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Incorporation of Amide Linked Thymidine Dimers into Oligodeoxynucleotides

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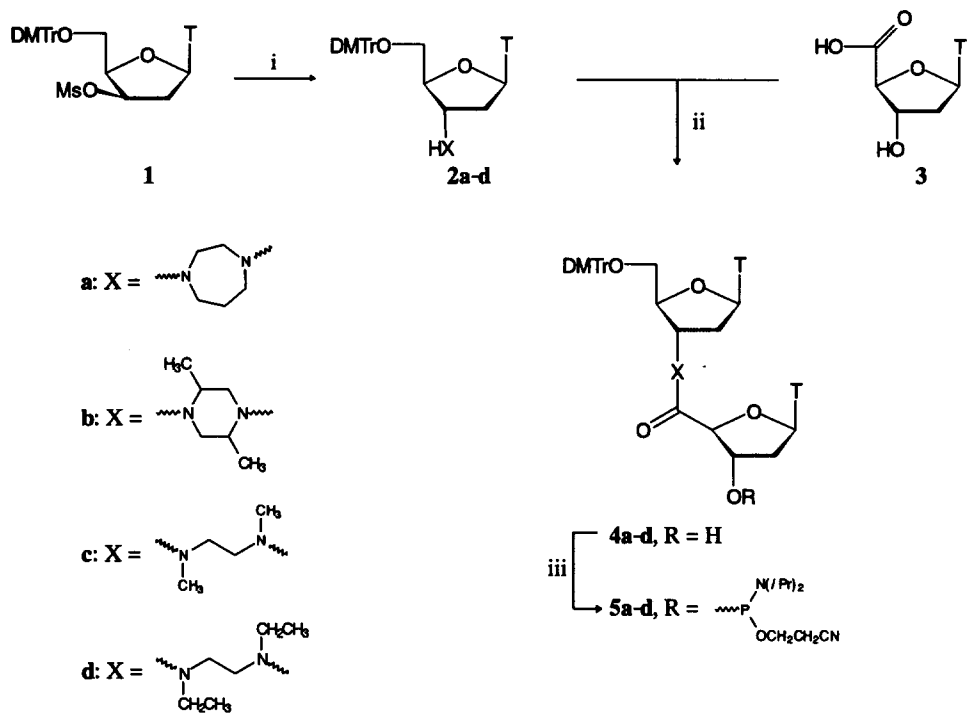
Abstract: The synthesis of thymidine dimers in which the phosphodiester linkage has been replaced by an amide linkage containing diamines (homopiperazine, 2,5-*trans*-dimethylpiperazine, *N,N*-dimethylethylenediamine and *N,N*-diethylethylenediamine) and their incorporation into oligodeoxynucleotides is described. The thermal and nucleolytic stability of the resulting oligodeoxynucleotide analogues are reported.

The possibility of controlling gene expression using antisense oligonucleotides has stimulated research in the area of modified oligonucleotides. In order to make these oligonucleotides useful in this context some major obstacles have to be overcome. Thus, they have to be stable towards nucleolytic degradation and be able to penetrate cell membranes while preserving the ability to bind specifically and strongly to a complementary oligonucleotide.[1] The research efforts have been put into four main categories, namely oligonucleotides containing a) modifications at the phosphorus atom,[2] b) modifications in the carbohydrate moiety,[3,4] c) modifications in which the phosphodiester linkage has been replaced by an achiral non-phosphorus moiety,[4] and d) modifications in which the entire sugar-phosphate backbone of the oligonucleotide has been replaced.[5] Substituting the phosphodiester linkage with an amide linkage leads to oligonucleotides having increased stability towards 3'-exonucleases and a thermal stability similar to unmodified oligonucleotides.[6,7]

Incorporation of piperazine in the internucleoside linkage has shown intriguing thermal behaviour.[7] Therefore we decided to incorporate other cyclic and acyclic diamines as internucleoside linkages in thymidine dinucleosides. The introduction of an amine group at 3'-*C* in a nucleoside is typically done by a S_N2 reaction.[8] To obtain the desired *erythro* configuration around 3'-*C* we chose to use nucleoside **1**[7] as starting material for synthesis of the key synthons **2a-d**. Heating **1** with the appropriate amine in the presence of pyridine at 150 °C for 72 h afforded 3'-amino-2',3'-dideoxynucleosides **2a-d** in moderate yields after purification by column chromatography (**2a**: 28%; **2b**: 21%; **2c**: 19%; **2d**: 21%).[9] Dimer formation was effected by dicyclohexylcarbodiimide (DCC)/*N*-hydroxysuccinimide (NHS) activation[10] of thymidine 5'-carboxylic acid (**3**)[11] followed by addition of **2a-d** affording **4a-d** (**4a**: 76%; **4b**: 53%; **4c**: 72%; **4d**: 81%).[12] The phosphoramidites **5a-d** were obtained by reacting **4a-d** with 2-cyanoethyl-*N,N*-diisopropylphosphoramido-chloridite in the presence of *N,N*-diisopropylethylamine hence giving **5a-d** after column chromatography and precipitation from hexane (**5a**: 97%; **5b**: 87%; **5c**: 83%; **5d**: 72%).



Reagents: i) 20–50 eq. diamine, pyridine, 150 °C, 72 h; ii) 1 eq. **3**, 1 eq. NHS, 1 eq. DCC, DMF, –20 °C then r.t., 60 h; iii) 3.0 eq. $NCCH_2CH_2OP(Cl)N(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2, r.t., 1 h; Ms = methanesulfonyl, DMTr = 4,4'-dimethoxytrityl, T = thymin-1-yl

The phosphoramidites **5a–d** were incorporated into different oligodeoxynucleotides on a Pharmacia Gene Assembler Special® DNA synthesizer using a standard protocol for unmodified phosphoramidites apart from an increase in coupling time from 2 to 12 minutes. The composition of the synthesized oligodeoxynucleotides with the sequence **A** were verified by matrix assisted laser desorption mass spectrometry.[13] Oligomers containing the dinucleosides derived from **5a–d** gave relative molecular masses of 5050.55 Da (calc. 5050.50 Da) for **5a**, 5064.2 Da (calc. 5064.5 Da) for **5b**, 5038.1 Da (calc. 5038.5) for **5c**, and 5067.2 Da (calc. 5066.5) for **5d**. Due to the homogenous trityl assays during synthesis of the modified oligodeoxynucleotides we consider this a verification of the composition of all the synthesized oligodeoxynucleotides.

Oligodeoxynucleotides modified at the 3'-end were tested for their stability, by UV-measurements, towards the 3'-exonuclease snake venom phosphodiesterase as described in the literature.[14] All modified linkages showed a 15 to 20 fold increase in stability when compared to unmodified DNA.

The denaturing temperature of the duplexes formed by the modified oligonucleotides and their DNA (Table 1) and RNA (Table 2, sequences **A** and **B**) complements were measured.[15] For all duplexes sharp transitions were detected. It is evident from Table 1 that incorporation of a dinucleoside (T*T) containing a cyclic internucleoside linkage (in **5a**) does not destabilize the duplex to the same extend as one with a similar

acyclic linkage (in **5c** and **5d**). Comparing with the results from oligonucleotides containing piperazine[7] in the internucleoside linkage this characteristic become even more pronounced.[16] As expected, the steric bulk of the two methyl groups on 2,5-*trans*-dimethylpiperazine is not tolerated very well. As the steric bulk is increased on the acyclic linkages (in **5c** and **5d**) the denaturing temperature is likewise lowered, but apparently the increased bulk is better tolerated in these flexible linkages compared to the 2,5-*trans*-dimethylpiperazine linkage (in **5b**). When the thermal stability is measured against complementary RNA (Table 2) the more flexible linkages (**5c** and **5d**) display an increased stability when compared to **5b** and an oligodeoxynucleotide containing a piperazine linked dinucleoside.[16] Albeit when compared to duplexes with DNA, all the modified oligonucleotide:RNA duplexes show a lower thermal stability.[17]

Table 1. Denaturing temperature measured against complementary DNA

	T_m (°C)	ΔT_m (°C)			
Sequence (5'→3')	w.t.	**5a**	**5b**	**5c**	**5d**
A CACCAACT*TCTTCCACA	61	−2.6	−6.8	−3.2	−3.6
B CACCAACT*TCT*TCCACA	61	−2.8	−7.0	−3.2	−3.8

w.t.: wild type DNA; ΔT_m: change per modification in denaturing temperature compared to the wild type DNA

Table 2. Denaturing temperatures measured against complementary RNA

	T_m (°C)	ΔT_m (°C)			
Sequence (5'→3')	w.t.	**5a**	**5b**	**5c**	**5d**
A CACCAACT*TCTTCCACA	60	−4.0	−8.8	−4.0	−4.0
B CACCAACT*TCT*TCCACA	60	−3.8	−8.4	−5.6	−4.0

w.t.: wild type DNA; ΔT_m: change per modification in denaturing temperature compared to the wild type RNA

In conclusion, incorporation of a rigid cyclic structure in the internucleoside linkage is well accommodated in a duplex with complementary DNA. On the contrary, the novel modified oligodeoxynucleotides synthesized do not exhibit the necessary thermal stability towards RNA to be useful in controlling gene expression *in vivo*. However, as excellent stability towards 3'-exonucleolytic degradation were induced with the diamine linked thymidine dimers, we are currently in progress of synthesizing further analogues designed for optimal binding to RNA-targets.

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9. Selected data for **2a–d**. **2a**: ^{1}H NMR ($CDCl_3$) δ: 1.76–2.96 (m, 5 x CH_2), 3.99 (br s, H–4'), 6.08–6.11 (m, H–1'), 7.68 (s, H–6). FAB MS: 627 $(M+H)^+$. **2b**: ^{1}H NMR ($CDCl_3$) δ: 1.39 (s, $CHCH_3$) 1.46 (s, $CHCH_3$), 2.33–2.83 (m, CH_2, 2 x $\underline{CH}$–CH_3), 3.00–3.14 (m, CH_2), 4.04–4.14 (m, H–4'), 6.12–6.28 (m, H–1'), 7.71–7.78 (m, H–6). FAB MS: 641 $(M+H)^+$. **2c**: ^{1}H NMR ($CDCl_3$) δ: 2.22 (s, NCH_3), 2.57 (s, NCH_3), 2.70–2.73 (m, CH_2), 2.88–2.90 (m, CH_2), 4.13 – 4.15 (m, H–4'), 6.15 (t, H–1'), 7.64 (s, H–6). FAB MS: 615 $(M+H)^+$. **2d**: ^{1}H NMR ($CDCl_3$) δ: 0.99 (t, $NCH_2\underline{CH_3}$), 1.15 (t, $NCH_2\underline{CH_3}$), 2.51–2.55 (m, CH_2), 2.73–2.82 (m, CH_2), 4.01–4.08 (m, H–4'), 6.15 (t, H–1'), 7.67 (d, H–6). FAB MS: 643 $(M+H)^+$.
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12. Selected data for **4a–d** (a denotes the "upper" and b the "lower" nucleoside). **4a**: ^{13}C NMR ($CDCl_3$) δ: 45.04, 46.67, 47.96, 48.08, 52.69 (5 x CH_2–piperazino), 81.70 (C–1'a), 82.50 (C–1'b), 85.01, 85.23, 86.68 (C–4'a, C–4'b, C–Ar_3), 135.41 (C–6a, C–6b), 163.86, 164.38 (C–4a, C–4b), 169.92 (C–5'b). FAB MS: 865 $(M+H)^+$. **4b**: ^{13}C NMR ($CDCl_3$) δ: 39.36, 42.34, 54.35, 54.48 (CH_2–piperazino, CH–piperazino), 81.43, 82.00, 82.91, 84.99, 85.75 (C–1'a, C–1'b, C–4'a, C–4'b, C–Ar_3), 135.48 (C–6a, C–6b), 163.90, 164.00 (C–4a, C–4b), 168.54, (C–5'b). FAB MS: 879 $(M+H)^+$. **4c**: ^{13}C NMR ($CDCl_3$) δ: 39.21, 39.0 (2 x NCH_3), 45.94 (2 x NCH_2), 82.89 (C–1'a, C–1'b), 85.45 (C–4'a, C–4'b), 135.42 (C–6a, C–6b), 164.00, 164.20 (C–4a, C–4b), 170.78 (C–5'b). FAB MS: 853 $(M+H)^+$. **4d**: ^{13}C NMR ($CDCl_3$) δ: 39.21, 39.0 (2 x NCH_3), 45.94 (2 x NCH_2), 82.89 (C–1'a, C–1'b), 85.45 (C–4'a, C–4'b), 135.42 (C–6a, C–6b), 164.00, 164.20 (C–4a, C–4b), 170.78 (C–5'b). FAB MS: 853 $(M+H)^+$.
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15. The melting experiments were performed at a concentration of 2.5 μM for each strand in a medium salt buffer: pH=7.2, 1 mM EDTA, 10 mM Na_2HPO_4, 140 mM NaCl.
16. A piperazine amide linked thymidine dinucleoside incorporated into a oligonucleotide with the sequence A showed the following melting behaviour: towards a complementary DNA strand ΔT_m = –1.6; towards a complementary RNA strand ΔT_m = –6.8.
17. MM+ calculations on 6–mer DNA:DNA or DNA:RNA duplexes containing **5a** and **5c**, using Hyperchem™, suggest that the 5 atom linkages are longer than the phosphodiester linkage. This causes a distortion of the base pairing in the duplexes. However no clear pattern were seen between the thermal behaviour of the duplexes and the molecular modeling results.

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