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A new approach to stereospecific synthesis of P-chiral phosphorothioates. Preparation of diastereomeric dithymidyl-(3′-5′) phosphorothioates†

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A new method for stereospecific synthesis of P-chiral phosphorothioates based on intramolecular nucleophile catalysis was developed.

Antisense oligodeoxynucleotides (AOs) are short, single-stranded DNA fragments that may inhibit the translation of mRNAs into proteins by forming complexes with a complementary mRNA target.[1] Among various kinds of oligonucleotide analogues that have been investigated for the purpose of antisense and antigene therapies,[2] it is oligodeoxynucleoside phosphorothioates (PS-oligos) that, due to their ability to induce the RNase H promoted degradation of target mRNA and the enhanced resistance to nuclease degradation *in vivo*, became the main focus of medical research.[3]

A perennial problem of oligonucleoside phosphorothioates synthesis is chirality of the phosphorus center that manifests itself in different chemical, biochemical, and biological properties of the diastereomeric species.[3] Despite the efforts of many research groups during the past years, the solution to the problem of stereocontrolled synthesis of PS-oligos has eluded a fully acceptable solution. Early attempts to control stereochemistry at the phosphorus center by using diastereomerically pure nucleoside phosphoramidites were not successful[4] due to the profound tendency of tervalent phosphorus compounds to epimerize under mild acidic conditions. To remedy this problem, a handful of *stereoselective* methods for the formation of P-chiral internucleotide linkages were developed. All of them made use of a special type of phosphoramidites in which a phosphorus atom is part of relatively rigid cyclic systems and which usually bear chiral, bulky auxiliaries attached to the phosphorus center.[5]

As to a *stereospecific* formation of P-chiral internucleoside phosphorothioate linkages, there are only two methods available for this purpose: the oxathiaphospholane method based on P(v) derivatives developed by Stec *et al.*[6] and the *N*-acyl-oxazaphospholidine approach based on tervalent P(iii) compounds elaborated by Beaucage *et al.*[7] Both methods are highly stereospecific and afford products of diastereomeric purity > 99%. The possible drawbacks of these methods, however, are that both require very strong bases to effect the elongation of an oligonucleotidic chain, and the preparation of the requisite starting materials is usually long and rather complicated. The inherent high reactivity of both classes of compounds make also their separation into individual diastereomers a delicate task and put severe limitations on possible recycling of the monomers.

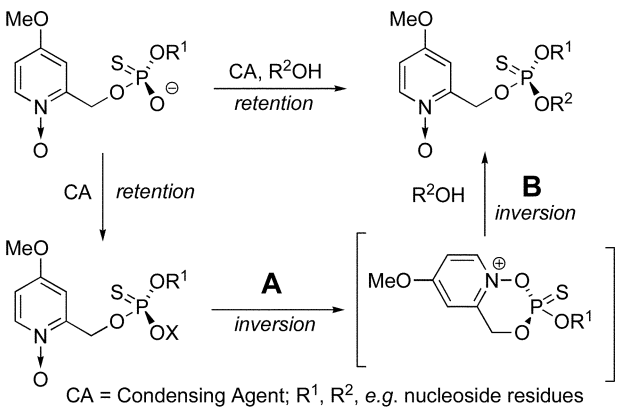
We argued that a viable approach to a stereospecific synthesis of oligonucleoside phosphorothioates could be a condensing agent promoted reaction of nucleosides with phosphorothioate diesters bearing a nucleophile catalytic group. There are several features which make this approach appealing. Firstly, by using phosphorothioate diesters we would benefit from an easy to handle and configurationally stable starting material, that also should be easy to prepare. Secondly, a powerful nucleophile catalysis provided by the catalytic group should secure efficient formation of an internucleotide bond and, due to the intramolecular character of this phenomenon, also prevent erosion of configuration at the phosphorus center during the condensation step. Thirdly, the method would share other advantages of the underlying phosphotriester chemistry, *e.g.* possibility of using various condensing agents, lower sensitivity to the presence of adventitious water and compatibility with both solution and solid-phase chemistry.

A mechanistic proposition for the whole transformation that utilizes 1-oxido-2-picolyl functionality as an intramolecular catalytic group, is outlined in Scheme 1. Activation of a phosphorothioate diester by a condensing agent (CA) will produce a reactive intermediate, which in two stereospecific steps involving (i) the intramolecular elimination **A** of a leaving group and (ii) the nucleophilic substitution **B** by a hydroxylic component, collapses to the product. Since both steps **A** and **B** are expected to proceed with inversion of configuration, the product of the reaction, a phosphorothioate triester, should be formed with an overall retention of configuration at the phosphorus center.

While the rate enhancement due to intramolecular catalysis exerted by a 1-oxido-2-picolyl group attached to a phosphorus centre finds literature precedents,[8,9] the use of a catalytic phosphate protecting group as means of securing stereochemical integrity of the phosphorus center during S_N2(P) reactions has until now not been investigated.

Our first objective was thus to find out if the underlying principle of the method, *i.e.* that the intramolecular catalysis provided by the 1-oxido-2-picolyl group would result in improved reaction kinetics while preventing epimerization at the phosphorus center, is sound. As a model system for our studies we chose a reaction of diastereomerically pure 5′-*O*-*tert*-butyldiphenylsilyl-thymidin-3′-yl 1-oxido-4-methoxy-2-picolyl phosphorothioates **4a** and **4b** with 3′-*O*-*tert*-butyldiphenylsilylthymidine in the presence of a condensing agent (Scheme 2).

To secure simple, efficient, and general access to the key intermediate, 4-methoxy-1-oxido-2-picolyl phosphorothioate diesters of type **4**, we developed a dedicated reagent, 4-methoxy-1-oxido-2-picolyl-(9-fluorenemethyl) phosphorothioate **2**, ena-



Scheme 1

† Electronic supplementary information (ESI) available: synthesis and characterization of compounds **2** through **6**. See http://www.rsc.org/suppdata/cc/b3/b311912b/

‡ Deceased.

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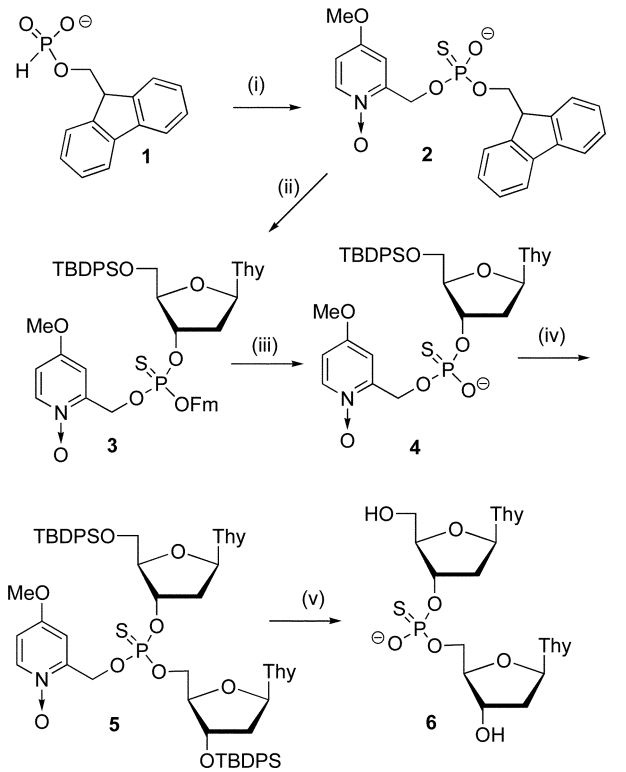
bling transfer of the phosphorothioate and 4-methoxy-1-oxido-2-picolyl moieties to a hydroxylic component in one step. It sports a 9-fluorenemethyl group as a lipophilic handle to facilitate chromatographic separation of the P-chiral phosphorothioate triesters **3**, and its removal can be effected *via* β-elimination without affecting the stereochemical integrity of the phosphorus center of the produced phosphorothioate diesters **4**. The reagent is a stable, white solid, that can be prepared by condensation of 9-fluorenemethyl phosphonate **1**[10] with 4-methoxy-2-pyridinemethanol 1-oxide,[8,9] followed by *in situ* sulfurisation with elemental sulfur (total yield 63%).

Preparation of separate diastereomers of the starting material **4**, nucleoside phosphorothioate diesters, required for stereospecific synthesis, commences with thiophosphorylation of 5′-*O*-*tert*-butyldiphenylsilylthymidine with reagent **2** in the presence of 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane (NEP-Cl). The reaction was uneventful and produced almost quantitatively (^{31}P NMR) nucleoside phosphorothioate triesters **3** as a 6 : 4 mixture of R_P and S_P diastereomers. Separation of the diastereomeric mixture by silica gel column chromatography furnished after one run **3a** (faster moving diastereomer, *ca.* 50%), **3b** (slower moving diastereomer, *ca.* 20%), and a mixture of **3** (*ca.* 25%). Due to the stability of phosphorothioate triesters **3**, the mixed fractions can be subjected to re-chromatography or stored for later separation.

To obtain nucleoside phosphorothioate diesters **4**, separate diastereomers of phosphorothioate triesters **3** were subjected to deprotection with *tert*-butylamine. The removal of 9-fluorenemethyl group from **3a** and **3b** was rapid and clean, and afforded after silica gel chromatography, diastereomerically pure **4a** (*ca.* 70%) and **4b** (*ca.* 80%), respectively.



Scheme 2 *Reagents and conditions*: (i) 1. 4-methoxy-2-pyridinemethanol 1-oxide + NEP-Cl/py, 2. H_2O, 3. S_8; (ii) 5′-TBDMS-T + NEP-Cl/py; (iii) t-$BuNH_2$/py; (iv) 3′-TBDMS-T + NEP-Cl/py; (v) 1. TEA–PhSH, 2. F^-. Abbreviations: Thy, thymin-1-yl; TBDMS, t-butyldimethylsilyl; NEP-Cl, 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane.

^{31}P NMR experiments allowed us to survey a wide range of reaction conditions for the formation of triesters **5a** and **5b**. The condensation of separate diastereomers **4a** or **4b** in dichloromethane containing pyridine (3 equiv.) with 3′-*O*-*tert*-butyldimethylsilylthymidine (1.5 equiv.) promoted by a mild condensing agent NEP-Cl[12] (3 equiv.) was fast (*ca.* 15 min), quantitative and completely stereospecific (> 99%).[11] Thus, **4a** (δ_P = 57.78 ppm) afforded exclusively **5a** (δ_P = 67.90 ppm), while **4b** (δ_P = 57.94 ppm) gave exclusively diastereomeric product **5b** (δ_P = 67.24 ppm). No sulfur activation was observed during the course of the condensation. The diastereomers **5a** and **5b** were subjected separately to total deprotection and absolute configurations at the phosphorus centre in dinucleoside phosphorothioates **6a** and **6b** were determined as R_P and S_P, respectively, by enzymatic digestion.[13,14]

In conclusion, we have shown that an intramolecular catalytic group can secure stereochemical integrity of the phosphorus center during $S_N2(P)$ reactions. On this basis we developed a new method for stereospecific synthesis of dinucleoside phosphorothioate diesters, that can be extended to the synthesis of other chiral phosphorothioates. The thiophosphorylating reagent **2** is stable, readily accessible, and the method offers a new avenue in the synthesis of P-chiral thiophosphates.

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