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CHEMICAL SYNTHESIS OF DIMER RIBONUCLEOTIDES CONTAINING INTERNUCLEOTIDIC PHOSPHORODITHIOATE LINKAGES

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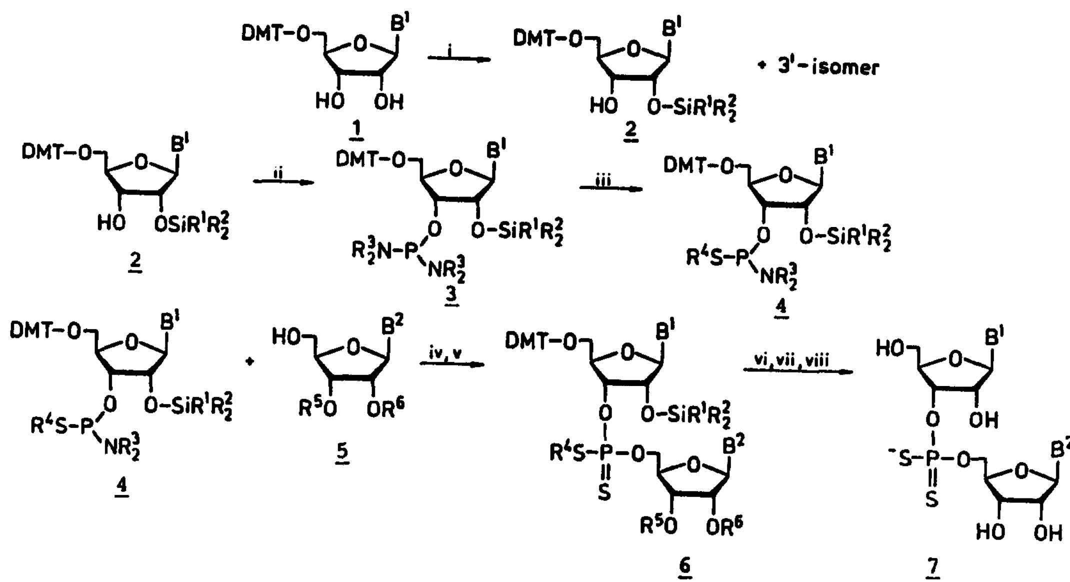
Ribonucleosides, chlorobis(amino)phosphines and thiols react *via* phosphorothioamidites to form phosphorothioites. Oxidation with sulphur gives ribonucleoside phosphorodithioate triesters which after deprotection yields the phosphorodithioate ribonucleoside analogues.

Recently, chemical synthesis of oligodeoxyribonucleotides containing internucleotidic phosphorodithioate linkages[1] or other internucleotidic phosphate analogues[2] have attracted substantial attention. Initial biological testings have shown that these so called antisense oligonucleotides could be useful as potential therapeutic agents against for example cancer and viral diseases.[3,4]

Likewise, relatively small RNA molecules are known to regulate gene expression[5] and accordingly we believe that synthetic backbone-modified ribonucleotides are attractive candidates as new antisense oligonucleotides or as pathogen-specific ribozymes.[6] So far, we are aware of only one report on synthetic modified oligoribonucleotides used as anti-HIV reagents, *i.e.* 2'-O-Methyl-RNA oligomers.[7] We are especially interested in ribonucleotides containing internucleotidic phosphorodithioates since they are achiral at phosphorus and supposedly chemically and enzymatically more stable than their unmodified counterparts. Until now, the few reports on phosphorodithioate ribonucleotides have been limited to uridine cyclic 2'-,3'-phosphorodithioate[8] and adenosine cyclic 3'-,5'-phosphorodithioate.[9]

This paper describes one of the preparative routes to the title compounds which we have investigated.[10] Uridine was used without base protection, adenosine and guanosine were protected with the phenoxyacetyl-group as described[11] and cytidine was benzoylated by a standard procedure.[12] For the protection of the 2'-hydroxyl we have used the *tert*-butyldimethylsilyl (TBDMS-) group for adenosine, cytidine and uridine and triisopropylsilyl (TIPS-) for guanosine. When their syntheses were carried out according to the procedures reported by Ogilvie *et al.* compounds **2a-d** were obtained in moderate yields after column chromatography.[13]



Synthesis of Diribonucleotide Phosphorodithioate Derivatives. (i) TBDMS-Cl or TIPS-Cl; (ii) Cl-P(NR^3_2)$_2$ + DIPEA; (iii) R^4SH; (iv) Tetrazole; (v) S_8; (vi) $ZnBr_2$ or H^+; (vii) conc. NH_3 in methanol; (viii) TBAF in THF. Abbreviations: DMT, dimethoxytrityl; R^1, *tert*-butyl or *iso*-propyl; R^2, methyl or *iso*-propyl; NR^3_2, dimethylamino or pyrrolidino; R^4, 2-cyanoethyl or 2,4-dichlorobenzyl; R^5 and R^6, benzoyl. B is N6-phenoxyacetyl adenine, N4-benzoyl cytosine, N2-phenoxyacetyl guanine or uracil.

The ribonucleoside phosphorothioamidites **4a-f** were obtained in a one-pot synthesis through the reaction of the appropriate chlorobis(amino)phosphine (1.3 mmol) in anhydrous chloroform or acetonitrile with the protected ribonucleoside **2a-d** (1 mmol) in the presence of diisopropylethylamine (DIPEA, 1.6 mmol). After 30 min the appropriate thiol (1.4 mmol) is added. The reaction is stirred for 30 min at room temperature and stopped by extraction with aq. $NaHCO_3$. The organic phase is dried over $MgSO_4$, filtered and evaporated to give a gum which is dissolved in ethyl acetate and precipitated in cold, degassed hexane. This method is similar to that described for deoxyribonucleoside phosphorothioamidites.[1d] However, in the case of silyl-protected ribonucleosides additional care should be taken. Addition of the phosphitylating agent should be co-incidental with the addition of DIPEA, or performed in a reverse order since, model experiments have shown that DMT-U-2'-TBDMS is isomerized to the thermodynamically stable mixture of the 2'- and 3'-TBDMS uridine derivatives when treated with a mixture of acetonitrile/DIPEA (2:1, v/v) for 15 minutes at room temperature. This behavior has recently been discussed in more detail.[14]

Unlike the corresponding deoxyribonucleoside phosphorothioamidites, the ribonucleoside derivatives are relatively stable to silicagel column chromatography. Most derivatives were purified successfully on degassed silicagel columns packed and eluted with degassed and anhydrous grades of $EtOAc/CHCl_3/NEt_3$ (45/45/10, v/v/v) containing 20 % distilled petroleumether. However,

phosphorothioamidites having the S-(2,4-dichlorobenzyl) group in combination with the dimethylamino-function did not tolerate these conditions as well as the corresponding S-(2-cyanoethyl) derivatives. We found, in agreement with Dahl et al.,[19] that S-(2-cyanoethyl) phosphorothioamidites derivatives have more favourable stabilities especially towards oxidation. Generally, the yields of column purified ribonucleoside phosphorothioamidites were 70-95%. The ^{31}P NMR data of a series of column purified phosphorothioamidites are reported in Table 1.

Table 1. ^{31}P NMR of Ribonucleoside Phosphorothioamidites

Compound	Base (B)	amidite	R	^{31}P NMR (δ)[a]
4a	A^{pha}	dimethylamino	2-cyanoethyl	177.9, 171.8
4b	C^{bz}	dimethylamino	2-cyanoethyl	177.5, 174.0
4c	G^{pha}	dimethylamino	2-cyanoethyl	177.9, 171.5
4d	U	dimethylamino	2-cyanoethyl	178.7, 172.5
4e	U	dimethylamino	2,4-dichlorobenzyl	177.7, 174.4
4f	U	pyrrolidino	2,4-dichlorobenzyl	173.9, 168.2

[a] ^{31}P NMR spectra were recorded in CH_3CN on a Jeol FX-90Q with 85% aqueous H_3PO_4 as an external standard.

The ribonucleoside phosphorothioamidite **4b** (0.7 mmol) was reacted with the 5'-unprotected ribonucleoside derivative **5b** (0.8 mmol) in the presence of tetrazole (2.4 mmol) in degassed acetonitrile (2 ml). The reaction mixture was stirred for 10 min at room temperature, quenched by adding sulphur (0.5 mmol S_8, 6 atomic eq.) and the stirring continued for 1 hour. The fully protected dinucleoside phosphorodithioate was purified by silicagel chromatography (EtOAc/petrolether; 60/40, v/v) to give **6bb** (C-C dimer) in 45 % yield. Similarly the dimer **6cb** (G-C dimer) was obtain in 51 % yield. ^{31}P-NMR (CH_3CN), δ 96.3/97.0 ppm (**6bb**), δ 94.7/96.3 (**6cb**).

The dimer **6bb**, was deprotected as follows. The fully protected dicytidyl phosphorodithioate was detritylated with anhydrous $ZnBr_2$ (10 eq.) in 2 ml anhydrous acetonitrile/nitromethane (1/1, v/v). After 1 h the deprotection was complete (TLC analysis) and the reaction mixture was quenched by extraction with NH_4HCO_3 (5 ml, 5 % aq. solution) and extracted with dichloromethane.[15] The organic layer was dried with $MgSO_4$, filtered and evaporated to an oil, which was purified by short column silica chromatography (dichloromethane/petrolether; 80/20, v/v, with a 0-8% methanol gradient). Further deprotection was accomplished by dissolving the partly deprotected dinucleotide in ammonia-saturated methanol (1 ml) and leaving it at room temperature overnight. After evaporation, the residual oil was dissolved in tetrabutylammonium fluoride in THF (TBAF, 1 ml, 1M). The reaction mixture was left for 4 hours, evaporated to dryness and redissolved in ether/water. The aquous phase was washed with ether and evaporated to give the unprotected dicytidyl phosphorodithioate **7bb** (δ(D_2O)114.7 ppm, >95% pure according to ^{31}P-NMR).

Unprotected dicytidyl phosphorodithioate (**7bb**, 0.03 OD_{260}) was phosphorylated with T4-polynucleotide kinase (20 u) and [γ-^{32}P]ATP for 1 hour at 37°C. This derivative was shown (PEI-plates, eluted with 1 M KH_2PO_4, pH 4.0) to be stable to snake venom phosphordiesterase and

RNase A under conditions where the parent dinucleotide was fully degraded. Similarly, we investigated the stability of the C-C-dimer to bases by reverse phase HPLC analysis. When treated with conc. aq. ammonia at 5°C for 16 hours, virtually no degradation was observed, whereas conc. aq. ammonia at 55°C for 16 h lead to some, but far from total degradation. This is a remarkable stability compared to natural RNA derivatives and might allow the use of base-labile 2'-hydroxyl protecting groups.

This report outlines a method for the chemical synthesis of phosphorodithioate analogues of ribonucleotides. So far ribonucleoside thiophosphoramidites have been the most efficient trivalent phosphorus synthons since some are stable during column chromatography purification and at the same time sufficiently reactive in the presence of an acidic catalyst such as tetrazole. Thus, ribonucleotides containing phosphorodithioates could be synthesized via a chemical pathway largely identical to existing chemical methods to synthesize oligodeoxy- and ribonucleotides and therefore, their synthesis is prone to automation. Since the phosphorodithioate linkage is biophysically very similar to the internucleotide linkage in natural RNA, we anticipate that this analogue, due to its higher chemical and enzymatical stability, will be very useful for investigating biological processes involving RNA and enzymatically active RNA molecules.

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