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Synthesis and Hybridization Properties of Amide-Linked Thymidine Dimers Incorporated into Oligodeoxynucleotides

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Abstract: *Thymidine dimers, connected by amide or N-methyl amide linkages, have been prepared. The dimers have each been incorporated three times into normal strands of DNA by solid phase synthesis. Thermal denaturation studies indicated that these modifications caused little or no destabilization of the DNA:RNA duplex.*

Antisense oligonucleotides, specifically designed to bind to complementary RNA, can be used to inhibit gene expression.[1] This approach to rational drug design is an increasingly important field of research.[2] In order to be pharmacologically useful, these nucleotides must be chemically modified to facilitate cell wall penetration and to increase resistance to enzymatic degradation. These changes, however, should not adversely affect binding affinity towards target RNA. The first generation of modified nucleotides, the phosphorothioates[3] and the methylphosphonates,[4] are presently being subjected to clinical trials.[5] As this work progresses, new problems are bound to arise that can not be solved by any one nucleotide modification.

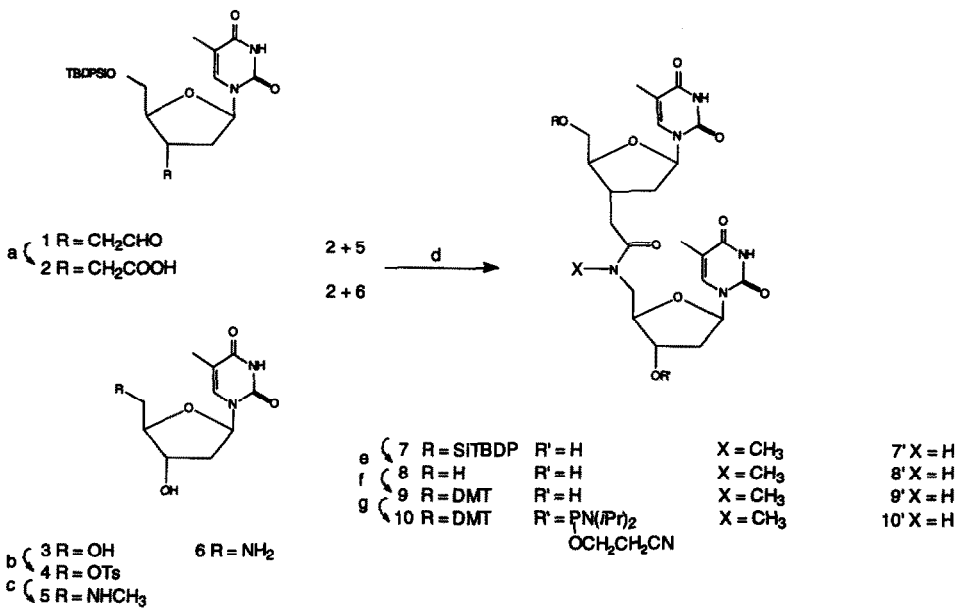
Of particular interest is a new class of nucleotide analogs, consisting of a peptide chain, carrying nucleoside bases at suitable intervals.[6] This major modification of the natural nucleotide has resulted in a strongly increased affinity for DNA. However, the sequence specific selectivity of such a compound is diminished.[7]

Our research has focused on the development of less dramatically modified nucleotides, replacing only the 3'- 5'-phosphate linkage with amides. The amide moiety has the advantage that it is nonionic, much less polar than the phosphate, and can be made even more lipophilic by N-alkylation.

We describe here the synthesis of two amide-linked thymidine dimers; one, methylamide **TmaT 8**, the other H-amide **TaT 8'** (Scheme 1). Each dimer was incorporated three times within strands of regular DNA, making 12-mers **11** and **11'**.

The nucleoside carboxylic acid **2** and 5'-methylamino-5'-deoxythymidine **5** and 5'-amino-5'-deoxythymidine **6**[8] were the key components in the formation of the dimers (Scheme 1). The methyl ester corresponding to carboxylic acid **2**, with the 5'-hydroxyl group protected as the benzyl ether, had been previously prepared in our laboratory in a 9-step total synthesis.[9]

SCHEME 1



Experimental conditions: (a) 2 eq PDC, DMF, 5h, 80%; (b) 6 eq TsCl, pyridine, 0°, 1h, 75%; (c) 40% aqueous NH_2CH_3 (22 eq), 55°, 2h, 83%; (d) 1 eq BOP, 2 eq TEA, DMF, 0.5 h, 80% (7, 7'); (e) 3 eq nBu_4NF, THF, 45 min, 89% (8), 65% (8'); (f) 2.5 eq DMTCl, 0.5 eq DMAP, pyridine, 3h, 90% (9) or 2.5 eq DMTCl, 2.5 eq TEA, pyridine, 1h, 92% (9'); (g) 2 eq 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, 5 eq TEA, CH_2Cl_2, 1h, 94% (10), 86% (10').

10 —solid phase synthesis→ d(5'-GpCpGpTmaTpTmaTpTmaTpGpCpT-3') 11

10' —solid phase synthesis→ d(5'-GpCpGpTaTpTaTpTaTpGpCpT-3') 12

By using aldehyde **1**,[10] made in four steps from thymidine as starting material, we were able to considerably shorten this synthesis. Aldehyde **1**, protected as the 5'-*t*-butyldiphenylsilyl ether, was oxidized in 80% yield to acid **2** under mild, neutral conditions using pyridinium dichromate in DMF. The synthesis of 5'-methylamino-5'-deoxythymidine **5** was achieved as follows. The 5'-hydroxyl group of thymidine was selectively tosylated and subsequently displaced by methylamine in aqueous solution at 55 °C, at atmospheric pressure, to give methylamine **5** in 83% yield. Benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) peptide coupling reagent[11] was used to effect the condensation of **2** and **5** and of **2** and 5'-amino-5'-deoxythymidine **6**, both in 80% yield. Subsequent removal of the silyl protecting group with tetra-*n*-butylammonium fluoride afforded amide nucleoside dimers **TmaT** (methylamide linked thymidine) **8** and **TaT** (H-amide linked thymidine) **8'**. The structures of **8** and **8'** were verified by ^1H and ^{13}C NMR and FAB HRMS. The spectral data of **TmaT 8** showed the existence of two (cis and trans) amide rotamers in a

ratio of 1 : 2. Only one rotamer was observed for **TaT 8'**. The assignment of the NMR signals was based on information obtained from COSY, NOESY and HETCOR experiments.

The 5'-hydroxyl group of the dimers was then reprotected with dimethoxytrityl chloride (in the presence of 4-dimethylaminopyridine for the TmaT dimer, triethylamine for the TaT dimer) and the 3' position functionalized as the phosphoramidite, in order to be incorporated into a normal DNA sequence.[12]

The synthesis of modified DNA 12-mers **11** and **12** was carried out in an Applied Biosystems DNA 381 synthesizer, using standard procedures,[13] except for an increase in coupling time to 2 minutes for the couplings that involved the dimers. The concentration of the dimers solution was 0.15 M in acetonitrile. After reversed phase purification on C-18 SEP-PAK^{TM} (Waters, Millipore), 12-mers **11** and **12** were subjected to electrophoresis on 16% polyacrylamide non-denaturing gel. Both sequences showed a single band.

To establish their ability to anneal to complementary RNA,[14] thermal denaturation studies of 12-mers **11** and **12** were performed at pH 6.9, in a 1.0 M NaCl, 10 mM phosphate buffer. In both cases the T_m measurements gave melting curves of a well-defined sigmoid shape, comparable to the unmodified DNA-RNA melting curve. The results of these studies indicated that modified nucleotide sequence **12**, containing three **TaT** dimers, binds to complementary RNA as strongly as does natural DNA (Table 1). The hybridization characteristics of methylamide sequence **TmaT 11** are very similar to those of the H-amide nucleotide **TaT 12**. While the extra methyl groups on this sequence lowered the T_m slightly, they are expected to impart greater lipophilicity, facilitating cell wall penetration.

TABLE 1

Hybridization data

Sequences	T_m (RNA[1]) $^oC^a$	T_m (RNA mismatch[2]) $^oC^a$	T_m (DNA[3]) $^oC^a$
Tmat **11**	65.8	60.4	62.8
TaT **12**	66.8	60.0	65.7
Unmodified DNA12-mer	67.9	57.1	74.2

1) Complementary RNA sequence (3'-CGCAAAAAACGA-5').

2) Complementary RNA sequence with one mismatched base (3'-CGCAAAUAACGA-5').

3) Complementary DNA sequence d(3'-CGCAAAAAACGA-5')

a) experimental error is $\pm$ 0.5^{o}C

The Watson - Crick base pair specificity of the modified sequences was tested by performing the same thermal denaturation studies, but substituting one uracil base for an adenine base in the complementary RNA strand. This one base mismatch resulted in a lowering of the melting temperature by 6.9 ^{o}C for the **TaT** sequence **12** and by 5.4 ^{o}C in the case of **TmaT** sequence **11**, while the unmodified DNA:RNA duplex was destabilised by 10.8 ^{o}C under the same conditions.

Modified sequences **11** and **12** were also found to be capable of annealing to complementary DNA at only slightly lower temperatures than the comparable normal DNA 12-mer (Table 1).

In summary, we have prepared modified oligonucleotides, replacing several phosphate linkages by either H-amide or methylamide connections. The H-amide nucleosides hybridize to RNA as strongly as does normal DNA, with excellent selectivity. The methylamides are also capable of annealing to RNA at comparable temperatures, while preserving a high level of Watson - Crick base pair specificity.

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