in the sample. It varied from 3–18 hours. In parallel experiments G^{2'}pG and a product mixture derived from the oligomerization of 2-MeImpG in the presence of poly(C) (3) were digested under the same conditions.

RESULTS

The yields of the major products from the reaction of 0.01M [^{14}C]-labelled ribonucleoside-5'-phosphorimidazolidates (U, A, G) and 0.1M of 4 are shown in Fig. 1. These products, which move slightly behind the corresponding dinucleoside monophosphates on paper in system S₂ were degraded by ribonuclease T₂ and pancreatic ribonuclease but were resistant to spleen phosphodiesterase. These findings are as anticipated for a 3'-5'-linked dinucleoside phosphoramidate in the light of the previously reported sensitivity of the phosphoramidates to enzymatic degradation (14) and are inconsistent with a structure involving a 2'-5'-phosphodiester bond.

The reactivities of the three phosphorimidazolidates are similar, but decrease somewhat in the order G>A>U. When 4 was replaced by uridine in the above experiments, only traces of the phosphodiester were found. In free solution the rate of reaction of the aminonucleoside is one to two orders of magnitude greater than the corresponding reaction of uridine.

We have studied the oligomerizations of 2-MeImpG_{NH₂} and ImpG_{NH₂} on poly(C) and poly(dC) templates at 0°C under a variety of conditions. The

Fig. 1 Yields of oligomers from the incubation of 0.01M 2-MeImp[^{14}C]N with 0.1M UNH_2 or U at room temperature in 0.1M 2-MeIm·HCl buffer (pH 7.45). -Δ-Δ- 2-MeImp[^{14}C]G; -○-○- 2-MeImp[^{14}C]A; -□-□- 2-MeImp[^{14}C]U; -■-■- 2-MeImp[^{14}C]G, 0.1M U.

efficiency of production of material remaining at the origin of paper chromatograms developed in system S_2 for typical reactions using ^{14}C -labelled derivatives is illustrated in Fig. 2. This material corresponds to oligomers at least 3 units in length. It is clear that 2-MeImpG NH_2 , like 2-MeImpG, con-

Fig. 2 Efficiency of oligomer formation in the condensation of 0.1M 2-MeImpG NH_2 or 2-MeImpG on 0.05M poly(dC) or poly(C) template. Reaction conditions: 0°C, 0.4M EPPS-Na buffer (pH 7.9 at room temperature), 0.6M NaCl and 0.2M MgCl_2 . -○-○- 2-MeImpG NH_2 on poly(dC); -●-●- 2-MeImpG on poly(dC); -Δ-Δ- 2-MeImpG NH_2 on poly(C); -▲-▲- 2-MeImpG on poly(C); -□-□- 2-MeImpG NH_2 , no template; -■-■- 2-MeImpG, no template.

Fig. 3 HPLC elution profiles of the products from the condensation of 2-MeImpGNE₂ or ImpGNE₂ on poly(dC) or poly(C) templates at 0°C under various conditions. Reaction conditions: 0°C, for 14 days.

- non-template directed oligomerization of 0.1M 2-MeImpGNE₂ in 0.4M EPPS-Na (pH 7.9 at room temperature), 0.6M NaCl and 0.2M MgCl₂.
- 0.05M poly(C) and 0.1M 2-MeImpGNE₂ in 0.2M HEPES buffer (pH 7.4 at room temperature), 1.2M NaCl and 0.2M MgCl₂.
- 0.05M poly(dC) and 0.1M 2-MeImpGNE₂ in EPPS-Na buffer (pH 7.9 at room temperature), 0.6M NaCl and 0.2M MgCl₂.
- 0.04M poly(dC) and 0.04M ImpGNE₂ in 2,6-lutidine-HCl buffer (pH 7.7 at room temperature), 0.4M NaNO₃ and 0.4M Mg(NO₃)₂.

The numbers above selected peaks are the lengths of the corresponding 3' to 5'-linked oligo(G(N))s. Conditions for HPLC elution with linear NaClO₄ gradient (pH 12, 0 to 0.04M, 60 minutes) UV absorption monitored at 254nm.

Fig. 4 HPLC elution profiles of the products from the condensation of 0.1M 2-MeImpGNH₂ on 0.05M poly(C) template in 0.4M EPPS-Na buffer (pH 7.9 at room temperature), 0.6M NaCl and 0.2M MgCl₂ at 0°C, for 14 days.

a) aliquot incubated in 0.05M TRIS-HCl (pH 7.6) at 37°C for 24 hours.

b) The same amount incubated at 37°C for 24 hours with dinucleotide nucleotidohydrolase (see Methods).

The numbers above selected peaks are the lengths of the corresponding 3' to 5'-linked oligo(G(N))s. Condition for HPLC elution with linear NaClO₄ gradient (pH 12, 0 to 0.03M, 45 minutes) UV absorption monitored at 254nm.

tinues to react until the template is fully occupied by oligomers. In some cases the yield of oligomers may even exceed the capacity of the template, presumably because shorter oligomers are displaced by growing longer chains. The rate of formation of oligomeric material is somewhat greater from 2-Me-*ImpGNH*₂ than from 2-Me*ImpG* under the same reaction conditions.

The distribution of oligomeric products as revealed by HPLC is almost independent of the choice of buffer (2,6-lutidine, HEPES, EPPS), the concentration of NaCl (up to 1.2M), and the pH in the range 7.5-8.5. Typical HPLC elution profiles for the oligomers obtained from 2-Me*ImpGNH*₂ on poly(C) and poly(dC) are shown in Fig. 3 b. and c. Almost identical results are obtained under a variety of conditions; the reaction on poly(dC) gives oligomers up to 12 or 13 long with good isomeric specificity, while the reaction on poly(C) gives oligomers up to 9 or 10 long with less good specificity. The last detectable oligomer obtained in a control non-template reaction is the pentamer (Fig. 3 a). When *ImpGNH*₂ is substituted for 2-Me*ImpGNH*₂ in the above reactions, the pattern of products is little changed (Fig. 3 d).

After prolonged digestion with ribonuclease T₁, the pure di-, tetra- and hexanucleotide analogues isolated from the column were completely degraded, establishing that these substances are 3'-5'-linked phosphoramidates. The rates of degradation were much less for the phosphoramidates than for the cor-

responding phosphodiesterases.

The results of incubating total reaction mixtures with dinucleotide nucleotidohydrolase are suggestive. In independent experiments using $G^{2'}pG$ and oligo(G)s as substrates we showed that this enzyme preparation, in addition to its pyrophosphatase action, completely degrades oligomers containing 2'-5'- and 3'-5'- phosphodiester bonds. This activity may be intrinsic to the pure enzyme or may be due to an unspecific phosphodiesterase present in the preparation (about 2.5% in the sample used). In either case the enzyme preparation permits a striking discrimination between phosphoramidate and phosphodiester bonds. All of the side products in reaction mixtures from 2-MeImpG $_{NH_2}$ were degraded by the enzyme, but the major peaks were left untouched (Fig. 4 a. and b). This strongly suggests that the minor peaks correspond to compounds containing 2'-5'- phosphodiester bonds and pyrophosphate bonds. The resistance of the major peaks to degradation shows that the hydrolase does not attack 3'-5'-linked phosphoramidate bonds.

DISCUSSION

Reactions between amines and nucleoside-5'-phosphorimidazolides in aqueous solution are substantially faster than the corresponding reactions with primary or secondary alcohols (13, 15). It is not surprising, therefore, that a 3'-amino-3'-deoxyuridine (4), reacts with nucleoside 5'-phosphorimidazolides to give 60-70% of the dinucleoside phosphoramidate under conditions that do not permit the synthesis of an appreciable amount of the phosphodiester from the corresponding nucleoside (Fig. 1). In a similar way, the self-condensation of ImpG $_{NH_2}$ or 2-MeImpG $_{NH_2}$, in the absence of a template, yields oligomers up to the pentamer (Fig. 3 a) under conditions that do not permit the formation of significant amounts of oligomers from ImpG or 2-MeImpG.

A comparison of the template-directed reactions of G $_{NH_2}$ and G reveals a much more complex relationship. In general, 5'-phosphorimidazolides of G with substituents on the imidazole ring react relatively inefficiently and non-regiospecifically on a poly(C) or a poly(dC) template. The products are short oligomers, and a variety of isomers incorporating 2'-5'- and 3'-5'- phosphodiester linkages in comparable amounts are obtained for each oligomer length. Only one derivative, 2-MeImpG, is known to react efficiently and regiospecifically. It yields long oligomers which are almost exclusively 3'-5'-linked (2, 16).

The reactions of 2-MeImpG and 2-MeImpG $_{NH_2}$ on poly(C) or poly(dC) are both extremely efficient, in the sense that the number of G residues incorporated

into polymers equals or even somewhat exceeds the number of C residues present in the template (Fig. 2). The accumulation rate of oligomeric products is somewhat faster in the amino- than in the ribonucleoside.

HPLC analysis reveals that the reaction of 2-MeImpG_{NH₂} on poly(C) is not highly specific with respect to isomers, and the oligomeric products obtained do not extend beyond the 10-mer (Fig. 3 b). The final product distribution is independent of the nature of the buffer (HEPES, EPPS or lutidine), or of the salt concentrations employed. The reaction of 2-MeImpG_{NH₂} on poly(dC) is somewhat more specific and yields oligomeric products up to 12-13 units in length. Again, the nature of the final products depends little on the nature of the buffer or the precise composition of the reaction mixture. The products from the reactions of ImpG_{NH₂} have almost the same distribution as those obtained with 2-MeImpG_{NH₂} both on poly(C) and poly(dC) templates. This contrasts sharply with the corresponding result obtained with the parent nucleotide derivatives (2, 16).

The formation of oligomeric products with high yield on a template is possible only if the condensation reaction on the template competes effectively with the hydrolysis of the activated nucleotide. The distribution of molecular sizes, on the other hand, is determined both by the rate of the condensation reaction and by the relative rates of chain initiation (dimer formation) and chain elongation. In the light of these simple considerations one can give a general interpretation of our findings.

The reaction of a secondary hydroxyl group with a phosphorimidazolidate is, in general, relatively inefficient. An efficient, regiospecific template-directed reaction in aqueous solution is, therefore, to be expected only if the hydroxyl group of one activated nucleotide is constrained to the optimum orientation relative to the phosphorimidazolidate of the next. The amino group is far more nucleophilic than the hydroxyl group and, therefore, can react efficiently with an activated phosphate even if the orientation on the template is far from optimal. The finding that 2-MeImpG_{NH₂} and ImpG_{NH₂} behave so similarly on poly(C) or poly(dC) templates suggest that neither substrate is optimally oriented on the template and that the efficiency of the reactions can be attributed to the intrinsic nucleophilicity of the amino group.

The absence of long oligomers amongst the products of the condensation of G_{NH₂} derivatives contrasts with our findings for 2-MeImpG (3). Since the efficiencies of the reactions are comparable, this must imply that initiation and elongation proceed at more nearly equal rates for the amino derivative

than for 2-MeImpG. This again may be attributed to the greater intrinsic nucleophilicity of the amino group and the consequent less sensitive dependence of the reaction rate on the precise orientation of the amino group with respect to the activated phosphate.

The conclusion that the template-directed reactions of 3'-amino-3'-deoxy-nucleotides are relatively efficient, even where the orientation of reaction groups is not optimal, has important consequences for studies of molecular replication. The oligomerization of the 2-methylimidazole derivatives of standard nucleotides, other than pG, on copolymer templates is less efficient than the oligomerization of 2-MeImpG on poly(C) (4). This, we believe, reflects the dependence of the geometry at the growing point of an oligomer chain on the nature of the last bases of the growing oligomer and the incoming monomer. The use of 2-methylimidazole derivatives is optimal only for the joining of 2-MeImpG to oligo(G)s. The substitution of 3'-amino-3'-deoxy-nucleosides for the normal nucleosides should lead to a much less sensitive dependence on oligomer sequence. Preliminary studies with short oligonucleotide templates confirm that this is the case, and show that rapid, isomerically specific reactions are more easily realized with aminonucleotides than with the parent nucleotides.

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6. Abbreviations: GN_3 , 3'-azido-3'-deoxyguanosine; GNH_2 , 3'-amino-3'-deoxyguanosine; ImpN, nucleoside 5'-phosphorimidazolidate; 2-MeImpN, nucleoside 5'-phosphor(2-methyl)imidazolidate; $\text{N}_{(\text{N})}\text{pN}$, dinucleotides containing (3'-5') phosphoramidate bond; TEAB, triethylammonium bicarbonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; DCE, 1,2-dichloroethane; Ph_3P , triphenylphosphine; TMSTF, trimethylsilyltrifluoromethanesulphonate; TEA, triethylamine; DMF, dimethylformamide; PC, paper chromatography; PE, paper electrophoresis; HPLC, high performance liquid chromatography; TLC,

- thin layer chromatography; MS-FAB, fast atom bombardment mass spectrometry.
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