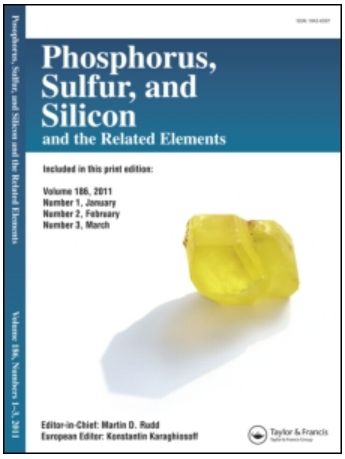

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Selenium-Derivatized Oligonucleotides

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Selenium-Derivatized Oligonucleotides

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Selenium-labelled macromolecules can be effectively analyzed by X-ray crystallography due to the phenomenon of multi-wavelength anomalous dispersion *(MAD). The originally developed nucleoside 2-selena-1,3,2-oxathiaphospholane derivatives allow for synthesis of fully modified, stereodefined oligomers (up to approximately 15-mers) with any combination of internucleotide phosphoroselenoate linkages of R_P or S_P absolute configuration as well as unmodified phosphate bonds, which, in general, cannot be achieved using enzymatic methods.*

Keywords Oxathiaphospholane method; phosphoroselenoate; PSe-diasteoisomer; PSe-oligonucleotide

INTRODUCTION

In our long-lasting research projects, selenium has been always present in an "intimate pair" with phosphorus. Phosphane selenides,[1] phosphoroselenonoates, and isomeric phosphoroselenoloates,[2] phosphoroselenocyanidates,[3] and phosphoroisoselenocyanates[4] were considered as excellent model compounds for studies on the mechanisms of nucleophilic substitution at phosphorus atom, molecular rearrangements, chemical transformations, and structural assignments. Direct selenium-phosphorus bond expanded the array of P-chiral organophosphates making them excellent model compounds for stereochemical studies on nucleophilic and metal-ion assisted substitution at P-atom, as well as intermediates for stereocontrolled conversions to other important classes of compounds, e.g., dinucleoside methanephosphonates.[5] Selected examples are shown below to demonstrate these early endeavors.

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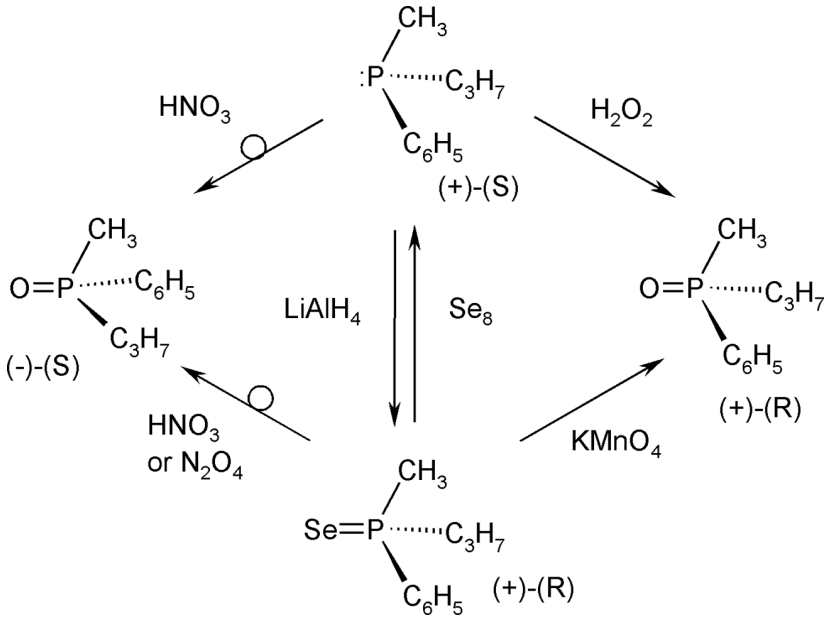
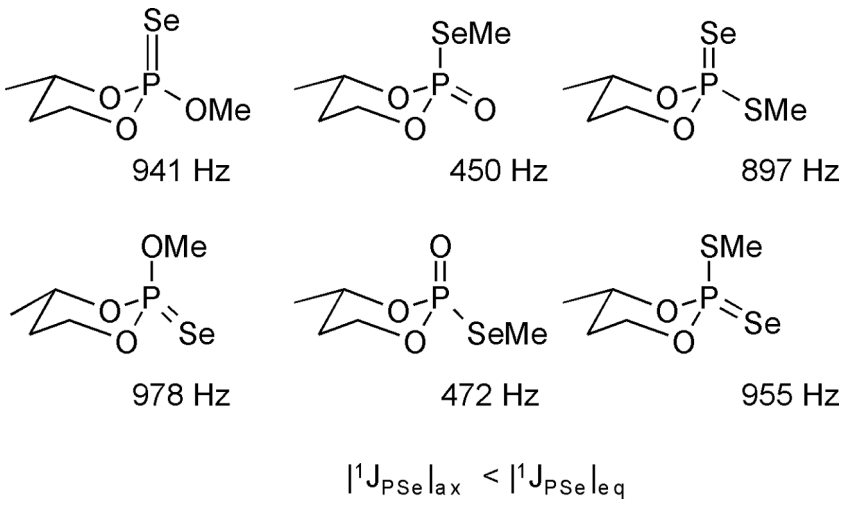
Trisubstituted phosphane selenides were obtained in enantiomerically pure form and were found to be configurationally stable; under treatment with $LiAlH_4$ they were reduced to parent phosphanes in a stereocontrolled manner (Scheme 1).[1]



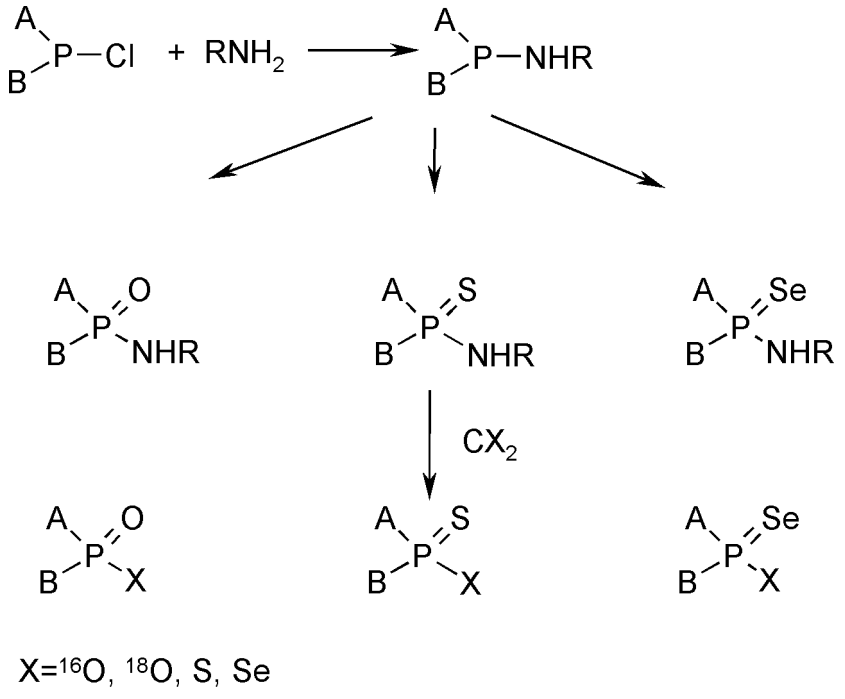
SCHEME 1

Since natural selenium contains ca. 7% of NMR active ^{77}Se isotope (spin number = 1/2), its derivatives are useful for NMR studies. Nuclear spin-spin coupling constants between directly bonded ^{31}P and ^{77}Se in several classes of organophosphorus compounds (with selenium linked to a phosphorus atom through a single or double bond) were measured,[2] and this type of information has been used for structural assignments. Numerous experiments revealed that for diastereomeric cyclic phosphoroselenoate compounds with an exocyclic selenium atom in either *axial* or *equatorial* positions (Scheme 2) the corresponding coupling constants $^1J_{PSe}$ are distinctly different and the absolute value of $^1J_{PSe}$ (*ax*) is always lower than $^1J_{PSe}$ (*eq*).[6]

Enantiomerically and diastereomerically pure phosphoroselenoates, as well as phosphorothioates and isotopically labelled phosphates, became much more easily accessible after implementation of Wadsworth-Emmons-Horner Reaction for PN→PX conversion in starting phosphoranilidates,[7] as well as in their phosphorothioate- and phosphoroselenoate congeners (Scheme 3).

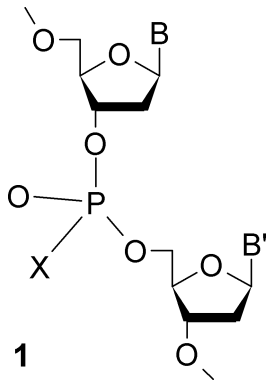


SCHEME 2



SCHEME 3

The abovementioned examples document our long-lasting interest in stereochemical aspects of phosphoroselenoates, extended also on phosphoroselenoate congeners of oligonucleotides.[8] Although the first diribonucleotide phosphoroselenoates were synthesized in solution by Ogilvie in 1980,[9] our adaptation of the Caruthers' and - Beaucage's solid phase phosphoramidite approach[10] to the synthesis of oligo(nucleoside phosphoroselenoate)s opened an access to this class of valuable congeners of oligonucleotides.[8] In retrospective analysis, we have to mention that oligo(nucleoside phosphoroselenoate)s have appeared to be toxic to numerous cell lines;[11] thus, endeavors towards stereocontrolled synthesis of oligo(nucleoside phosphoroselenoate)s (*vide infra*) were abandoned. We focused our efforts on the development of stereocontrolled synthesis of phosphorothioate analogues of DNA (PS-Oligos, **1**, X = S) (Inset 1).



1

For many applications, including so called *antisense* or *antigene* strategies, PS-Oligos—the ionic analogues of DNA with enhanced nucleolytic stability—were highly demanded, although their use for *in vivo* studies also appeared to be limited by non-specific binding to certain proteins[12] and immune-related side effects.[13,14] Importantly, despite of the fact that PS-Oligos are isoelectronic with natural DNA, the presence of a sulfur atom affects the properties of internucleotide bonds, mostly due to different steric requirements of sulfur atom (P—S vs. P—O bond length), different affinity towards metal ions ("soft" sulfur vs. "hard" oxygen), and changes in negative charge distribution in the phosphorothioate anion. Therefore, the hydration pattern of PS-Oligos is different from that of natural oligonucleotides, obstructing the evaluation of results on kinetics of "rescue effects" of thiophilic metal ions, and making analysis of direct or water-mediated contacts between metal ions and phosphate groups much more difficult. However, this is a calculated and accepted toll paid for increased stability and preserved good

hybridization properties of phosphorothioate oligonucleotide probes. It is important to note that PS-Oligos are P-chiral species and the shortest such modified DNA fragments, i.e., dinucleotides, consist of the mixture of two P-diastereomers of either R_P or S_P absolute configuration. For oligonucleotides consisting of n nucleoside units and containing $n-1$ modified internucleotide bonds, the number of existing P-diastereomeric forms N is given by the equation $N = 2^{n-1}$. This means, that for oligonucleotides as long as decamers, the product obtained on non-stereocontrolled way (e.g., standard phosphoramidite[8] or H-phosphonate methods) exists as the mixture of $2^9 = 512$ diastereomers and the content of given single diastereomer drops below 0.2% (1/512).[15] Short PS-Oligos prepared with a non-stereocontrolled or partially stereoselective mode, can be separated into diastereomeric species by means of chromatographic techniques,[16] but this method cannot be considered general as the efficiency of separation depends upon the sequence of nucleobases and the composition of the buffered eluent. At the time being, several phosphorothioate analogues of DNA (as random mixtures of all possible P-diastereomers) are evaluated in clinical studies[17] (Table 1), while one compound—*Vitravene*® (fomivirsen sodium)—targeting cytomegalovirus IE-2 gene has been accepted by FDA for the treatment of CMV -induced retinitis. Properties of antisense probes and discussion of the mechanism of their action can be found in several excellent reviews.[18–22]

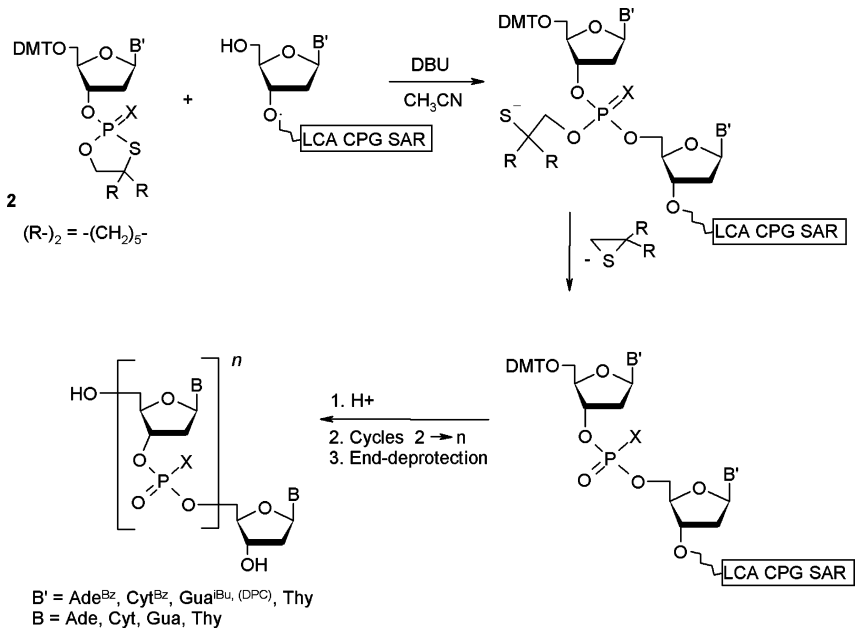
Since virtually all biomolecules the oligonucleotides interact with, are chiral and exist in single stereochemical forms, each diastereomer of a P-chiral oligonucleotide may interact with them in a slightly different way. Therefore, stereochemical aspects should be taken into consideration in a design of P-chiral oligonucleotides to assure their most favorable biological activity. Similar functional alterations and stereochemical consequences are seen in another class of compounds, closely related to phosphorothioates, consisting of phosphoroselenoate oligonucleotides (PSe-Oligos, **1**, X = Se), where one of the oxygen atoms of internucleotide bond is replaced with selenium—the downstairs neighbour of sulfur in the Periodic Table. Solid-phase syntheses of PSe-Oligos were performed using a phosphoramidite or H-phosphonate approach with "selenation" of the P^{III} intermediate.[11,23–28] Although it has been found that stereorandomal PSe-Oligos have a diminished hybridization capability to complementary DNA and RNA templates, as compared with both the unmodified and phosphorothioate oligomers,[11] they have been evaluated as research tools for studies of interactions with metal ions. Although the enzymatic synthesis of stereodefined PSe-Oligo based on the use of DNA polymerase, which, rather unexpectedly, accepted both S_P and R_P diastereomers of TTPαSe, has

TABLE I Phosphorothioate Analogues of DNA (As Mixtures of All Possible P-Diastereomers) Evaluated in Clinical Studies[17]

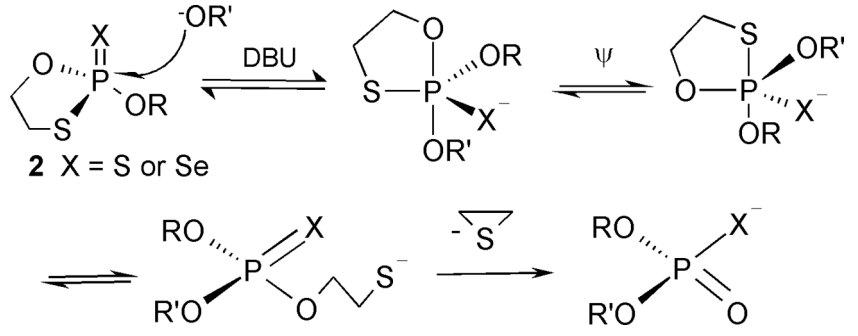
Company	Drug	Target	Disease or indication	Clinical status
Isis	ISIS 301012	ApoB	High cholesterol	Phase 2
Pharmaceuticals	ISIS 113715	PTP-1B	Diabetes	Phase 2
AVI BioPharma	Resten-NG	C-Myc	Re-stenosis	Phase 2
	AVI-4065	Hepatitis C virus	Hepatitis C	Phase 1b
	AVI-4557	Cytochrome P450	Drug metabolism	Phase 1
	AVI-4020	West Nile virus	West Nile virus	Phase 1
Genta	Genasensea	Bcl-2	Solid tumors, blood cancers	Phase 3
	G4460a	C-Myb	Solid tumors	Phase 1
Lorus	GTI-2040a	R2 subunit of RNR	Renal cell carcinoma, acute myeloid leukemia	Phase 2
Therapeutics	GTI-2501a	R1 subunit of RNR	Prostate and kidney tumors	Phase1/2
Topigen	TPI-ASM8	Chemokine receptor-3/IL3,5	Asthma	Phase 2
	TPI-1100	Phosphodiesterases PDE4 and PDE7	COPD	Pre-IND
Oncogenex	OGX-011a	Clusterin	Prostate and breast cancer, non-small cell lung cancer	Phase 2
VIRxSYS	VRX496	HIV	Chronic HIV infection	Phase 1

been published,[29] stereocontrolled method for chemical synthesis, free of limitations typical for enzymatic reactions, was highly demanded. Such a method has been developed in our Laboratory.[30] It is based on an oxathiaphospholane approach (Scheme 4, its mechanism is shown on Scheme 5), originally developed for to the solid phase synthesis of stereodefined phosphorothioate oligonucleotides[31,32] and employs separated diastereomerically pure 5′-*O*-DMT-nucleoside-3′-*O*-(2-selena-4,4-pentamethylene-1,3,2-oxathiaphospholane) monomers 2 (X = Se) instead of their 2-thio congeners (2, X = S). A set of deoxyribonucleoside monomers **2** (B′ = Ade^{Bz}, Cyt^{Bz}, $Gua^{iBu,DPC}$ and Thy) was synthesized as depicted in Scheme 6.

The monomers **2** were obtained with satisfactory yield (45–60%) and in their ^{31}P NMR spectra, the resonances in the range of 99–100 ppm accompanied by satellite doublets resulting from the direct ^{31}P-^{77}Se spin-spin interactions ($^{1}J(P,Se) = 945–954$ Hz) were found. The monomers were then chromatographically separated into pure P-diastereoisomers. Correlation of chromatographic mobility of *fast*- and

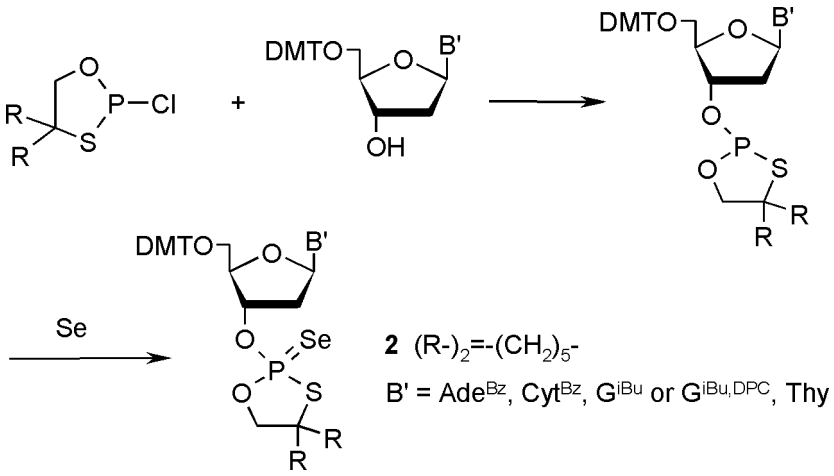


SCHEME 4



SCHEME 5

slow-**2** with the respective R_P and S_P absolute configuration of resulting internucleotide phosphoroselenoate bond in d($N_{PSe}T$) (N = dA, dC, T or dG) has been achieved enzymatically using R_P-specific snake-venom phosphodiesterase (svPDE) and S_P-specific nuclease P1 (nP1).[23] It was found that for all four nucleobases *fast*-eluting monomers were precursors of dinucleotides with R_P absolute configuration of phosphorus atom. Then, 2-selena monomer *fast*-2 (B′ = Ade^{Bz}) was used for elongation at the 5′-end of natural oligomer d(AGCGGTCGGC) synthesized



SCHEME 6

by phosphoramidite approach (using the DBU-resistant sarcosinyl-succinoyl linker[33]) to yield 5′-*O*-DMT-d(A_{PSe}AGCGGTCGGC) with R_P absolute configuration of phosphoroselenoate internucleotide bond. Figure 1 shows a spectrum from MALDI TOF analysis of resulting

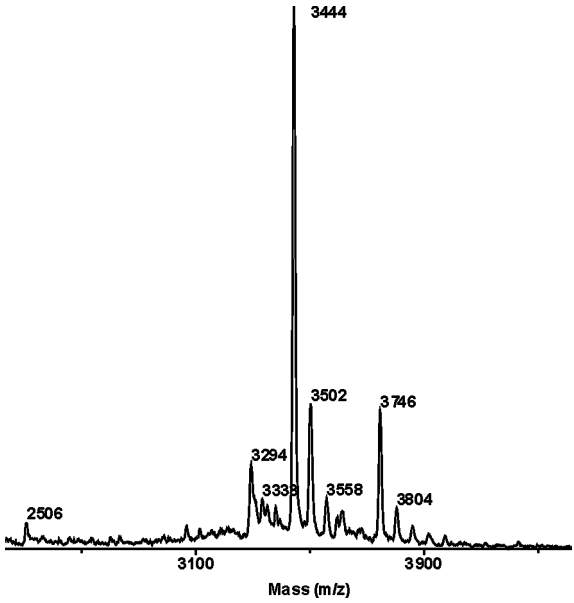


FIGURE 1 MALDI TOF spectrum for 5′-*O*-DMT-d(A_{PSe}AGCGGTCGGC) with R_P absolute configuration of phosphoroselenoate internucleotide bond.

oligomer. In the spectrum, a molecular ion m/z 3746 is observed, whereas a signal at m/z 3444 corresponds to the compound detritylated upon action of acidic matrix used in the experiment. This synthetic approach can be used for synthesis of diastereomerically pure Se-labelled oligonucleotides for X-ray analysis.

It must be emphasized that the developed method allows for the synthesis of fully modified, stereodefined oligomers (up to approximately 15-mers) with any combination of internucleotide phosphoroselenoate linkages of R_P or S_P absolute configuration as well as unmodified phosphate bonds, which, in general, cannot be achieved using enzymatic methods. Obtained PSe-Oligos were used for investigations on thermodynamic stability of their complexes with DNA and RNA templates. Such the complexes of stereodefined phosphorothioate DNA analogues have been analyzed thoroughly, and it has been found that, usually, PS-DNA/DNA and PS-DNA/RNA complexes have lower thermal stability as compared to unmodified congeners.[34] However, we have also found that this rule is not valid for certain homopurine [All-R_P-PS]-DNA oligomers interacting with complementary (in the Watson-Crick sense) RNA templates. Detailed analysis, including titration and fluorescence quenching experiments, showed that if the homopurine sequences of at least six nucleotides are palindromic, parallel triplexes RNA/PS-DNA/RNA are formed, while only duplexes RNA/DNA are observed for [PO]-, [Mix-PS]- and [All-S_P-PS]-DNA.[35] It was interesting to check whether the same phenomenon could be seen for corresponding PSe-Oligos. For that purpose two stereoregular dodecamers [All-R_P-PSe]- and [All-S_P-PSe]-dA_{12}were synthesized. They were analyzed by MALDI TOF MS and PAGE, and, then, melting temperatures of their complexes with complementary 2′-OMe-RNA templates were measured. Obtained data confirmed the stereodependence of melting temperatures, and for a series of [PX]-dA_{12} mixed with 2′-OMe-U_{12} at a molar ratio 1:2, melting temperatures of 35°C, 53°C and 58°C were found for [PO]-, [All-R_P-PS]- and [All-R_P-PSe]-, respectively, whereas for [All-S_P-PSe]-dA_{12} Tm of only 28°C was assigned. Undoubtedly, the highest thermal stability of the triplex [All-R_P-PSe]-dA_{12}/2 × U_{12} (a relevant melting curve has good S-shape indicating high cooperativity of the transition, Figure 2) was found with surprise.

In our interpretation, this finding reflects astonishing functionality of phosphoroselenoate moieties, where selenium atoms present in proper spatial orientation confer the oligomers unique hybridization and thermodynamic properties. Important characteristics of those interactions are expected to be obtained from analysis of charge distribution in phosphoroselenoates moieties. Ongoing project aiming the assignment of the order of P-Se bond in internucleotide

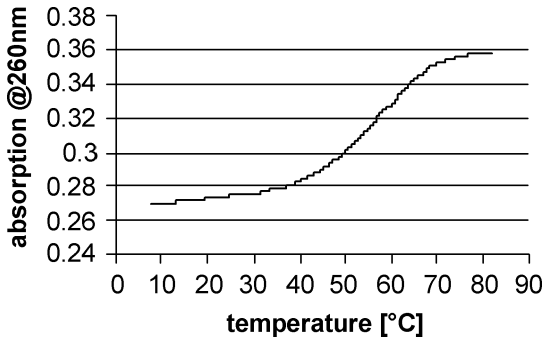


FIGURE 2 Melting curve for complex of [All-S_P-PSe]-dA_{12} with 2′-OMe-U_{12} template at pH 7.2.

phosphoroselenoate linkages involves the measurements of coupling constants ^{31}P-^{77}Se, the method recalled at the beginning of this account[2] and initiated more than 35 years ago.

With conclusions presented in this account, one has to admit that the role of selenium in biological systems is of increasing interest.[36] Analogues of proteins and oligonucleotides, with sulfur or oxygen atoms replaced with selenium, have been evaluated as research tools for studies of interactions with metal ions, and selenium-labelled macromolecules can be effectively analyzed by X-ray crystallography due to the phenomenon of multiwavelength anomalous dispersion (MAD).[37] Although a selenium atom can be introduced into an oligonucleotides in numerous ways, like via incorporation of 2′-SeMe derivatives of nucleosides (relevant methods are under continuous development),[37–40] the internucleotide phosphoroselenoate moiety offers, besides relevance for X-ray structural studies, other possibilities for non-reductionistic "in-solution" investigation of structural polymorphism and dynamics of nucleic acids.

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