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SYNTHESIS AND CHARACTERIZATION OF A CARBAMATE-LINKED OLIGONUCLEOSIDE

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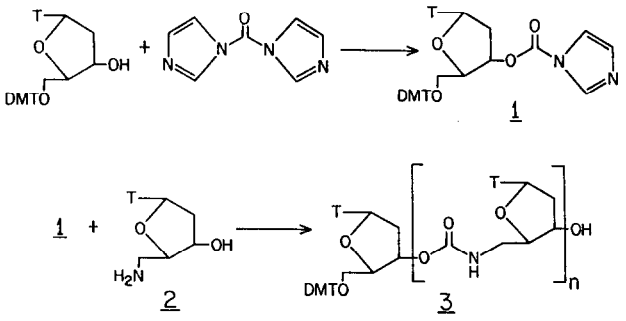
Summary: Reaction of 5'-O-dimethoxytritylthymidine with 1,1'-carbonyldiimidazole gave the 3'-O-carbonylimidazolide, which was condensed in high yield with 5'-amino-5'-deoxythymidine to produce a dinucleoside containing a 3'-O-5'-N-carbamoyl linkage. Four repetitions of carbonyl imidazolide formation and condensation produced a carbamate-linked hexamer. Hydrolytic and thermal denaturation studies did not indicate the presence of intramolecular base stacking.

Oligonucleotide analogues containing non-ionic internucleoside linkages are of interest due to their potential as anti-viral agents, as repressors of gene expression at the transcriptional and translational levels, and as models for the study of nucleic acid structure and function. Some of the analogues reported to date contain methylphosphonate (1), carbonate (2), oxyacetamide (3) and carbamate (3,4) internucleoside linkages. The oxyacetamide and carbamate analogues are attractive due to their stability over a wide pH range and their resistance to enzymatic degradation. Furthermore, in contrast to the methylphosphonate analogues, they possess linkages which are achiral, thus avoiding the difficult separation of diastereomers during their synthesis and purification.

The synthesis of a 3'-O-5'-N-carbamate-linked dimer of thymidine was reported in 1974 by Gait and co-workers (3). The compound was obtained in 38% yield from the condensation of the 3'-O-(2,2,2-trichloroethyl)-carbonate of 5'-O-tritylthymidine with 5'-amino-5'-deoxythymidine followed by detritylation. In 1977, Mungall and Kaiser described the synthesis of a trimer containing 3'-O-5'-N-carbamoyl moieties. This synthesis was accomplished by reaction of 5'-amino-5'-deoxythymidine with the 3'-O-(4-nitrophenyl)-carbonate of 5'-O-tritylthymidine (4). The isolated dinucleoside carbamate was derivatized at the 3' position with 4-nitrophenylchloroformate and condensed as above to give, upon detritylation, the trimer in 30% overall yield.

To investigate the properties of these unique nucleic acid analogues, the synthesis of a carbamate-linked hexamer of thymidine was undertaken. The synthetic strategy is illustrated below. Reaction of 5'-O-dimethoxytritylthymidine with 1.1 equivalents of 1,1'-carbonyl-diimidazole (CDI) in tetrahydrofuran, followed by aqueous extraction, resulted in a 97% yield of the pure 3'-O-carbonylimidazolide, 1 (5). No symmetrical 3',3'-dinucleoside carbonate was produced. The use of CDI (6) to introduce the 3' activated carbonyl group avoided the difficulties associated with the handling of highly reactive chloroformates. Furthermore, compound 1 was stable in pyridine, a solvent known to decompose the 3'-O-(4-nitrophenyl)-carbonate of 5'-O-tritylthymidine (4).

Compound 1 was reacted in pyridine with 1.1 equivalents of 5'-amino-5'-deoxythymidine, 2, (7) for 20 hours at 20°C. Excess 2 was removed by silica gel chromatography to provide the



pure dinucleoside carbamate, 3 (n=1), in 87% yield. The presence of the 3'-O-5'-N-carbamoyl linkage was confirmed by 200 MHz ^{1}H NMR spectroscopy (8). A one flask procedure for 3'-O-carbonylimidazolide formation and condensation was developed to minimize product loss (9). Four repetitions of the procedure resulted in a 40% yield (based on 1) of the 5'-O-dimethoxytrityl hexamer, 3 (n=5). The dimethoxytrityl group was removed by dissolving 3 (n=5) (34 μmol) in 10.3 mL of 80% aqueous acetic acid for 20 minutes at 20°C. The orange solution was concentrated at reduced pressure and coevaporated three times with acetonitrile/water, 1/2 (V/V). The resulting white solid was dissolved in 50% aqueous tetrahydrofuran and applied to Whatman 3MM paper. Descending chromatography was performed with n-butanol/acetic acid/water, 6/2/2 (V/V/V), as the mobile phase. The product was visualized with ultraviolet light and eluted from the paper with water/conc. NH_4OH, 9/1 (V/V), to give 32 μmol of the hexamer. The structure of the carbamate hexamer was confirmed by fast atom bombardment mass spectrometry and 470 MHz ^{1}H NMR spectroscopy (10). The compound is not readily soluble in water or many organic solvents and adheres tenaciously to glassware. Prior to optical studies, trace impurities were removed by reversed-phase HPLC (11).

Extinction coefficients were determined by base-catalyzed hydrolysis of the hexamer to its parent nucleosides (12). In contrast to a carbamate-linked dimer of thymidine, which has 8% hypochromicity (3), the carbamate hexamer is not hypochromic. The corresponding phosphodiester-containing molecule, dT-dT$_4$-dT, has 10.4% hypochromicity (13). In addition, there was no change in absorbance when the carbamate hexamer was heated in 10 mM sodium phosphate, pH 7.0, from 0°C to 85°C (14). The lack of an observed hypochromicity indicates that base stacking is not occurring (15). Although the carbamate linkage is about 0.5 Å shorter than a phosphodiester linkage (3), a CPK model of the carbamate polymer can be placed in strain-free conformations in which the bases are stacked, and the atoms of the linkages are arranged to maximize delocalization of the nitrogen atom lone pair electrons. In this arrangement, the carbamate carbonyl oxygen is trans planar to the carbamido proton and cis planar to the 3' carbon of the deoxyribose ring. Thus, the apparent lack of base stacking in the carbamate hexamer is not due to an intrinsic steric limitation.

Model building studies also revealed that the carbamate polymer might form base-paired helices with complementary nucleic acid sequences. To test this possibility, equimolar mixtures of the carbamate hexamer and A-A$_4$-A or dA-dA$_4$-dA in 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0, were cooled to 0°C and the absorbance at 260 nm was recorded at one degree intervals as the solutions were heated to 85°C at a rate of 0.5°C per minute. The absorbance versus temperature profiles of the mixtures were identical, within experimental error, to the

sum of the profiles of the individual components obtained under the same experimental conditions. Thus, as determined by ultraviolet spectroscopy in this buffer, there was no interaction between either A-A_4-A or dA-dA_4-dA and the carbamate-linked polymer of thymidine. An explanation of the lack of hypochromicity of the carbamate polymer and its apparent inability to base pair with complementary oligonucleotide sequences requires further structural analysis. In particular, the conformation of the carbamoyl moieties in the backbone as well as the solvated structure of the polymer must be determined.

The use of CDI as a carbonyl synthon affords clean, high yield reactions in the synthesis of oligonucleoside carbamates, and should find application in the synthesis of other carbamate polymers. Future work will include development of methods for the solid-phase synthesis of carbamate oligonucleotide analogues containing all four bases, as well as investigation of synthetic routes to other non-ionic nucleic acid analogues.

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5. 5'-O-dimethoxytritylthymidine (1.5 mmole) was coevaporated three times with dry tetrahydrofuran and taken up in 6.0 mL of dry tetrahydrofuran. 1,1'-Carbonyldiimidazole (1.65 mmole) was added and the reaction was stirred for 4.0 hours at 20°C. The mixture was concentrated, taken up in 100 mL of ethyl acetate, and extracted with two 100 mL portions of 5% aqueous NaH_2PO_4. The organic layer was dried over Na_2SO_4, filtered and concentrated to give 1 as a white foam, 933 mg, 97% yield. 200 MHz ^{1}H NMR in $CDCl_3$ (δ,TMS=0.00): 8.90(S,1H,N-H) 8.16(q,1H,Im) 7.62(d,1H,H6) 7.10(q,1H,Im) 6.50(dd,1H,H1') 5.65(br d,1H,H3') 4.32(m,1H,H4') 3.80(s,6H,OCH_3) 3.56(m,2H,H5',5") 2.80-2.49(m,2H,H2',2") 1.44(d,3H,CH_3)
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8. Compound 1 (0.5 mmole) and 5'-amino-5'-deoxythymidine, 2 (0.55 mmole), were coevaporated with 2.0 mL of dry pyridine, and taken up in 2.0 mL of dry pyridine. The reaction mixture was stirred for 20 hours at 20°C, concentrated and dissolved in 50 mL of $CHCl_3$. The chloroform solution was extracted with 50 mL of 5% aqueous NaH_2PO_4 and the aqueous layer back-extracted three times with 50 mL portions of $CHCl_3$. The combined organic layers were dried over Na_2SO_4 and concentrated. The resulting glass was dissolved in $CHCl_3$ and purified by centrifugal chromatography. The silica plate (2mm thickness) was washed with $CHCl_3$ and eluted with $CHCl_3/CH_3OH$, 24/1 (V/V), followed by $CHCl_3/CH_3OH$, 7/93 (V/V). The product fractions were concentrated to give a white foam, 354 mg, 87% yield. 200 MHz ^{1}H NMR in $CDCl_3$ (δ,TMS=0.00): 7.61(s,1H,H6) 6.47-6.22(m,2H,5'H1',OH) 5.94(br t,1H,3'H1') 5.42(m,1H,5'H3') 4.46(m,1H,3'H3') 4.07(br s,1H,5'H4') 3.92(m,1H,3'H4') 3.76(s,6H,OCH_3) 3.67-3.14(br m,4H,H5'5") 2.57-2.19(br m,4H,H2',2") 1.86(s,3H,3'CH_3) 1.29(s,3H,5'CH_3)
9. Representative one-flask procedure for 3'-O-carbonylimidazolide formation and condensation: 3 (n=2), (0.25 mmole) was coevaporated with then dissolved in 1.0 mL of dry pyridine.

1,1'-Carbonyldiimidazole (0.5 mmole) was added and the reaction was stirred for 2.0 hours at 20°C. Water (3.0 mmole) was added, stirring was continued for 0.5 hr and then the reaction was concentrated. After three coevaporations from pyridine, the mixture was taken up in 1.0 mL of dry pyridine and 2 (0.275 mmole) was added. The reaction was stirred 20 hours at 20°C. Following concentration, the mixture was coevaporated with $CHCl_3$, taken up in $CHCl_3/CH_3OH$, 20/1 (V/V), and applied to a column (2.5 X 6 cm) of Merck silica gel 60G packed in the same solvent. The column was washed with 60 mL of loading solvent and eluted with 60 mL of $CHCl_3/CH_3OH$, 9/1 (V/V), followed by 60 mL $CHCl_3/CH_3OH$, 4/1 (V/V). The product fractions were combined and concentrated to give 3 (n=3), 320 mg, 96%. 200 MHz ^1H NMR in $CDCl_3/CD_3OD$, 7/1 (V/V), (δ,TMS=0.00): 7.62(s,2H,H6) 7.04(s,2H,H6) 6.38(t,1H,5'H1') 6.15(t,2H,H1') 6.03(t,1H,3'H1') 5.40(br s,1H,5'H3') 5.30-5.06(br m,2H,H3') 4.33(br m,1H,3'H3') 4.21(s,1H,5'H4') 4.06(m,2H,H4') 3.89(br m,1H,3'H4') 3.79(s,6H,OCH_3) 3.46(br s,8H,H5',5") 2.56-2.16(m,8H,H2',2") 1.91(br s,9H,CH_3) 1.31(br s,3H,5'CH_3)

10. FAB in the negative ion mode (glycerol/thioglycerol, 2/1, (V/V)) M-1=1578.5 ± 1. 470 MHz ^1H NMR in D_2O/tetrahydrofuran-d_8, 1/1 (V/V), (δ, external 1% TSP/D_2O=0.00): 7.91(s,1H,H6) 7.64(s,4H,H6) 7.60(s,1H,H6) 6.31(dd,1H,3'H1') 6.24(br t,5H,H1') 5.25(d,1H,5'H3') 5.16(br s,4H,H3') 4.37(br m,1H,3'H3') 4.14(br s,5H,H4') 3.98(dd,1H,3'H4') 3.85(dd,2H,5'H5',5") 3.58-3.38(br m,10H,H5',5") 2.52-2.27(br m,12H,H2',2") 2.22-2.12(br s,18H,CH_3) These assignments were confirmed by DQF COSY.
11. Preparative isolation was performed by reversed-phase chromatography on a 5x0.4 cm column of 5 μm, ODS-Hypersil (Shandon, U. K.). The carbamate hexamer was loaded in a mixture of 17% solvent A in solvent B and the hexamer was eluted from the column by running a gradient from 17% to 55% A in B over 17 min at a flow rate of 1.0 mL per min. Solvent A was 20% aqueous acetic acid. Solvent B was 50% aqueous acetonitrile.
12. The carbamate hexamer (approximately 5 A_{266} units) was dissolved in 50 μL of 1.4 N NaOH. The sample was heated in a sealed tube at 70°C for 4 hours. The reaction mixture was adjusted to pH 7.0 with dilute phosphoric acid and taken to a volume of 10.00 mL. The ultraviolet absorbance of this solution was used to determine the percent hypochromicity (15). The assumption was made that no change in the extinction coefficient of 5'-amino-5'-deoxythymidine had occurred upon polymerization. The hydrolysis products were separated on a 5 X 0.4 cm column of 5 μm ODS-Hypersil eluted isocratically with 20 mM sodium phosphate, pH 6.8. Thymidine, 5'-amino-5'-deoxythymidine and a trace of thymine (<1%) were the only products detected. The nucleoside ratio was determined by integration of peak areas. The values were corrected for the differential loss of nucleoside material on the column. The ratio of 5'-amino-5'-deoxythymidine to thymidine was 5.06 ± 0.15. The hypochromicity measurement and HPLC analysis were performed six times. The average extinction coefficients for the carbamate hexamer in 10 mM sodium phosphate, pH 7.0, using previously reported extinction coefficients (7,16) for the parent nucleosides are: ε_{254}=42,900 ± 1,800, ε_{260}=52,600 ± 2,300, ε_{266}=56,200 ± 2,400.
13. dT-dT_4-dT was synthesized by solid-phase phosphoramidite methods as described by J. M. Coull, H. L. Weith and R. Bischoff, Tetrahedron Lett. 27, 3991-3994 (1986). For the procedure used to determine the hypochromicity see J. G. Nadeau and P. T. Gilham, Nucleic Acids Res., 13, 8259-8274 (1985).
14. A 7.9 μM solution of the purified carbamate hexamer, in 10 mM sodium phosphate, pH 7.0, was heated from 0°C to 85°C at a rate of 0.8°C per min. The absorbance at 260 nm was recorded at intervals of 1.0°C and corrected for thermal expansion of the solution (17).
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