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Achiral Internucleoside Linkages 3: CH_2-NH-CH_2 Linkage

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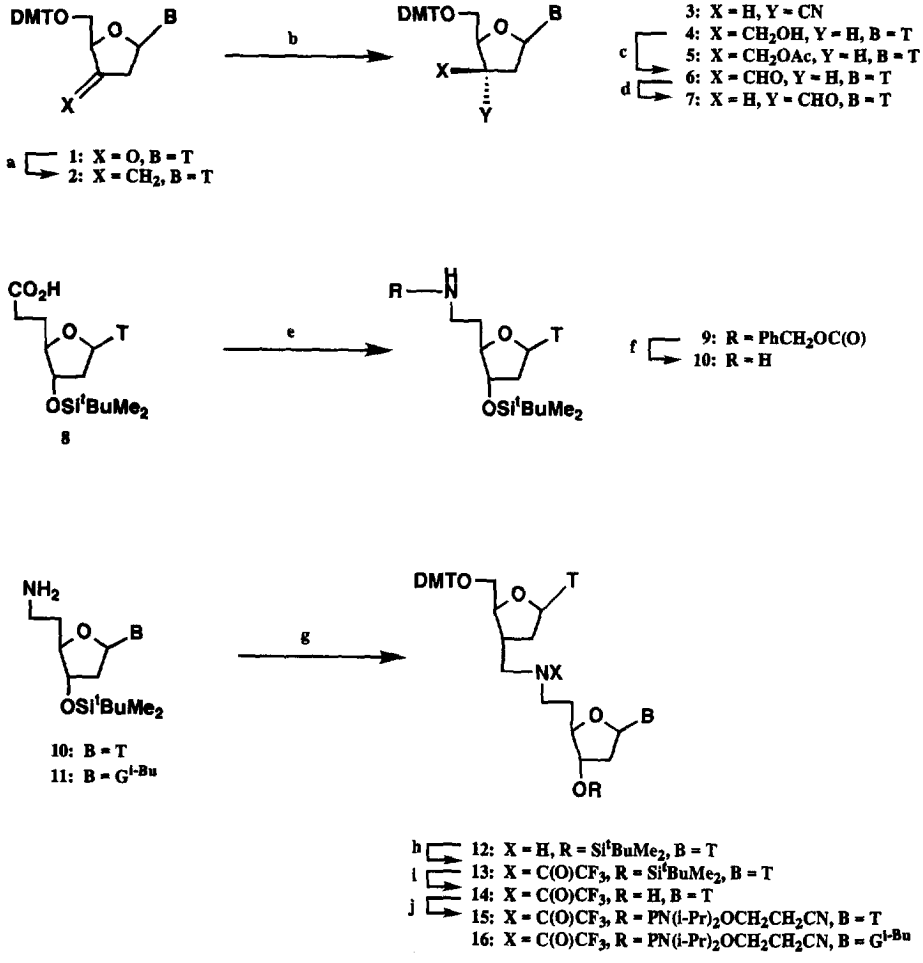
Abstract: A stereoselective method for the synthesis of 3'-α- or 3'-β- carbon substituted pyrimidine nucleosides was developed. The synthesis of the CH_2-NH-CH_2 linkage is described. The linkage offers enhanced resistance to 3'-exonuclease.

Syntheses of oligonucleotides with modified internucleoside phosphate linkages have recently emerged as an important area in the antisense approach to regulate gene expression. Modified oligonucleosides are expected to have enhanced nuclease resistance and improved pharmacokinetic properties.[1,2] In this connection, we have recently described the syntheses, hybridization and nuclease resistance studies of 5'-NH-CH_2-CH_2-3' and 5'-CH_2-CH_2-NH-3' linkages.[3] In the present communication, we describe the synthesis of dimers containing 5'-CH_2-NH-CH_2-3' linkage, their incorporation into oligonucleoside strands, and hybridization and nuclease resistance studies on these modified strands.

Scheme 1 outlines the synthesis of modified dinucleosides **15** and **16** which are ready for incorporation into a predetermined DNA sequence. The nucleoside aldehyde **7** and nucleoside amine **10** were the key subunits in the formation of dimers (**Scheme 1**). Initially, attempts to synthesize aldehyde **7** from the known cyanothymidine[4] **3** by DIBAL-H reduction proceeded in only 30% yield. Further, introduction of a cyano group at the 3'-position of thymidine under radical conditions was critically dependent on the steric bulk of the 5'-protecting group.[5,6] Therefore, an alternative method is needed for the synthesis of **7** in a stereopredictive manner. Hydroboration-oxidation of readily available 3'-methylene-3'-deoxythymidine[7,8] **2** produced primary alcohol **4** stereoselectively in 85% yield. Stereochemical assignment of **4** was based on NOE measurement of **5.** Irradiation of methylene group attached to 3'-carbon of **5** gave a 7% positive NOE on the 5'-methylene hydrogens suggesting the approach of borane from the α-face. The alcohol **4** was converted to the 3'-β aldehyde **6** in quantitative yield using Dess-Martin periodane. Epimerization of 3'-β aldehyde **6** under DBU catalyzed conditions afforded a 15:1 mixture of aldehydes favoring the required 3'-α-aldehyde **7.** Stereochemical assignment of **7** was confirmed by chemical correlation. Spectral properties of **7** were in complete agreement with the aldehyde derived from the known cyano compound. Methylenation of 3'-keto-5'-O-dimethoxytrityl-N^4-benzoyl-2'-deoxycytidine with CH_2Br_2-$TiCl_4$-Zn provided the 3'-methylenecytidine derivative in 50% yield. Under similar conditions 3'-keto-5'-O-dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine and 3'-keto-5'-O-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine were also converted to 3'-methylene compounds but only in 10-15% yield. Observed depurination under the strong acidic conditions was responsible for low yield. Attempted methylenation of 3'-keto-5'-O-dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine with CH_2I_2-$TiCl_4$-Zn or Tebbe reagent did not improve the yield.

The synthesis of amine **10** was accomplished from the known carboxylic acid[9] **8** by Curtius rearrangement. Treatment of carboxylic acid with diphenylphosphorylazide-triethyl amine followed by addition

Scheme 1[a]: Synthesis of CH_2-NH-CH_2 Linkage



[a]Reagents and conditions: (a) CH_2Br_2-$TiCl_4$-Zn, THF, 92%; (b) BH_3•THF then H_2O_2, NaOH, 85%,; (c) Dess-Martin periodane, CH_2Cl_2; (d) 0.05 eq DBU, $CHCl_3$, 94% from 4; (e) $Ph_2P(O)N_3$, Et_3N, THF then $PhCH_2OH$, 86%; (f) H_2, Pd/C, 98%; (g) 7 + 10, $Na(OAc)_3BH$, $ClCH_2CH_2Cl$, 81%; (h) $(CF_3CO)_2O$-Et_3N, CH_2Cl_2; (i) nBu_4NF, THF, 91% from 12; (j) $EtN(i\text{-}Pr)_2$, 2-cyanoethyl N,N-diisopropyl-chlorophosphoramidite, CH_2Cl_2, 79%.

of benzyl alcohol produced the carbamate **9** in 86% yield. The benzyl carbamate group was removed using 10% Pd on carbon to give amine **10**. The corresponding 5'-homologated A^{Bz}, $G^{i\text{-}Bu}$ and C^{Bz} amine derivatives were synthesized in the same manner in 70-78% yield.

Reductive amination of aldehyde **7** with the amine **10** was carried out with $Na(OAc)_3BH$[10] and the desired dimer **12** was isolated in 81% yield. Then, the aliphatic nitrogen was protected as a trifluoroacetamide[3] by treatment with $(CF_3CO)_2O$-Et_3N. Subsequent removal of the silyl protecting group with $^nBu_4NF$ afforded the alcohol **14** in 91% yield. Compound **14** was chosen as a model for the deprotection of trifluoroacetamide. The deprotection was accomplished in almost quantitative yield by treating with ammonia at 60°C for 6 h. The secondary hydroxyl was transformed to the required phosphoramidite **15** by reacting with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite. A 5'-T-CH_2-NH-CH_2-G-3' heterodimer (**16**) was synthesized using amine **11** in the above synthetic sequence. Phosphoramidite **15** was incorporated into 11-mer polythymidine sequences **18, 19** and **20.** The coupling yields with **15** and **16** were consistently in the range of 96-99% in automated DNA synthesis. The oligosequences were analyzed by negative FAB-MS and the expected deprotonated ions $(MW-1)^-$ were observed.

Polythymidine strands containing a single incorporation of the modified dimer **15** were chosen to probe the effect of the dimer on duplex stability since changes observed in T_m with poly T/deoxyribo-poly A sequences are expected to be more pronounced than in duplexes containing mixed base sequences.[11] Thermal melting curves of strands **18** and **19** with a deoxyribo-A_{11} sequence showed a characteristic single sigmoid transition. ΔT_m resulting from incorporation of CH_2-NH-CH_2 linkage is approximately -3.5 to -5.0°C with ssDNA which is comparable to ΔT_m resulting from incorporation of other internucleoside linkages.[3, 12, 13]

Table 1: Thermal Melting and Nuclease Resistance Data

Oligomer	T_m(°C)[a]	ΔT_m(°C)	$T_{1/2}$ in min
17: d-$T_pT_pT_pT_pT_pT_pT_pT_pT_pT_pT$	32.5	-	3
18: d-T-**CH_2-NH-CH_2**-$T_pT_pT_pT_pT_pT_pT_pT_pT_pT$	29.0	-3.5	-
19: d-$T_pT_pT_pT_pT_pT_pT_pT_pT$**T-$CH_2$-NH-$CH_2$-**$T_pT$	27.5	-5.0	36
20: d-$T_pT_pT_pT_pT_pT_pT_pT$**T-CH_2-NH-CH_2-**T_pT**-CH_2-NH-CH_2-**T_pT	b	-	229

(a) Melting Temperatures (T_m) measured at 5.0 μM oligomer concentration containing 100 mM NaCl, 10 mM Na_2HPO_4 (pH= 7.0) and 0.1 mM EDTA. (b) No duplex formation > 25°C.

Nuclease stability of oligonucleosides **19** and **20** was evaluated in 10% fetal bovine serum containing media (RPMI 1640) at 37°C with time. Oligonucleosides were purified from the serum by extraction and analyzed by HPLC using an anion exchange column. Results were analyzed for peak retention times and area. HPLC analysis of oligothymidylates **19** and **20** revealed secondary product peaks which were stable over much longer periods of time than the unmodified strands. These results were indicative of exonuclease cleavage of the 3'-terminal thymidylate residue to the point of modification, with the remainder of the strand offering enhanced resistance to further hydrolysis by the enzyme. Thus, oligonucleosides **19** and **20** were 12- and 76- fold more stable than unmodified strand, respectively.

In conclusion, a stereoselective method was developed for the construction of carbon-carbon bond at the 3'-position of pyrimidine nucleosides. An efficient and practical synthesis of the CH_2-NH-CH_2 internucleoside linkage was demonstrated. Oligonucleosides containing CH_2-NH-CH_2 hybridized to the ssDNA with ΔT_m's corresponding to other linkages reported in the literature. Incorporation of the modified linkage showed enhancement of nuclease resistance by a factor of 12 to 75 depending on the number of dimer incorporations.

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