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Gene Silencing Activity of siRNA Molecules Containing Phosphorodithioate Substitutions

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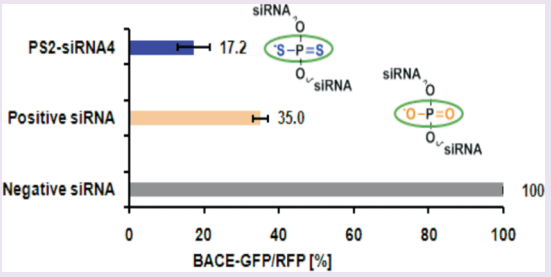
Supporting Information

ABSTRACT: Chemically synthesized small interfering RNAs (siRNAs) have been widely used to identify gene function and hold great potential in providing a new class of therapeutics. Chemical modifications are desired for therapeutic applications to improve siRNA efficacy. Appropriately protected ribonucleoside-3′-yl *S*-[*β*-(benzoylmercapto)-ethyl]pyrrolidino-thiophosphoramidite monomers were prepared for the synthesis of siRNA containing phosphorodithioate (PS2) substitutions in which the two non-bridging oxygen atoms are replaced by sulfur atoms. A series of siRNAs containing PS2 substitutions have been strategically designed, synthesized, and evaluated for their gene silencing activities. These PS2-siRNA duplexes exhibit an A-form helical structure similar to unmodified siRNA. The effect of PS2 substitutions on gene silencing activity is position-dependent, with certain PS2-siRNAs showing activity significantly higher than that of unmodified siRNA. The relative gene silencing activities of siRNAs containing either PS2 or phosphoromonothioate (PS) linkages at identical positions are variable and depend on the sites of modification. 5′-Phosphorylation of PS2-siRNAs has little or no effect on gene silencing activity. Incorporation of PS2 substitutions into siRNA duplexes increases their serum stability. These results offer preliminary evidence of the potential value of PS2-modified siRNAs.



RNA interference (RNAi) has emerged as a novel mechanism that is activated in mammalian cells by small interfering RNAs (siRNAs), which hold great potential in providing a new class of therapeutics.[1] Although unmodified siRNAs have been used with success for gene silencing, chemical modifications of one or both strands are desired for research and pharmaceutical applications in order to enhance potency and to improve pharmacokinetic properties.[2−4] A variety of chemical modifications have been evaluated for their effects on RNAi activity including selected phosphate backbone modifications such as phosphoromonothioates (PS)[5,6] and boranophosphates.[7] These modifications involve substitution of a single non-bridging phosphate oxygen atom with either a sulfur atom or a borane group. This renders the internucleotide linkage nuclease resistant, but unlike natural RNA, both the PS- and borane-modified phosphorus centers become chiral. Synthesis of the PS-RNA by standard phosphoramidite methodology generates a mixture of unresolvable diastereomeric oligomers possibly having variable biochemical, biophysical, and biological properties.[8] While stereocontrolled synthesis of P-chiral PS[9,10] or boranophosphates[11] represents one possible solution to this problem, another lies in the synthesis of modifications that are achiral at phosphorus.

The substitution of both non-bridging phosphate oxygen atoms with sulfur atoms gives rise to a phosphorodithioate (PS2) internucleotide linkage, which like natural phosphorodiester linkages of RNA is achiral at phosphorus. The PS2 substitution is a very attractive RNA analogue because it is a closely related mimic of natural RNA and should have other biochemical and biophysical properties similar to phosphorodiester linkages of RNA. In addition, it has been shown that dinucleoside phosphorodithioates (PS2-dimers) have high nuclease resistance.[12] Moreover, a hammerhead ribozyme with one PS2 substitution at the cleavage site was evaluated for its cleaving rate to the expected product.[13] Further, PS2-dimers have shown a great resistance to alkaline degradation when compared to natural RNA derivatives.[12] Over the past 20 years, the only reported PS2-RNA chemistries are the thiophosphoramidite solution-phase synthesis of PS2-dimers,[12] the H-phosphonothioate solid-phase syntheses of a 12-mer oligoribonucleotide containing a single PS2 substitution,[13] and

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a 15-mer oligoribouridylate phosphorodithioate bearing exclusively PS2 substitutions.[14]

In this paper, we report the synthesis of protected ribonucleoside-3′-yl *S*-[*β*-(benzoylmercapto)ethyl]pyrrolidinothiophosphoramidite monomers **1a−d** (Figure 1) and their

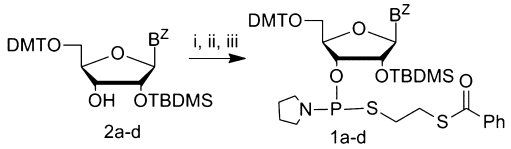

Figure 1. Synthesis of thiophosphoramidites **1a−d**: (i) tris-(pyrrolidino)phosphine, 1*H*-tetrazole; (ii) 1-(trimethylsilyl)imidazole; (iii) ethanedithiol monobenzoate, 1*H*-tetrazole. Abbreviations: B^Z = Ade^{Bz} (a), Cyt^{Ac} (b), Gua^{Ac} (c), Ura (d); DMT = dimethoxytrityl; Ph = phenyl; TBDMS = *tert*-butyldimethylsilyl.

use for the solid-phase preparation of RNA molecules containing between one and four PS2 substitutions. These modified RNAs are used as components of a novel type of siRNA molecule. We envisaged that the addition of PS2 modifications to certain positions of the siRNA duplex would increase nuclease resistance and enhance RNAi activity. Moreover, we characterize the gene silencing activity of these PS2-siRNA duplexes. Finally, we analyze their conformational structures and evaluate the serum stability of PS2-siRNAs. To the best of our knowledge these are the first studies evaluating biological properties of siRNA duplexes containing the PS2 substitutions.

RESULTS AND DISCUSSION

Preparation of Protected Ribonucleoside Thiophosphoramidites. The PS2-RNAs were synthesized using a solid-phase thiophosphoramidite method because of the speed and simplicity of this approach. Currently, the only practical method for solid-phase synthesis of PS2-DNA uses commercially available 2′-deoxyribonucleoside thiophosphoramidites (www.glenresearch.com).[15,16] The appropriately protected ribonucleoside-3′-yl *S*-[*β*-(benzoylmercapto)ethyl]pyrrolidinothiophosphoramidite monomers **1a−d** (Figure 1) were prepared in ca. 75−85% yield.

We chose to use the *β*-(benzoylmercapto)ethyl sulfur protecting group because this is in the context of PS2-containing DNAs.[17] In addition, it was reported to help reduce the PS contamination in the final products when compared to the use of *β*-cyanoethyl or 2,4-dichlorobenzyl.[17] Moreover, the *β*-(benzoylmercapto)ethyl sulfur protecting group was reported to have better stability in solution than the 2,4-dichlorobenzyl group. Like the *β*-cyanoethyl group, the removal of the *β*-(benzoylmercapto)ethyl group can also be conveniently carried out during a simple base-deprotection treatment to give the final RNA products. The ^{31}P NMR spectra of these thiophosphoramidites **1a−d** showed the expected two singlets between *δ* 160 and *δ* 180 ppm for two diastereoisomers (Supplementary Figure 1S). Since the 2′-deoxyribonucleoside thiophosphoramidites were reported to be degraded and rearranged to the Arbuzov-type product[16,17] on silica gel during column chromatography, the ribonucleoside thiophosphoramidites were used in the PS2-RNA synthesis without further purification. This approach was successful, indicating that further purification of the thiophosphoramidites is unnecessary.

Synthesis of RNAs Containing PS2 Substitutions. In our previous studies, we identified and verified an active unmodified siRNA sequence that effectively silences the BACE1 (*β*-site APP-cleaving enzyme 1) in cellular models (siRNA-1 in Supplementary Table 1S).[18] We chose this sequence for initial tests of siRNA molecules containing the PS2 substitutions. Thus, 21 siRNAs (PS2-3−24, Supplementary Table 1S) were designed to contain up to four PS2 substitutions strategically placed at positions considered important for silencing activity. In addition, PS2-siRNAs containing 5′-phosphate were chemically synthesized to evaluate the gene silencing effect of the 5′- hydroxyl and 5′-phosphate of PS2-siRNAs since the 5′-phosphate plays a critical role for RNAi activity.[19−21] The PS2-RNAs (Supplementary Table 1S) were synthesized on an Expedite 8909 DNA/RNA Synthesizer using commercial 5′-DMT-2′-*O*-TBDMS nucleoside (A^{Bz}, C^{Ac}, G^{Ac}, and U) phosphoramidite monomers as well as thiophosphoramidites **1a−d** as described in Methods. The average stepwise coupling efficiency of all phosphoramidites including thiophosphoramidites was about 97% as estimated by the DMT-cation assay. After deprotection, all of the modified RNAs were isolated by FPLC according to a previously described protocol for purifying PS2-DNAs.[22] The PS2-RNAs were desalted using reverse-phase HPLC to yield the PS2-RNA final products. The structures of the PS2-RNAs were confirmed by ESI-MS. The purity of all RNAs and correctness of double strand structures of PS2-siRNAs were assessed by denaturing 20% polyacrylamide (PAGE) and agarose gel electrophoresis analyses, respectively.

RNAi Activity of PS2-siRNAs. The impact of introducing the PS2 substitution(s) in the siRNA duplex was investigated in a GFP/RFP dual fluorescence assay (DFA).[23] The gene silencing results are summarized in Supplementary Table 1S and Figure 2 (the level of a target gene expression is presented as the % of negative control). Since the PS2-modified duplexes exhibit slightly decreased thermal stability (Supplementary Table 1S), the introduction of the PS2 modification(s) at the 3′-end of the sense strand was hypothesized to favor duplex unwinding and therefore enhance gene silencing activity. Thus, we designed PS2-3 having one PS2 substitution at position 20 and PS2-4 having two PS2 substitutions at positions 19 and 20 of the sense strand (counting from the 5′-end). Interestingly, PS2-3 showed slightly lower RNAi activity compared to that of wild-type siRNA-1 (39.4% *versus* 35.0%), while PS2-4 showed significantly enhanced gene silencing activity (17.2% *versus* 35.0%; $p < 0.001$). PS2-6 having four PS2 substitutions at positions 5, 10, 15, and 20 showed a significant decrease in RNAi activity when compared to the wild-type siRNA-1 (76.7% *versus* 35.0%, $p < 0.001$). PS2-7 having three PS2 substitutions at positions 5, 12, and 20 (two identical positions with PS2-6) showed gene silencing activity comparable to that of the wild-type siRNA-1, while PS2-8 having two PS2 substitutions at position 10 (the sense strand cleavage site) and position 20 showed moderately reduced RNAi activity when compared to siRNA-1. In contrast, PS2-9 having two PS2 substitutions at positions 3 and 12 showed significantly better gene silencing when compared to siRNA-1 (18.7% *versus* 35.0%, $p < 0.001$). Therefore, we conclude that the PS2 substitution effects in the sense strand are position-dependent. This has been shown in previous studies where various chemical modifications were added to siRNAs to determine the biochemical properties required for RNAi.[2,4,23−27]

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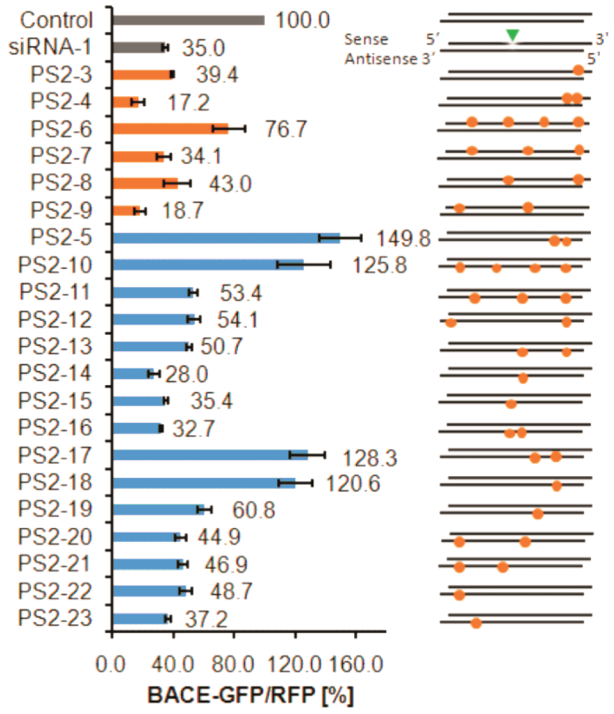

Figure 2. Gene silencing activity of PS2-siRNAs. Right panel: schemes of siRNA duplexes, where the colored orange balls represent PS2 substitutions. Left panel: PS2-siRNA activities were tested in a DFA as described in Methods. BACE-GFP/RFP levels were normalized to cells transfected with negative control siRNA in each experiment (Control). The orange bars represent PS2 modifications in the sense strand, and the blue bars represent PS2 modifications in the antisense strand. Error bars represent the standard deviation. The results are also tabulated in Supplementary Table 1S.

The antisense strand of siRNA has been reported to be more sensitive to chemical modifications than the sense strand.[2,5,26−28] This is most likely due to the importance of this strand for incorporation into the RISC, nucleation and binding with target mRNA, as well as mRNA cleavage.[29−31] To determine the impacts of PS2 modification(s) in the antisense strand, PS2 modifications at several positions were also examined. Modified siRNAs PS2-11, 12, 13, 19, 20, 21, 22, and 23 exhibited equal or moderately reduced gene silencing activity when compared to the wild-type siRNA-1. These PS2-siRNA duplexes containing one to three PS2 substitutions decreased the BACE1 expression to the range of 37.2−60.8% *versus* 35.0% for the wild-type siRNA-1. Next, we evaluated the gene silencing activity of PS2-14, 15, and 16 siRNAs where PS2 substitutions are opposite to the cleavage site of the target mRNA, which is between the 10th and 11th residues from the 5′-end of the antisense strand.[32] Based on a model derived from the crystal structure of a bound antisense strand,[20,33] these phosphates do not directly interact with the Ago2 protein. Our experiments showed that the PS2 substitutions for these phosphates have similar or slightly enhanced gene silencing activity (Figure 2, Supplementary Table 1S) when compared with the wild-type siRNA-1. Importantly, PS2-14 having only one PS2 substitution between nucleosides 10th and 11th (just opposite to the cleavage site) slightly improved the gene silencing activity when compared to the wild-type siRNA-1 (28.0% for PS2-14 *versus* 35.0%; $p = 0.05$). Interestingly, the BACE1-GFP expression level for PS2-16 (32.7% reading), having two PS2 substitutions at both the 10th and 11th positions, is close to the mean value of the BACE1-GFP expression levels for PS2-14 (28.0% reading) and PS2-15 (35.4% reading) having a single PS2 substitution at the 10th and 11th position, respectively. It appears that the PS2 substitutions for these phosphates have a potential to improve the gene silencing activity. Thus, on the basis of the above results, one could expect some positive effects on RNAi activity for siRNAs having PS2 substitutions on the antisense strand.

SiRNA duplexes PS2-5, 10, 17, and 18 containing one to four PS2 substitutions in the antisense strand showed no gene silencing activity toward BACE1 in the assay depicted in Figure 2. These results may be due to a couple of plausible factors. The recent *Archaeoglobus fulgidus (Af*Piwi*)*/siRNA duplex crystal structure[33] indicates that the phosphate groups from the first phosphate (between the first and second bases from the 5′ end) to the fourth phosphate in the antisense strand closely interact with the residues conserved in the Piwi and Ago subfamilies. Since PS2 has a stronger affinity for protein residues than phosphate in general,[34,35] PS2 substitutions at these four phosphates might alter the interaction between the four phosphates and residues of the Piwi protein, which could possibly inhibit processes in which this part of the siRNA is engaged.[33,36] In addition, the coordination of one of the oxygen atoms of the second phosphate (between the second and third bases) to a magnesium ion[33] is vital for RNAi activity. This interaction is probably disrupted when the sulfur atom is substituted for the oxygen atom because the soft sulfur atom of the PS2 is known to coordinate poorly with hard magnesium cations.[19,30,31,33,37] Since PS2-5, 10, 17, and 18 all contain at least one PS2 substitution from the first to the fourth phosphate of the antisense strand, their gene silencing activity could be affected by the altered affinity between the siRNA duplex and the Piwi protein. As well, the gene silencing activity of PS2-5 and 10 could further be affected by the disruption of a magnesium cation−phosphate interaction. In the following paragraph, we observed that PS-5 and PS-10 show partial recovery of RNAi activity when directly compared with their counterparts PS2-5 and PS2-10, respectively. These results confirm the importance of the second and third phosphates of the antisense strand in the RNAi process. More systematic studies would be required to fully determine the influence of each phosphate of the antisense strand on the silencing properties of the siRNA duplex.

Rana and co-workers reported that RNAi activity absolutely requires A-form helix formation between target mRNA and its guiding antisense strand.[4,21] To determine the overall structures of PS2-siRNAs, CD spectra were collected for all of the PS2-siRNA duplexes 3−23 and compared with the CD spectrum of the wild-type siRNA-1. All of the CD spectra of the PS2-siRNAs are similar to the spectra of the unmodified duplex and are consistent with the typical A-type structure of double-stranded RNA (a maximum of the positive Cotton effect at 268 nm and crossover point at 240 nm). This result is in contrast with earlier studies that demonstrated significant structural perturbations from PS2 substitutions in DNA duplexes.[34] Since the CD spectra indicate that PS2-siRNA is globally similar in structure to the wild-type siRNA (Figure 3), the observed different RNAi activity caused by the PS2 substitution is likely to depend on the interaction of the phosphate functions with metal ions and amino acid residues of the RISC proteins as well. Our results suggest that PS2 substitutions have crucial interactions with the active RISC complex.

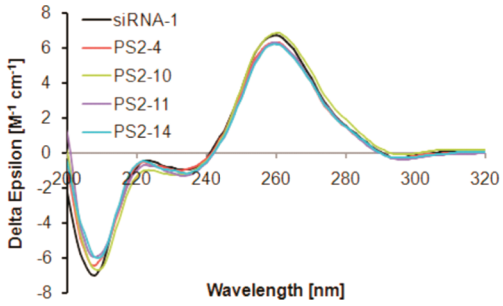

Figure 3. Circular dichroism (CD) spectra of wild-type siRNA (siRNA-1) and PS2-siRNAs: PS2-14, 4, 11, and 10 with 1, 2, 3, and 4 PS2 substitutions, respectively.

Phosphorodithioate (PS2) *versus* Phosphoromonothioate (PS) Substitutions. PS modifications have been used extensively to increase the stability of antisense oligonucleotides[38,39] and were also examined for their RNAi activity.[4−6,40] However, since the phosphorus center with PS substitution is chiral, the PS-siRNA synthesized by the standard phosphoramidite chemistry results in a mixture of unresolvable diastereomeric oligomers. These may potentially have variable biochemical, biophysical, and biological properties. Since the PS2-siRNAs are achiral at phosphorus, we hypothesized that the introduction of PS2 linkages into siRNA might be able to improve the RNAi activity when compared to PS-siRNA. We have therefore synthesized several PS-siRNAs (PS-4, 5, 9, 10, and 16) in which the PS2 linkages of PS2-4, 5, 9, 10, and 16 were replaced by PS linkages. We then compared gene silencing activity of the PS2- and PS-model siRNAs in HeLa cells using a DFA assay (shown in Figure 4). We found that both PS2-4 and

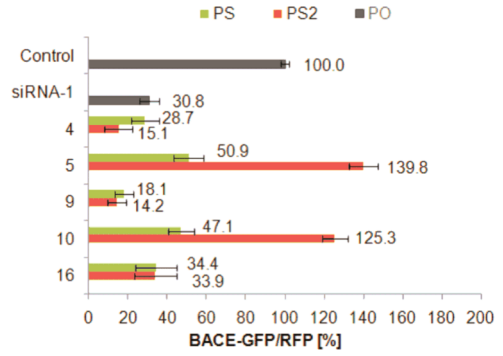

Figure 4. Silencing activity of PS2-siRNAs and PS-siRNAs targeting the BACE1 gene. Percent of BACE-GFP fusion gene expression in cells treated with unmodified or modified siRNA at 5 nM. The results of each individual experiment were normalized to the negative control siRNA. Error bars represent the standard deviation. PS2-siRNA is represented by red bars, and PS-siRNA is represented by green bars. Unmodified and control siRNA are represented by light black bars.

PS2-9 showed statistically significant increase in gene silencing activity ($p < 0.001$) when compared with that of PS-4 and PS-9. The decreased gene silencing activities for the PS-siRNAs may be caused by the mixture of isomers that may have variable biological effects. Both siRNA PS2-16 and PS-16 have RNAi activity very similar to that of the wild-type siRNA-1. This observation may indicate that the interactions between these phosphates and the protein are not critical. The most interesting results were observed from PS-5 and PS-10, which show partial recovery of RNAi activity when compared with their counterparts PS2-5 and PS2-10, respectively. These results indicate the importance of the interactions between the phosphates of the antisense strand and the Argonaute protein in the RNAi process.

5′-Terminal Phosphate of the PS2-siRNA. During the processing of long dsRNA by Dicer, endogenous siRNAs are generated with phosphate groups at their 5′- ends. Synthetic siRNAs usually display the 5′-OH group, and to become incorporated into the RISC and mediate target mRNA cleavage, the guide strand of the duplex has to be phosphorylated. The importance of the 5′-terminal phosphate of the guide strand has been demonstrated by recent crystallographic structural studies of the Ago2 guide strand binding pocket.[20,30] The 5′-end phosphate is critical for the binding of the guide strand with Ago2 and its incorporation into the RISC. Additionally, it has been shown that chemical or enzymatic pre-phosphorylation of the guide strand is generally unnecessary because the synthetic siRNAs are phosphorylated by endogenous kinases directly after transfection into the cells.[41,42] However, some chemical modifications introduced at the 5′-ends of the RNA duplex can interfere with the endogenous kinase activity and disturb the phosphorylation process. In these cases the chemical pre-phosphorylation of the guide strand at the 5′-end can rescue the siRNA activity.[42] Because our PS2-5 duplex has PS2 modifications located near the 5′-end of the antisense strand (at the second and third positions), we wondered whether the loss of activity is caused by inhibition of kinase activity by the presence of the PS2 substitution. To test our suspicions, we chemically synthesized variants of the PS2-5 siRNA having a 5′-terminal phosphate on just the antisense strand (PS2-5-P) or on both the sense and antisense strands (PS2-5-PP; Supplementary Table 2S). We transfected these phosphorylated PS2-5 siRNAs into HeLa cells and assessed the RNAi activity in comparison to nonphosphorylated PS2-5 siRNA. We did not observe any improvement of the PS2-5 activity (Figure 5). Similar studies were performed using phosphorylated versions of the PS2-4, 9, and 16, as well as siRNA-1. The 5′-phosphorylation had little or no effect on the activity of any of these siRNAs. These results suggest that the free 5′-hydroxyl of the wild-type siRNA or PS2-siRNA duplex is first phosphory-

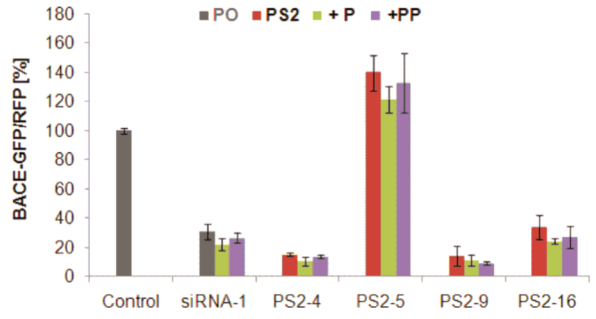

Figure 5. Effect of 5′-end phosphorylation of siRNAs on BACE1 gene silencing. Percent of BACE-GFP gene expression was determined in cells treated with unmodified or modified siRNA at 5 nM concentration. The results of each individual experiment were normalized to negative control siRNA without 5′-end phosphate. Error bars represent the standard deviation. Native siRNAs without 5′-end phosphate are represented by light black bars. PS2-siRNAs are represented by red bars. SiRNA-1 and PS2-siRNAs with 5′-end phosphate (P) at the antisense strand are represented by green bars. SiRNA-1 and PS2-siRNAs with 5′- end phosphates (PP) at both the sense and antisense strands are represented by purple bars.

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lated by endogenous kinases and then enters the RNAi pathway, which is consistent with the published results.[12] The results also demonstrated that the free 5′-hydroxyl of the PS2-siRNA duplex is sufficient for RNAi activity in mammalian cells.

Stability of PS2-siRNAs in Serum. The identification of highly nuclease-resistant siRNAs is a key concern for *in vivo* applications, and great efforts have been applied to enhance stability by chemical modification.[4,18,27,43,44] Since the above experiments showed that PS2-siRNAs such as PS2-4 and PS2-9 could effectively enhance RNAi activity compared to that of the wild-type siRNA-1, it was of interest to study the serum stability of PS2-siRNAs as well as to compare it with serum stability of the PS-siRNAs. To carry out such studies, unmodified or modified dsRNAs (Supplementary Table 3S) were incubated in 10% fetal bovine serum (FBS) at 37 °C for up to 24 h. At various time points, siRNAs were extracted, separated on 4% agarose gels under nondenaturing conditions, stained with ethidium bromide, and quantified. The observed stability of the PS2-siRNA duplexes was consistently higher than that of their counterpart PS-siRNAs, while nuclease stability of both PS2-siRNA and PS-siRNA was higher than that of the unmodified siRNA (Figure 6). The increased nuclease stability of the PS2-siRNAs should have implications for *in vivo* application, *e.g.*, in mouse models or in therapeutic applications of siRNA. However, like other studies for most chemistries, we find the PS2-siRNA serum stability to be positively correlated with the level of the RNA modifications, but highly modified siRNAs generally displayed poor silencing. The number of PS2 modification added to siRNA to increase serum stability must be chosen prudently in order to maintain or possibly improve high RNAi silencing activity.

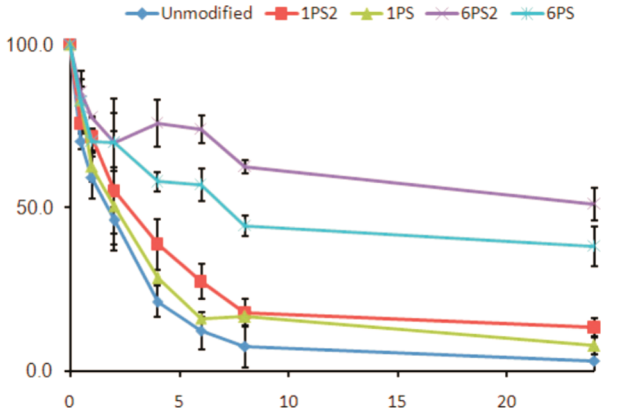

Figure 6. Serum stability of PS2-siRNA and PS-siRNA in comparison to unmodified siRNA. The sequences of PS2-siRNA and siRNAs, listed in the Supplementary Table 3S, were incubated with 10% fetal bovine serum (FBS) in PBS buffer at 37 °C. Aliquots were collected after 0.5, 1, 2, 3, 4, 6, 8, and 24 h, separated in 4% agarose gel, and analyzed using G-box gel visualization system (SynGene). Graph represents results from two independent experiments. Horizontal axis represents time (h). Vertical axis represents remaining siRNA (%). Bars represent the standard deviation.

Summary. In this report we present a method of using thiophosphoramidites with a β-(benzoylmercapto)ethyl protecting group[16,17] on sulfur for the first solid-phase synthesis of the PS2-RNAs. By automating this process, 21 PS2-siRNAs containing from one to four PS2 linkages were prepared, and their identity and purity were confirmed by gel electrophoresis and mass spectrometry. CD spectra indicated an A-form helical structure comparable to that of the unmodified siRNA counterpart. The siRNAs containing PS2 substitutions in either the sense strand or the antisense strand were evaluated for their gene silencing activity in the DFA system. The PS2 modifications were poorly tolerated in the seed region of the antisense strand but were well tolerated in the central part of the antisense strand. In contrast, the sense strand tolerated most of the PS2 modifications located in all tested positions, with significantly improved gene silencing activity in several positions, *e.g.*, PS2-4 (PS2 in positions 19 and 20) and PS2-9 (PS2 in positions 3 and 12). 5′-Phosphorylation of the PS2-siRNAs did not substantially improve gene silencing activity. This means that PS2 modification does not disturb the endogenous phosphorylation process. The biophysical properties of the PS2-siRNA modification, reflected in its enhanced serum stability, will hopefully translate to improved *in vivo* potency.

METHODS

Preparation of the Thiophosphoramidites 1a–d. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl) ribonucleosides (**2a–d**, 1.0 mmol, Figure 1) were phosphitylated by means of tris-(pyrrolidino)phosphine (130 μL, 1.0 mmol)[17,35] in the presence of 1*H*-tetrazole (0.35 mmol) in anhydrous dichloromethane (15 mL) containing a spatula of 3 Å molecular sieves for 15 min at RT. Trimethylsilylimidazole (15 μL, 0.1 mmol) was then added to the reaction mixture, and the reaction was stirred for 5 min. The resulting bis(pyrrolidino)phosphite intermediates were converted into the desired thiophosphoramidites (**1a–d**) by treatment *in situ* with monobenzoylethanedithiol (220 μL, 1.3 mmol)[17,35] and additional 1*H*-tetrazole (2.7 mmol). After an aqueous workup to remove 1*H*-tetrazole and tetrazolide salts, the fully protected RNA thiophosphoramidites (**1a–d**, Figure 1) were isolated by precipitation from hexane in 75–85% yield at more than 90% purity as assessed by ^{31}P NMR (Figure 1S). **1a** (B^Z = Ade^{Bz}): yield, 85%, ^{31}P NMR, δ = 166.7, 174.2 ppm. **1b** (B^Z = Cyt^{Ac}): yield, 75%, ^{31}P NMR, δ = 168.4, 175.2. ppm. **1c** (B^Z = Gua^{Ac}): yield, 87%, ^{31}P NMR, δ = 163.8, 175.3 ppm. **1d** (B^Z = Ura): 77%, ^{31}P NMR, δ = 170.01, 177.1 ppm.

Synthesis of RNA Containing PS2 Substitutions. All PS2-modified RNAs were synthesized on 1000 Å controlled pore glass T support (resulting in a thymidine deoxyribonucleoside at the 3′ end). The standard 2′-*O*-TBDMS RNA phosphoramidites were used for incorporation of A, C, G, and U residues. Each standard nucleotide was coupled using approximately 120 μL of a 0.1 M solution of the appropriate phosphoramidite in anhydrous acetonitrile. Each PS2 substitution was coupled using approximately 250 μL of a 0.15 M solution of the appropriate thiophosphoramidite in anhydrous acetonitrile, except that G thiophosphoramidite (**1c**) was prepared in anhydrous acetonitrile containing 10% anhydrous dichloromethane. All coupling times were 10 min. A 0.05 M solution of EDITH reagent in anhydrous acetonitrile was used as a sulfurizing agent to oxidize the internucleosidic thiophosphite to the phosporodithiotriester.[45] A solution of 0.02 M I_2 in THF/pyridine/water was used to oxidize internucleosidic phosphites to phosphorotriesters. The average stepwise coupling efficiencies of all thiophosphoramidites were about 97% as estimated by the dimethoxytrityl cation assay. After completion of the synthesis, the solid support was suspended in ammonium hydroxide/methylamine (AMA) solution (prepared by mixing 1 volume of ammonium hydroxide (28%) with 1 volume of 40% aqueous methylamine) and heated at 65 °C for 15 min to release the product from the support and to complete the removal of all protecting groups except the TBDMS group at the 2′-position. The solid support was filtered, and the filtrate was concentrated to dryness. The obtained residue was resuspended in 115 μL of anhydrous DMF and then heated for 5 min at 65 °C to dissolve the crude product. TEA (60 μL) was added to each solution, and the solutions were mixed gently. TEA·3HF (75 μL) was added to each solution, and the tubes were then sealed tightly and incubated at 65 °C for 2.5 h. The reaction

was quenched with 1.75 mL of DEPC-treated water. The samples were analyzed by UV−Vis spectroscopy, and the OD readings were recorded.

Purification and Characterization of RNAs and PS2-RNAs. Purification was performed on an Amersham Biosciences P920 FPLC instrument fitted with a Mono Q 10/100 GL column, based on a previously described procedure.[22] The buffers were prepared with DEPC-treated water, and their compositions were as follows: Buffer A: 25 mM Tris-HCl, 1 mM EDTA, pH 8.0; Buffer B: 25 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 8.0. The FPLC gradient profile was as follows: 0−100% B over 50 min, 100% B for 5 min, 100−0% B over 5 min. Purified fractions were pooled and desalted *via* RP-HPLC on an Amersham Biosciences P900 fitted with a PRP-1 Column (Hamilton Co., Reno, NV). The buffers used were as follows: Buffer A: 0.1 M NH_4OAc in DEPC-treated water, pH 7.0; Buffer B: HPLC grade acetonitrile. The gradient run was as follows: 0−35% B over 25 min, 35−0% B over 1 min. After desalting, the samples were frozen at −80 °C for 1 h, lyophilized, and dissolved in DEPC-treated water. All the RNA, PS-RNA and PS2-RNA oligonucleotides were characterized by denaturing polyacrylamide gel electrophoresis. Representative PS2-RNAs were further characterized by ESI-MS.

Preparation of SiRNA Duplexes. Assembly of siRNA duplexes was performed in phosphate buffered saline (PBS) by heating the equivalent mixture of RNA oligonucleotides coding the sense and antisense strands of siRNA at 95 °C for 2 min followed by slow cooling to room temperature (over 2 h). The assembly of the resulting duplexes was confirmed by a 4% agarose gel electrophoresis.

Circular Dichroism (CD) Measurements. CD spectra were recorded on a CD6 dichrograph (Jabin-Yvon, Longjumeau, France) using cells with 0.5 cm path length, 2 nm bandwidth, and 1−2 s integration time. Each spectrum was smoothed with a 25-point algorithm (included in the manufacturer's software, version 2.2.1) after averaging of at least three scans. The spectra from 200 to 340 nm were recorded at 25 °C in the same buffer (100 mM NaCl, 0.1 mM EDTA, 10 mM TRIS, pH 7.4) as in the melting experiments. The concentration of the two complementary RNA oligonucleotides was ca. 2 μM.

Cell Line Culture and Transfection Conditions. HeLa cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Polfa) at 37 °C and 5% CO_2 in 96-well black well plates with transparent bottom (Perkin-Elmer). Before transfection, the culture medium was replaced with fresh antibiotic-free medium. HeLa cells were cotransfected with 15 ng/well pDsRed2-N1 plasmid DNA (BD Biosciences), 70 ng/well pBACE-GFP-plasmid DNA,[23,44] and the appropriate siRNA at 5.0 nM final concentration in OPTI-MEM1 medium (Gibco). Transfection was performed using Lipofectamine 2000 (Invitrogen) at a 2:1 ratio (2 μL of Lipofectamine 2000 per 1 μg of nucleic acids). The cells were incubated for 6 h, and then the medium containing the transfection mixture was replaced with the fresh culturing medium with antibiotics. After 48 h incubation the cells were washed three times with PBS and lysed overnight with a 1:3 mixture of NP-40 buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris-HCl, pH 7, protease inhibitor cocktail, Roche) and PBS at 37 °C. Cell lysates were used for fluorescence determination. Mock transfection control was included for each experiment.

Dual Fluorescence Assay (DFA). Fluorescence values of BACE-GFP fusion protein and red fluorescent protein (RFP) were measured using a Synergy HT reader (BIO-TEK); data quantification was performed using KC4 software. Excitation and emission wavelengths were as follows: EGFP λ_{Ex} = 485/20 nm, λ_{Em} = 528/20 nm; RFP λ_{Ex} = 530/25 nm, λ_{Em} = 590/30 nm. The siRNA activity was calculated as the ratio of EGFP to RFP fluorescence values, averaged over four independent repetitions. The relative level of fluorescence (EGFP/RFP) in control cells transfected with pBACE-GFP, pDsRed2-N1 (BD Biosciences), and control nonsilencing siRNA[23] was taken as the reference (100%).

Serum Stability Assay. Unmodified (PO) and modified (PS2 or PS) siRNAs (100 pmols/reaction) were diluted in the PBS buffer (without Ca^{2+}/Mg^{2+}) with 10% FBS (Gibco) and incubated at 37 °C. Aliquots of 10 μL were collected after 0.5, 1, 2, 3, 4, 6, 8, and 24 h, diluted in 1x loading buffer (Fermentas), frozen in liquid nitrogen, and kept at −70 °C until analysis. A similar control reaction without FBS was performed for each siRNA as well. Samples were separated in 4% low melting agarose (NuSieve GTG Agarose, Cambrex) under nondenaturing conditions. Gels were analyzed using a G-box gel visualization system and quantified by GeneTools 4.00 software (SynGene).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 411*, 494−498.

(2) Shukla, S., Sumaria, C. S., and Pradeepkumar, P. I. (2009) Exploring chemical modifications for siRNA therapeutics: a structural and functional outlook. *ChemMedChem 5*, 328−349.

(3) Behlke, M. A. (2006) Progress towards in vivo use of siRNAs. *Mol. Ther. 13*, 644−670.

(4) Chiu, Y. L., and Rana, T. M. (2003) siRNA function in RNAi: a chemical modification analysis. *RNA 9*, 1034−1048.

(5) Braasch, D. A., Jensen, S., Liu, Y., Kaur, K., Arar, K., White, M. A., and Corey, D. R. (2003) RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry 42*, 7967−7975.

(6) Li, Z. Y., Mao, H., Kallick, D. A., and Gorenstein, D. G. (2005) The effects of thiophosphate substitutions on native siRNA gene silencing. *Biochem. Biophys. Res. Commun. 329*, 1026−1030.

(7) Li, P., Sergueeva, Z. A., Dobrikov, M., and Shaw, B. R. (2007) Nucleoside and oligonucleoside boranophosphates: chemistry and properties. *Chem. Rev. 107*, 4746−4796.

(8) Lebedev, A. V., and Wickstrom, E. (1996) The chirality problem in P-substituted oligonucleotides. *Perspect. Drug Discovery Des. 4*, 17−40.

(9) Wada, T., Fujiwara, S., Sato, T., Oka, N., and Saigo, K. (2004) Stereocontrolled synthesis of phosphorothioate RNA by the oxazaphospholidine approach. *Nucleic Acids Symp. Ser. (Oxford)*, 57−58.

(10) Sierzchala, A., Okruszek, A., and Stec, W. J. (1996) Oxathiaphospholane method of stereocontrolled synthesis of diribonucleoside 3′,5′-phosphorothioates. *J. Org. Chem. 61*, 6713−6716.

(11) Wada, T., Maizuru, Y., Shimizu, M., Oka, N., and Saigo, K. (2006) Stereoselective synthesis of dinucleoside boranophosphates by an oxazaphospholidine method. *Bioorg. Med. Chem. Lett. 16*, 3111−3114.

(12) Petersen, K. H., and Nielsen, J. (1990) Chemical synthesis of dimer ribonucleotides containing internucleotidic phosphorodithioate linkages. *Tetrahedron Lett. 31*, 911−914.

(13) Derrick, W. B., Greef, C. H., Caruthers, M. H., and Uhlenbeck, O. C. (2000) Hammerhead cleavage of the phosphorodithioate linkage. *Biochemistry 39*, 4947−4954.
(14) Greef, C. H., Seeberger, P. H., and Caruthers, M. H. (1996) Synthesis of phosphorodithioate RNA by the H-phosphonothioate method. *Tetrahedron Lett. 37*, 4451−4454.
(15) Wiesler, W. T., Marshall, W. S., and Caruthers, M. H. (1993) Synthesis and purification of phosphorodithioate DNA. *Methods Mol. Biol. 20*, 191−206.
(16) Yang, X., and Mierzejewski, E. (2010) Synthesis of nucleoside and oligonucleoside dithiophosphates. *New J. Chem. 34*, 805−819.
(17) Wiesler, W. T., and Caruthers, M. H. (1996) Synthesis of phosphorodithioate DNA via sulfur-linked, base-labile protecting groups. *J. Org. Chem. 61*, 4272−4281.
(18) Sierant, M., Kubiak, K., Kazmierczak-Baranska, J., Warashina, M., Kuwabara, T., and Nawrot, B. (2009) Evaluation of BACE1 silencing in cellular models. *Int. J. Alzheimer's Dis. 2009*, 1−10.
(19) Wang, Y., Sheng, G., Juranek, S., Tuschl, T., and Patel, D. J. (2008) Structure of the guide-strand-containing argonaute silencing complex. *Nature 456*, 209−213.
(20) Parker, J. S., Roe, S. M., and Barford, D. (2005) Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. *Nature 434*, 663−666.
(21) Rana, T. M. (2007) Illuminating the silence: understanding the structure and function of small RNAs. *Nat. Rev. Mol. Cell. Biol. 8*, 23−36.
(22) Yang, X., Hodge, R. P., Luxon, B. A., Shope, R., and Gorenstein, D. G. (2002) Separation of synthetic oligonucleotide dithioates from monothiophosphate impurities by anion-exchange chromatography on a mono-q column. *Anal. Biochem. 306*, 92−99.
(23) Sipa, K., Sochacka, E., Kazmierczak-Baranska, J., Maszewska, M., Janicka, M., Nowak, G., and Nawrot, B. (2007) Effect of base modifications on structure, thermodynamic stability, and gene silencing activity of short interfering RNA. *RNA 13*, 1301−1316.
(24) Allerson, C. R., Sioufi, N., Jarres, R., Prakash, T. P., Naik, N., Berdeja, A., Wanders, L., Griffey, R. H., Swayze, E. E., and Bhat, B. (2005) Fully 2′-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *J. Med. Chem. 48*, 901−904.
(25) Prakash, T. P., Allerson, C. R., Dande, P., Vickers, T. A., Sioufi, N., Jarres, R., Baker, B. F., Swayze, E. E., Griffey, R. H., and Bhat, B. (2005) Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *J. Med. Chem. 48*, 4247−4253.
(26) Nawrot, B., and Sipa, K. (2006) Chemical and structural diversity of siRNA molecules. *Curr. Top. Med. Chem. 6*, 913−925.
(27) Bramsen, J. B., Laursen, M. B., Nielsen, A. F., Hansen, T. B., Bus, C., Langkjaer, N., Babu, B. R., Hojland, T., Abramov, M., Van Aerschot, A., Odadzic, D., Smicius, R., Haas, J., Andree, C., Barman, J., Wenska, M., Srivastava, P., Zhou, C., Honcharenko, D., Hess, S., Muller, E., Bobkov, G. V., Mikhailov, S. N., Fava, E., Meyer, T. F., Chattopadhyaya, J., Zerial, M., Engels, J. W., Herdewijn, P., Wengel, J., and Kjems, J. (2009) A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. *Nucleic Acids Res. 37*, 2867−2881.
(28) Dowler, T., Bergeron, D., Tedeschi, A. L., Paquet, L., Ferrari, N., and Damha, M. J. (2006) Improvements in siRNA properties mediated by 2′-deoxy-2′-fluoro-beta-D-arabinonucleic acid (FANA). *Nucleic Acids Res. 34*, 1669−1675.
(29) Hall, A. H., Wan, J., Shaughnessy, E. E., Ramsay Shaw, B., and Alexander, K. A. (2004) RNA interference using boranophosphate siRNAs: structure-activity relationships. *Nucleic Acids Res. 32*, 5991−6000.
(30) Wang, Y., Juranek, S., Li, H., Sheng, G., Wardle, G. S., Tuschl, T., and Patel, D. J. (2009) Nucleation, propagation and cleavage of target RNAs in Ago silencing complexes. *Nature 461*, 754−761.
(31) Wang, Y., Juranek, S., Li, H., Sheng, G., Tuschl, T., and Patel, D. J. (2008) Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. *Nature 456*, 921−926.
(32) Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *EMBO J. 20*, 6877−6888.
(33) Parker, J. S., Roe, S. M., and Barford, D. (2004) Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *EMBO J. 23*, 4727−4737.
(34) Volk, D. E., Yang, X., Fennewald, S. M., King, D. J., Bassett, S. E., Venkitachalam, S., Herzog, N., Luxon, B. A., and Gorenstein, D. G. (2002) Solution structure and design of dithiophosphate backbone aptamers targeting transcription factor NF-kappaB. *Bioorg. Chem. 30*, 396−419.
(35) Yang, X., Fennewald, S., Luxon, B. A., Aronson, J., Herzog, N. K., and Gorenstein, D. G. (1999) Aptamers containing thymidine 3′-O-phosphorodithioates: synthesis and binding to nuclear factor-kappaB. *Bioorg. Med. Chem. Lett. 9*, 3357−3362.
(36) Parker, J. S., Parizotto, E. A., Wang, M., Roe, S. M., and Barford, D. (2009) Enhancement of the seed-target recognition step in RNA silencing by a PIWI/MID domain protein. *Mol. Cell 33*, 204−214.
(37) Ma, J. B., Yuan, Y. R., Meister, G., Pei, Y., Tuschl, T., and Patel, D. J. (2005) Structural basis for 5′-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. *Nature 434*, 666−670.
(38) Stein, C. A. (1996) Phosphorothioate antisense oligodeoxynucleotides: questions of specificity. *Trends Biotechnol. 14*, 147−149.
(39) Stein, C. A., and Cheng, Y. C. (1993) Antisense oligonucleotides as therapeutic agents--is the bullet really magical? *Science 261*, 1004−1012.
(40) Grunweller, A., Wyszko, E., Bieber, B., Jahnel, R., Erdmann, V. A., and Kurreck, J. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-O-methyl RNA, phosphorothioates and small interfering RNA. *Nucleic Acids Res. 31*, 3185−3193.
(41) Weitzer, S., and Martinez, J. (2007) hClp1: a novel kinase revitalizes RNA metabolism. *Cell Cycle 6*, 2133−2137.
(42) Weitzer, S., and Martinez, J. (2007) The human RNA kinase hClp1 is active on 3′ transfer RNA exons and short interfering RNAs. *Nature 447*, 222−226.
(43) Jackson, A. L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J. M., Lim, L., Karpilow, J., Nichols, K., Marshall, W., Khvorova, A., and Linsley, P. S. (2006) Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA 12*, 1197−1205.
(44) Sierant, M., Sobczak, M., Janicka, M., Paduszynska, A., and Piotrzkowska, D. (2010) Biological and physicochemical characterization of siRNAs modified with 2′,2′-difluoro-2′-deoxycytidine (gemcitabine). *New J. Chem. 34*, 918−924.
(45) Ma, M. Y., Dignam, J. C., Fong, G. W., Li, L., Gray, S. H., Jacob-Samuel, B., and George, S. T. (1997) Evaluation of 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) as a new sulfurizing reagent in combination with labile exocyclic amino protecting groups for solid-phase oligonucleotide synthesis. *Nucleic Acids Res, 25*, 3590−3593.