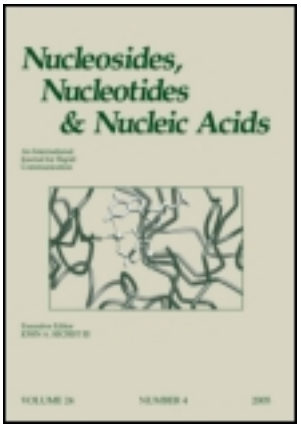

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SULFURIZATION OF DINUCLEOSIDE PHOSPHITE TRIESTERS WITH CHIRAL DISULFIDES

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□ *Sixteen chiral analogues of phenylacetyl disulfide (PADS) and 5-methyl-3H-1,2,4-dithiazol-3-one (MEDITH) were used to sulfurize five dithymidine phosphite triesters, each incorporating a β-cyanoethoxy or siloxy group. Each mixture of S_P:R_P phosphite triester diastereomers was combined with approximately one fourth of an equivalent of each of the sulfurizing reagents, and the R_{PS}:S_{PS} diastereomer ratios of the resulting phosphite sulfides or phosphorothioates were determined by reverse-phase HPLC. Diastereoselectivities and corresponding diastereomeric excess (de) values were calculated by correcting for the starting triester diastereomer ratios. The highest de values for R_{PS} and S_{PS} phosphorothioates were 14.7% and 7.9%, respectively, both using MEDITH analogues.*

Keywords Sulfurization; chiral disulfides; dinucleoside phosphite triesters; antisense; phosphorothioate oligonucleotides

INTRODUCTION

A variety of soluble sulfurization reagents[1,2] have been developed for the synthesis of phosphorothioate oligonucleotides, materials that have attracted intense interest as DNA analogues for antisense applications.[3,4] While the substitution on the phosphate backbone of a terminal oxygen with a sulfur atom gives an oligonucleotide that is more biologically stable yet still possesses biological activity, this substitution also gives a new stereogenic center at every phosphorus atom on the oligonucleotide. Standard DNA synthesis methods give rise to a random mixture of configurations at phosphorus before sulfurization occurs, and following stereoretentive sulfurization, a mixture of R_{PS} and S_{PS} diastereomers forms. These mixtures are still acceptable for pharmaceutical use, but efforts to develop efficient

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strategies for stereoselective phosphorylation to control stereochemistry at phosphorus have been reported for years.[5–10] However, each reported method evidently has flaws that prevent large-scale development, either in the synthesis of the chiral phosphorylating reagents or in the coupling efficiency.[11]

As part of a program to develop a new method for the synthesis of P-stereogenic phosphorothioates, we have begun to examine the sulfurization reaction. A recent report by Mikolajczyk's group described the use of chiral disulfides for the kinetic resolution of phosphines,[12] so a similar approach involving the synthesis of chiral analogues of reagents used for phosphorothioate synthesis seemed warranted. In principle, an achiral reagent could be used for oligonucleotide phosphite sulfurization, since the carbohydrate rings attached to the phosphorus atoms generate P-epimers that are diastereomers, rather than the enantiomers examined by Mikolajczyk. Starting with a mixture of phosphite epimers, the achiral sulfurization reagent could selectively give one diastereomer at early reaction, but a chiral sulfurizing reagent might do so more readily by double stereodifferentiation.[13]

We recently reported the synthesis of two classes of chiral disulfides, one based on phenylacetyl disulfide[14,15] [PADS, (1a)–(1d)] and the other on 5-methyl-3*H*-1,2,4-dithiazol-3-one[16] [MEDITH, (2a)–(2e); Figure 1.[2] We note that the usual name quoted in the literature, 3-methyl-1,2,4-dithiazolin-5-one, appears to us to be in error; the parent ring system is fully unsaturated (hence the "ol" ring termination) and the position of the unsaturation is indicated by giving the *H* the lowest available number.[17] Since the P-epimers do not interconvert at room temperature,[18] selective sulfurization alone will not suffice to give P-stereogenic phosphorothioates. Nevertheless, in this study, we report the results of screening 16 chiral disulfides with five dinucleoside phosphite triesters, including the synthesis of dinucleosides with different degrees of steric hindrance at the phosphite triester, analytical methods for measuring diastereomer ratios of the resultant phosphite sulfides, and the derivation of a means of calculation of the sulfurization selectivity.

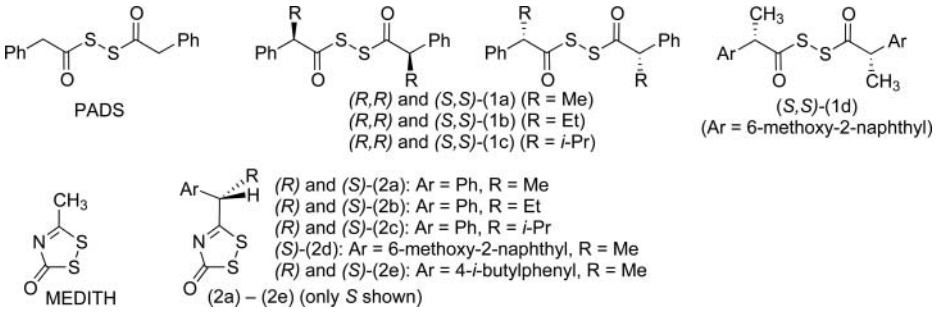
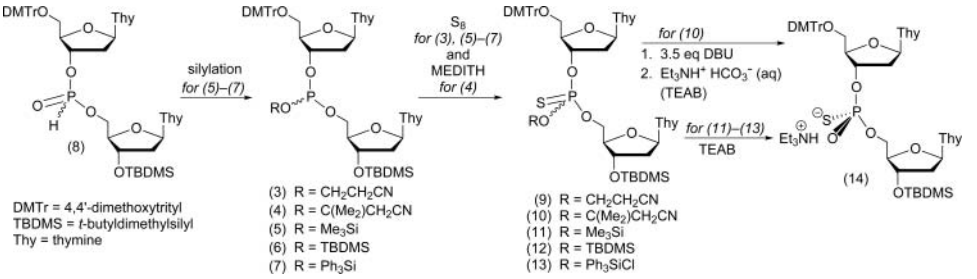


FIGURE 1 Chiral sulfurization reagents.

RESULTS AND DISCUSSION

Phosphite Triesters

Initial screening was planned using the readily available dithymidine phosphite triester (3), chosen since the β-cyanoethoxy group would normally be present using the phosphoramidite method of oligonucleotide synthesis (Scheme 1). However, stereodifferentiation at phosphorus could be difficult, since two of the alkoxy groups are primary and one is secondary. In order to circumvent this, we included the tertiary 1,1-dimethyl-2-cyanoethoxy protecting group in (4).[19,20] In addition, we tested the siloxy phosphite triesters (5)–(7), which are readily available via the *H*-phosphonate (8) method of oligonucleotide synthesis. In this way, the phosphite triester would consist of a 1° alkoxy group due to the 5′-thymidine, a 2° alkoxy group due to the 3′-thymidine, and a 3°-like alkoxy group due to the siloxy group, and thereby provide a higher degree of steric bulk around the phosphorus, which might allow greater selectivity for sulfurization.



SCHEME 1 Phosphite triesters for screening, sulfurization, and phosphorothioate formation for analysis.

Phosphite triester (3) was synthesized as previously described[18] and (4) was readily prepared in a comparable manner starting from 1,1-dimethyl-2-cyanoethanol[21] and $(i\text{-Pr}_2\text{N})_2\text{PCl}$. Conversion of (8) and close analogues to trimethylsiloxy phosphite triesters analogous to (5) using *N*,*O*-bis(trimethylsilyl)acetamide (BSA)[22–24] and other silylating reagents[23,25] is known; procedures for the synthesis of phosphite triesters (6) and (7) have not been reported, but the desired conversions were expected to proceed using *tert*-butyldimethylsilyl chloride (TBDMSCl) and Et_3N and $\text{Ph}_3\text{SiCl}/\text{Et}_3\text{N}$, respectively.[23,25,26] *H*-phosphonate (8) was prepared following literature procedures reported for the 5′-*O*-TBDMS analogue.[26] Initial experiments to find the best silylating conditions for the synthesis of phosphite triesters (5) and (6) (Scheme 1) were carried out by simultaneously treating 5 mg each of *H*-phosphonate (8) with 3 equivalents of BSA (the amount reported by Shaw[23]) and $\text{TBDMSCl}/\text{Et}_3\text{N}$ in anhydrous acetonitrile-d_3 at room temperature. Since *H*-phosphonate (8) is a mixture of diastereomers, only two peaks corresponding to the R_P and S_P diastereomers of phosphite triesters (5) and (6) were expected after complete silylation. However, after ~30

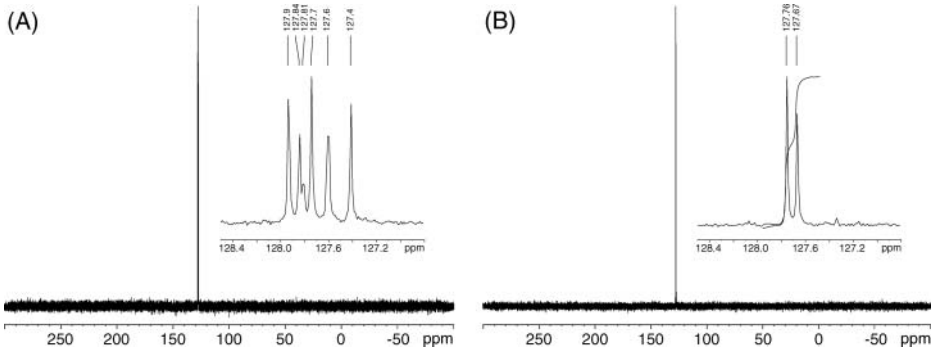


FIGURE 2 (A) ^{31}P NMR spectrum of the reaction mixture of (8) and 3 equivalents of BSA after 1 day, in the attempted synthesis of phosphite triester (5). (B) ^{31}P NMR spectrum of the reaction mixture of (8) and 5 equivalents of BSA after 20 minutes.

minutes reaction with BSA, the ^{31}P NMR spectrum of the reaction solution exhibited six peaks in the phosphite region near 127 ppm; no change was observed after 1 day (Figure 2A). Similarly, the ^{31}P NMR spectrum of the reaction with TBDMSCl/Et_3N exhibited several peaks in the phosphite triester region as well as in the *H*-phosphonate region (~8 ppm) after ~2 hours and was unchanged after 1 day. Surprisingly, an addition of 5 rather than 3 equivalents of BSA to (8) gave two peaks in the ^{31}P NMR spectrum after 20 minutes (Figure 2B) and similarly 5 equivalents of TBDMSCl or Ph_3SiCl with 10 equivalents of NEt_3 gave two peaks in the ^{31}P NMR spectra after 2 hours, each in virtually the same 56:44 R_P:S_P ratio as that seen for (8). We suggest that the source of the additional peaks in the ^{31}P NMR spectra is simply the partial silylation of the carbonyl moieties on the thymine rings; that is, silylation could occur at the 3′- and/or 5′-phosphorylated R_P and S_P nucleosides. However, once a large excess of silylating reagent is added, complete silylation can give only the R_P and S_P fully silylated P-epimers.

Analytical Methods for Determination of the Diastereomer Ratios

Each of the phosphite triesters (3)–(7) was converted to the corresponding phosphite sulfides (9)–(13) by treatment with excess sulfur [(9) and (11)–(13)] or MEDITH (10) at room temperature (Scheme 1). Sulfur is known to react in a stereoretentive manner with chiral phosphite triesters,[24,27] although the labels change according to the Cahn–Ingold–Prelog rules and the R_P epimer gives rise to the S_{PS} epimer upon sulfurization. Diastereomer assignments of the sulfides were made by comparisons of the observed ratios to those of the phosphite triesters from the ^{31}P NMR spectra, although the ratios for (3) and (9) are so close to 1:1 that the assignment must be viewed with caution; NMR and HPLC (*vide infra*) data may be found in the Experimental section. Because the ^{31}P

NMR sulfide peak separations were quite small, however, it was clear that they could not be integrated with sufficient precision to allow the detection of small sulfurization selectivities. We therefore sought to use HPLC[28] for the measurement of diastereomer ratios.

The diastereomers of (9) and (10) were examined first, and the diastereomers of (9) gave baseline separation by elution on a C18 column with 65:35 acetonitrile/water with an integrated ratio similar to that seen by NMR. However, we were unable to find any conditions for the separation of the diastereomers of (10). We did not attempt any HPLC analysis of the oxygen-sensitive phosphite triesters (3)–(7) or the water-sensitive siloxy ester sulfides (11)–(13), but for these sulfides as well as (10), conversion to phosphorothioate (14) was a potential alternative.

We expected that mild base treatment of (10) would give rise to stereospecific elimination of dimethylacrylonitrile,[29,30] and in fact, addition of 3.5 equivalents of diazabicycloundecene (DBU) followed by treatment with 2 M aqueous triethylammonium bicarbonate (TEAB) gave phosphorothioate (14) (Scheme 1). The diastereomer ratios of (4) and (10) measured by ^{31}P NMR were the same (45.5:54.5), and the diastereomers of (14) gave baseline separation on a C8 column with 55:45 acetonitrile:0.1 M triethylammonium acetate (TEAA), giving a diastereomer ratio of 45.66:54.34. In a similar manner, the siloxy ester sulfides (11)–(13) were treated with TEAB[25] to give the diastereomers of phosphorothioate (14) (Scheme 1), a reaction previously shown to be stereospecific,[27] in essentially the same ratio as that of the *H*-phosphonate (8) precursor; the HPLC ratio of the diastereomers of (14) in the final mixture was 43.44:56.56. Identification of (14) was confirmed by isolation from the reaction mixture and chromatography, giving material that matched the beautifully detailed NMR data in $CDCl_3$ reported by Stawinski and coworkers.[31] The HPLC ratios of (14) from (12) and (13) were the same within experimental error, as expected since these too were derived from the same sample of (8). Assignments of configurations of (4)–(7) and (10)–(14) were all determined on the basis of the reported assignments of (8),[27] and since we obtained the diastereomers of (8) in a ~45:55 ratio, it was easy to assign the products formed from (8) [that is, (11)–(14)] and the precursors of (11)–(14) [that is, (4)–(7) and (10)] on the basis of that 45:55 ratio. It should be noted that (4) and (10) were not derived from (8), but once the diastereomers of (14) were assigned, the corresponding diastereomers of (4) and (10) that gave rise to (14) could be assigned.

Reaction of Sulfurizing Reagents with Phosphite Triesters

Procedure

The phosphite triesters consist of a mixture of R_P and S_P diastereomers, which do not interconvert on the reaction time scale.[18] Addition of less

than one equivalent of a sulfurizing reagent to the mixture would then allow any selectivity for one P-epimer to be detected. The use of smaller amounts of sulfurizing reagent would allow a more accurate determination of selectivity, but since a reasonable amount of product was needed, approximately one fourth of an equivalent seemed to represent a good compromise. In order to increase the possibility of greater selectivity, the reactions were carried out at −32°C, necessitating a reaction time of 20 hours. After sulfurization, anhydrous *tert*-butylhydroperoxide (TBHP)[32] was added to quench the reaction in order to convert the remaining phosphite triester to the phosphite oxide.[26,33] The S_{PS}:R_{PS} diastereomer ratio determined by HPLC of the phosphite sulfide (9) after chiral sulfurization or of the phosphorothioate (14) after sulfurization and elimination (10) or hydrolysis [(11)–(13)] can then be compared with the R_P:S_P ratio of the starting phosphite triester—determined in the same way by complete, nonselective sulfurization with excess sulfur or MEDITH—to determine the selectivity of the reaction (*vide infra*).

Sulfurization Yields

While each screening reaction was carried out with ∼25 mol% of the sulfurizing reagent with respect to the amount of phosphite triester used, analysis of each reaction by ^{31}P NMR and/or HPLC showed that more than 30% of the phosphite triester had been sulfurized in ∼30% of the reactions. We ascribe this to weighing errors in the glove box, since there was no pattern that might suggest (for instance) sulfurization by more than one sulfur atom of the disulfides. A plot of the extent of sulfurization for each sulfurizing reagent for each triester shows that the extent of sulfurization for most of the reactions ranged from 20% to 40% (Figure 3). It can be seen that phosphite triester (3) is less reactive toward PADS, especially its chiral analogues, and phosphite triester (5) is generally more reactive to many sulfurizing reagents compared with phosphite triesters (6) and (7).

R_{PS}:S_{PS} Diastereomer Ratios by HPLC

The R_{PS}:S_{PS} diastereomer ratios determined by HPLC analysis of phosphite sulfide (9) and phosphorothioate (14) derived from phosphite triesters (10)–(13) are shown in Table 1. The first row displays data for reactions with excess sulfur [or for (4), excess MEDITH instead], and so gives the initial triester diastereomer ratio. The remaining data points are those for partial sulfurization, that is, addition of ∼25 mol% of the sulfurizing reagent. The data in Table 1 might in principle be corrected by taking into account the fact that the sulfurizing reagents were not all 100% enantiomerically pure,[2] but the correction was not necessary since the diastereomer ratio would only change by ∼0.15% at most.[34]

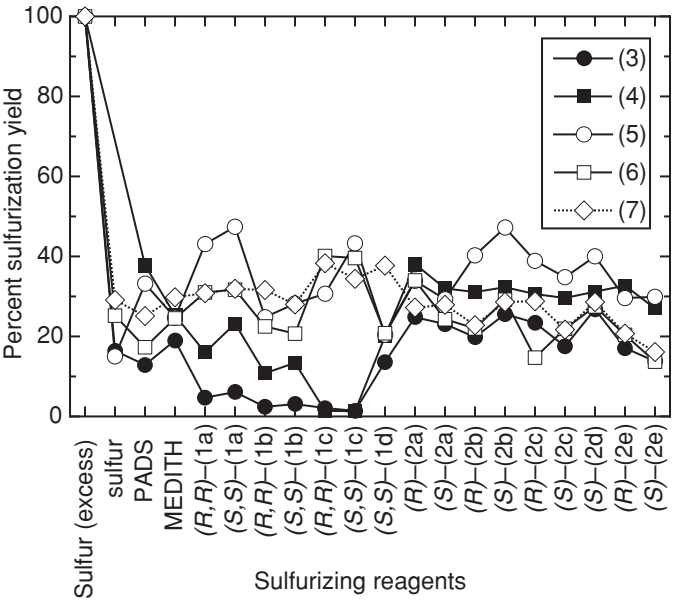


FIGURE 3 Extent of sulfurization of phosphite triesters (3)–(7) by ~25 mol% of sulfurizing reagent.

Derivation and Calculation of Selectivity of the Chiral Sulfurization Reactions

For a reaction in which a single isomer reacts to give two stereoisomers, the isomer ratio can be expressed as the enantiomeric excess (ee) if they are enantiomers, or diastereomeric excess (de) if they are diastereomers. Unlike a reaction that has a single isomer as the starting substrate, calculation of the selectivity of a reaction such as phosphite triester sulfurization, in which the starting substrate is a mixture of diastereomers, is neither standard nor intuitive. Since sulfurization of the two diastereomers of a phosphite triester occurs in a stereoretentive manner,[24,27] the R_P configuration almost certainly goes to the S_{PS} phosphorothioate and the S_P configuration goes to the R_{PS} phosphorothioate. The diastereoselectivities[35,36] for the R and S configurations are expressed, respectively, as $ds_{R(PS)}$ and $ds_{S(PS)}$. That is, if a sulfurizing reagent was 100% selective for the formation of S_{PS} from R_P, then in that case, $ds_{S(PS)} = 100\%$ and $ds_{R(PS)} = 0\%$, and we would only expect R_P to react leaving S_P untouched. This leads to Equation (1), and for the case of 100% selectivity, the equation can always be inverted to prevent a zero in the

$$(S_{PS}/R_{PS}) = (R_P/S_P) \times (ds_{S(PS)}/ds_{R(PS)}) \quad (1)$$

$$ds_{S(PS)} = 1 - ds_{R(PS)} \quad (2)$$

$$ds_{R(PS)} = R_{PS} \times R_P/[(S_P \times S_{PS}) + (R_{PS} \times R_P)] \quad (3)$$

$$de = ds_{S(PS)} - ds_{R(PS)} \quad (4)$$

TABLE 1 R_{PS}:S_{PS} diastereomer ratios for sulfurization reactions[a]

	Phosphite sulfide (9)[b] from (3)		Phosphorothioate (14)[c] from (4)		Phosphorothioate (14)[d] from (5)		Phosphorothioate (14)[d] from (6)		Phosphorothioate (14)[d] from (7)	
Sulfurizing reagent	R_{PS}	S_{PS}	R_{PS}	S_{PS}	R_{PS}	S_{PS}	R_{PS}	S_{PS}	R_{PS}	S_{PS}
Sulfur (excess)[e]	50.59	49.41	45.66[e]	54.34[e]	43.44	56.56	43.38	56.62	43.36	56.64
Sulfur	52.12	47.88			43.52	56.48	43.41	56.59	45.63	54.37
PADS	52.08	47.92	49.47	50.53	44.87	55.13	44.2	55.8	45.96	54.04
MEDITH	49.09	50.91	48.01	51.99	44.19	55.81	42.87	57.13	44.96	55.04
(*R*,*R*)-(1a)	49.89	50.11	49.74	50.26	42.72	57.28	40.86	59.14	45.42	54.58
(*S*,*S*)-(1a)	51.99	48.01	51.13	48.87	43.49	56.51	43.07	56.93	43.81	56.19
(*R*,*R*)-(1b)	46.85	53.15	51.59	48.41	42.32	57.68	40.46	59.54	46.42	53.58
(*S*,*S*)-(1b)	50.49	49.51	52.50	47.50	41.87	58.13	43.44	56.56	44.53	55.47
(*R*,*R*)-(1c)	49.57	50.53	45.04	54.96	48.96	51.04	46.37	53.63	43.57	56.43
(*S*,*S*)-(1c)	52.81	47.19	51.76	48.24	45.44	54.56	43.33	56.67	47.27	52.73
(*S*,*S*)-(1d)	51.35	48.65	51.31	48.69	44.01	55.99	41.62	58.38	40.24	59.76
(*R*)-(2a)[f]	51.13	48.87	48.50	51.50	43.12	56.88	45.96	54.05	48.33	51.68
					±0.56	±0.56	±0.48	±0.48	±0.47	±0.47
(*S*)-(2a)[g]	51.14	48.86	49.39	50.61	43.16	56.84	46.28	53.72	48.32	51.68
					±0.36	±0.36	±1.33	±1.33	±0.52	±0.52
(*R*)-(2b)	51.2	48.8	45.90	54.10	41.85	58.15	43.51	56.48	47.79	52.21
(*S*)-(2b)	51.24	48.76	50.41	49.59	43.73	56.27	45.82	54.18	49.03	50.97
(*R*)-(2c)[f]	50.11	49.90	45.03	54.97	40.68	59.32	44.57	55.44	45.55	54.46
	±0.25	±0.25			±0.44	±0.44	±0.48	±0.48	±0.32	±0.32
(*S*)-(2c)[f]	49.82	50.18	46.05	53.95	39.58	60.43	45.85	54.15	45.26	54.75
	±0.46	±0.46			±0.29	±0.29	±0.32	±0.32	±0.03	±0.03
(*S*)-(2d)	50.92	49.08	49.20	50.80	50.81	49.19	47.81	52.19	49.63	50.37
(*R*)-(2e)	50.91	49.09	48.68	51.32	42.05	57.95	44.94	55.06	45.36	54.64
(*S*)-(2e)	50.73	49.27	47.17	52.83	49.66	50.34	41.92	58.08	44.72	55.28

[a]Except for row one with sulfur (see footnote *e* also), all reactions were carried out with ~0.25 equivalents of sulfurizing reagent.

[b]Ratio determined by HPLC analysis of the reaction solution; see the Experimental Section for details.

[c]Ratio determined by conversion of the product sulfide to the phosphorothioate with DBU, followed by HPLC analysis; see the Experimental Section for details.

[d]Ratio determined by conversion of the product siloxy sulfide to the phosphorothioate by hydrolysis with $[Et_3NH][HCO_3]$, followed by HPLC analysis; see the Experimental Section for details.

[e]Excess sulfur gave complete conversion of (3) and (5)–(7) to the corresponding sulfides; for (4), excess MEDITH was used instead and similarly gave complete conversion to the corresponding sulfide.

[f]Average of two experiments where the average deviation is given.

[g]Average of three experiments where the average deviation is given.

denominator. Rearranging Equation (1) to solve for $ds_{S(PS)}/ds_{R(PS)}$ and substitution of $ds_{S(PS)}$ using Equation (2) give an expression for the diastereoselectivity $ds_{R(PS)}$ [Equation (3)], where S_P and R_P are the initial phosphite percentages and R_{PS} and S_{PS} are the observed phosphite sulfide percentages from S_P and R_P, respectively. Although it is not necessary, we found that the de of the reaction [Equation (4)] allowed the results to be more easily visualized; we have chosen that selectivity for the S_{PS} phosphite sulfide or phosphorothioate, derived from the more abundant R_P isomer of (4) and (8), although the less abundant (by a small amount) R_P isomer of (3), will give a positive de.

Using the R_{PS}:S_{PS} diastereomer ratios in Table 1 along with the initial S_P and R_P phosphite triester values taken from the excess sulfur reactions [or for (4), excess MEDITH], the diastereoselectivity ratios ($ds_{R(PS)}$:$ds_{S(PS)}$) were calculated using Equation (3) for $ds_{R(PS)}$ and Equation (2) for $ds_{S(PS)}$. The diastereoselectivities for (2a) and (2c) are the average values from the repeated reactions. The resultant de values are plotted in Figures 4–6.

Achiral Sulfurization Selectivity

The use of excess sulfur for the sulfurization of (3)–(7) gives by definition no selectivity. However, as seen in Figure 4, even the achiral sulfur, PADS, and MEDITH reagents, when not present in excess, gave small selectivities, particularly for (3), (4), and (7). In most cases, the selectivities favored the R_{PS} isomers and so gave negative de values—that is, the major isomer for (3) but the minor isomer for (4)–(7) were favored. Somewhat surprisingly, for instance, the least hindered phosphite triester (3) gave de values of approximately ±3% for the three achiral sulfurization reagents, while the most hindered triesters (4) and (7) less surprisingly gave the highest de values of −4.7% to −7.6% and −3.2% to −5.3%, respectively.

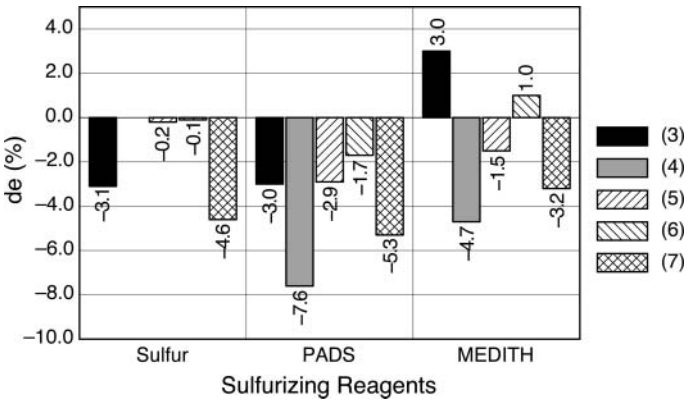


FIGURE 4 Diastereoselectivity excess (S_{PS} positive) for sulfurization of phosphite triesters (3)–(7) by achiral sulfurizing reagents.

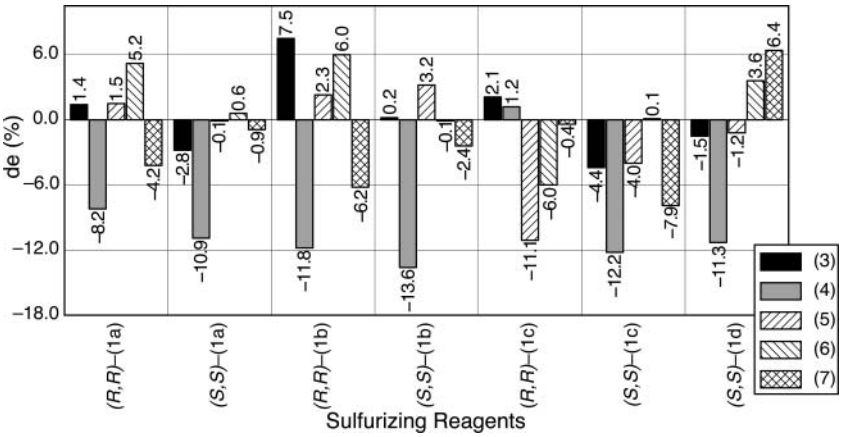


FIGURE 5 Diastereoselectivity excess (S_{PS} positive) for sulfurization of phosphite triesters (3)–(7) by chiral PADS analogues (1).

Chiral Sulfurization Selectivity

Separate plots of de for the PADS and MEDITH analogues (Figures 5 and 6) exhibit a range of de values up to +7.5% and −14.7%. For the PADS analogue sulfurizations, no discernable pattern of diastereoselectivity was observed except for the reactions of (4), where in most cases a large negative de was seen. The effect for (4) seems more due to the triester than to the sulfurizing reagents, since even achiral PADS gave a de of −7.6%, but an interesting effect was seen for isopropyl-substituted PADS analogue (1c): the (*R,R*) isomer gave a small de of +1.2% while the (*S,S*) isomer gave a large de of −12.2%. Otherwise, each of the other triesters gave bigger

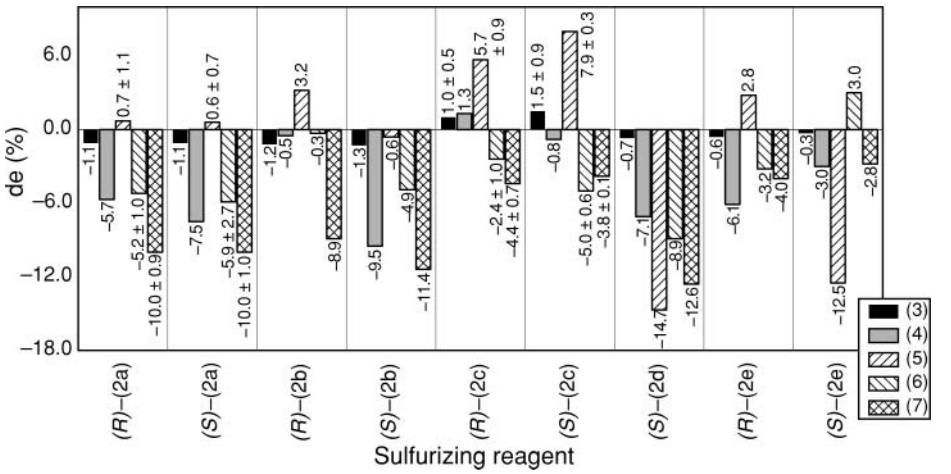


FIGURE 6 Diastereoselectivity excess (S_{PS} positive) for sulfurization of phosphite triesters (3)–(7) by chiral MEDITH analogues (2).

differences between the enantiomeric PADS analogues and so would seem to be more effective than (4) as screening choices. The highest de other than for (4) of −11.1% was observed for sulfurization of trimethylsiloxyphosphite (5) with the hindered isopropyl PADS (*R,R*)-(1c). The negative de indicates selectivity for the minor R_{PS} isomer, so the observed 49.0:51.0 R_{PS}:S_{PS} ratio of (14) from (11) must be referenced to the starting 43.4:56.6 S_P:R_P ratio of (5) to generate the (relatively) high de. The largest absolute difference in the observed diastereomers was the 40.2:59.8 R_{PS}:S_{PS} ratio seen for sulfurization of (7) by (*S,S*)-(1d), which translates into a de of +6.4%.

In contrast to the PADS data, the MEDITH analogues clearly exhibited a pattern for the pairs of enantiomeric disulfides (2a) and (2c)—that is, each pair gave a similar pattern of selectivities with the five phosphites, although the patterns were different for the different MEDITH analogues. For (2b) and (2e), the similarities were not strong, but the results nevertheless suggested that the chiral MEDITH analogues were racemizing under the reaction conditions, a concern since these compounds had been observed to racemize upon silica gel chromatography.[2] MEDITH analogue (2a) was tested for racemization, by running sulfurization reactions of (5) with excess *(R)* or *(S)*-(2a), and the unreacted (2a) was then analyzed by HPLC as previously described[2] to test for enantiopurity. Complete racemization was observed.

Because of the racemization of the MEDITH analogues, the sulfurization selectivities could arise from faster reaction with one enantiomer, or simply arise as a mixture of two selectivities. Interestingly, higher selectivities were observed than for the nonracemizing PADS analogues [which were tested in the same manner, using excess *(R,R)* and *(S,S)*-(1a) with (4) and (5), with the result that no racemization was found]. For instance, (*S*)-(2d) gave three of the highest de values of −14.7%, −8.9%, and −12.6% with (5)–(7), and (*S*)-(2e) [but not (*R*)-(2e)] gave a de of −12.5% for the least hindered siloxy phosphite (5). The most hindered siloxyphosphite, (7), gave the highest de values of −8.9% to −11.4% with (2a)–(2b), while (*S*)-(2c) gave the highest positive de of 7.9% ± 0.3% with the trimethylsiloxyphosphite (5) [that for (*R*)-(2c) was 5.7% ± 0.9%], and hence the highest absolute selectivity ratio of 39.6:60.4 R_{PS}:S_{PS}. No significant selectivity was seen for any of the MEDITH sulfurizations of β-cyanoethoxy phosphite (3).

EXPERIMENTAL SECTION

General

NMR spectra were recorded on a 400 MHz Bruker spectrometer with tetramethylsilane and 85% H_3PO_4 in CD_3CN, $CDCl_3$, and toluene-d_8 as external standards. High-resolution mass spectrometry was performed on an Agilent G6520A Q-TOF instrument. Air- and water-sensitive compounds were

handled in an inert atmosphere glove box under nitrogen. While oligonucleoside reagents do not require glove box use, we have found it to be extraordinarily convenient in maintaining nonoxidizing conditions for the more air-sensitive reagents, and in maintaining dry solvents for all reactions and NMR analyses. Amines, acetonitrile, 1,4-dioxane, dimethyformamide (DMF), and pyridine were distilled from calcium hydride under nitrogen, and diethyl ether and tetrahydrofuran (THF) were distilled from sodium benzophenone under nitrogen. Flash column chromatography was carried out on 230–400 mesh silica gel, and thin layer chromatography was carried out on aluminum-backed silica gel F (200 microns). Water (HPLC grade, submicrofiltered), dichloromethane, hexanes, and acetic acid were used as received. For the peak assignments in the ^{1}H NMR spectra of (3) and (4) that follow, the 3′-phosphorylated thymidine and the 5′-phosphorylated thymidine are labeled T1 and T2, respectively; see ref. [18] for details on the methods of assignment. Details of the syntheses of dinucleosides (3), (4), and (8) are given below because these procedures do not appear to be readily available.

Synthesis of 2-Cyanoethyl 5′-*O*-(4,4′-Dimethoxytrityl)Thymidin-3′-yl 3′-*O*-(Tertbutyldimethylsilyl) Thymidin-5′-yl Phosphite (3)

The synthesis of (3) was carried out on a 0.51 g scale of phosphoramidite as previously described,[18] but instead of adding Me_2SBH_3 to the crude mixture to effect separation of diastereomers, the mixture was taken into the glove box and purified by using a short column of silica gel (∼20 g of silica gel in a 60 mL coarse frit) and solvent mixture of THF/hexane (2:1). All fractions with R_f of 0.55 (THF/hexane 2:1) were evaporated under reduced pressure to afford (3) as a white foam (0.60 g, 94% yield). ^{1}H NMR (400 MHz, CD_3CN, two diastereomers): δ 9.37 (br s, NH, 4H), 7.48–7.26 (m, includes 4 × H-6, 22H), 6.91–6.88 (m, 8H), 6.30 (m, 2 × ^{T1}H-1′, 2H), 6.19 (m, 2 × ^{T2}H-1′, 2H), 5.03 (m, 2 × ^{T1}H-3′, 2H), 4.41 (m, 2 × ^{T2}H-3′, 2H), 4.12 (m, 2 × ^{T1}H-4′, 2H), 4.08–3.91 (m, 2 × ^{T2}H-4′, 2 × ^{T2}H-5′, 2 × ^{T2}H-5″, 2 × CH_2OP, 10H), 3.79 (s, 2 × CH_3O, 6H), 3.78 (s, 2 × CH_3O, 6H), 3.35 (m, 2 × ^{T1}H-5′, 2 × ^{T1}H-5″, 4H), 2.65 (br t, $J = 5.9$ Hz, 2 × CH_2CN, 4H), 2.44 (m, 2 × ^{T1}H-2′, 2 × ^{T1}H-2″, 4H), 2.19 (m, 2 × ^{T2}H-2′, 2 × ^{T2}H-2″, 4H), 1.83 (m, 2 × CH_3C-5, 6H), 1.52 (d, $J = 1.1$ Hz, CH_3C-5, 3H), 1.50 (d, $J = 1.1$ Hz, CH_3C-5, 3H), 0.91 (s, 2 × (CH_3)$_3$CSi, 18H), 0.12 (s, (CH_3)$_2$Si, 6H), 0.10 (s, (CH_3)$_2$Si, 6H). ^{31}P NMR (161 Mz, CD_3CN, two diastereomers): δ 140.41, 140.38; (toluene-d_8): δ 139.7 (S_P), 139.3 (R_P), 50.7:49.3; assigned from the NMR spectra of the isolated diastereomers.[18] HRMS (ESI): Calculated for $C_{50}H_{61}N_5O_{13}PSi$ [M-H]$^-$: 998.3773, found 998.3777.

Synthesis of 1,1-Dimethyl-2-Cyanoethyl 5′-*O*-(4,4′-Dimethoxytrityl)Thymidin-3′-yl 3′-*O*-(Tertbutyldimethylsilyl) Thymidin-5′-yl Phosphite (4)

The starting phosphoramidite, 1,1-dimethyl-2-cyanoethyl 5′-*O*-(4,4′-dimethoxytrityl)thymidin-3′-yl *N*,*N*-diisopropylphosphoramidite, was synthesized as follows: under a nitrogen atmosphere, a solution of 5′-*O*-(4,4′-dimethoxytrityl)thymidine[37] (0.6008 g, 1.103 mmol) in 4 mL CH_3CN was added dropwise over 2–3 minutes to a magnetically stirred solution of $(i\text{-}Pr_2N)_2POC(CH_3)_2CH_2CN$ (0.7837 g, 2.379 mmol, prepared in 90% yield using the reported procedure[38] from 1,1-dimethyl-2-cyanoethanol[21] and $(i\text{-}Pr_2N)_2PCl$) and *N*-methylimidazolium trifluoromethanesulfonate[39] (0.1578 g, 0.6796 mmol) in 10 mL CH_3CN. The reaction solution was stirred for 24 hours at room temperature, after which the solvent was evaporated on a vacuum line to give a gum. The gum was dissolved in 50 mL of CH_2Cl_2, washed with 15 mL saturated Na_2CO_3 solution, the organic layer was dried with anhydrous $MgSO_4$ and the solvent was evaporated on a vacuum line to give a gum. This material was eluted through a pad of silica gel (20 g) with ethyl acetate, and the UV-active material with $R_f = 0.9$ (EtOAc) was collected and the solvent evaporated on a vacuum line to give 0.85 g (100% yield) of product as a slightly yellow foam, which was used in the next step.

The synthesis of (4) was then carried out in the same manner as described for the synthesis of (3), starting from 0.793 g (1.03 mmol) of the above phosphoramidite. The crude compound was taken out of the glove box and purified by column chromatography (~28 g of silica gel in a 30 mm diameter column) using a mixture of ethyl acetate/hexane (3:1). All fractions with $R_f = 0.38$ (ethyl acetate/hexane 3:1) were evaporated under reduced pressure to afford (4) as a white foam (0.60 g, 60% yield). ^{1}H NMR [400 MHz, $CDCl_3$, two diastereomers; T^1 and T^2 assigned by comparison to (3)]: δ 8.34 (br s, NH, 4H), 7.59–7.24 (m, includes 4 × H-6, 22H), 6.85–6.83 (m, 8H), 6.41 (m, 2 × ^{T1}H-1′, 2H), 6.22 (m, 2 × ^{T2}H-1′, 2H), 4.97 (m, 2 × ^{T1}H-3′, 2H), 4.34 (m, 2 × ^{T2}H-3′, 2H), 4.18 (m, 2 × ^{T1}H-4′, 2H), 4.07–3.92 (m, 2 × ^{T2}H-4′, 2 × ^{T2}H-5′, 2 × ^{T2}H-5″, 6H), 3.79 (s, 4 × CH_3O, 12H), 3.42 (m, 2 × ^{T1}H-5′, 2 × ^{T1}H-5″, 4H), 2.54 (m, 2 × CH_2CN, 4H), 2.55–2.31 (m, 2 × ^{T1}H-2′, 2 × ^{T1}H-2″, 4H), 2.29–2.08 (m, 2 × ^{T2}H-2′, 2 × ^{T2}H-2″, 4H), 1.89 (d, J = 1.0 Hz, CH_3C-5, 3H), 1.88 (d, J = 1.0 Hz, CH_3C-5, 3H), 1.510, 1.505, 1.49, 1.47 (4s, C(CH_3)$_2$OP,12H), 1.45 (d, J = 1.0 Hz, CH_3C-5, 3H), 1.44 (d, J = 1.0 Hz, CH_3C-5, 3H), 0.89 (s, (CH_3)$_3$CSi, 9H), 0.88 (s, (CH_3)$_3$CSi, 9H), 0.075, 0.070 (2s, (CH_3)$_2$Si, 6H), 0.062, 0.058 (2s, (CH_3)$_2$Si, 6H). ^{31}P NMR (161 MHz, $CDCl_3$, two diastereomers): δ 135.15 (S_P), 134.75 (R_P), 45.5:54.5; assigned by conversion to (14).

Synthesis of 5′-*O*-(4,4′-Dimethoxytrityl)Thymidin-3′-yl 3′-*O*-(Tertbutyldimethylsilyl) Thymidin-5′-yl-(*H*-Phosphonate) (8)

Compound (8) was synthesized by following the literature procedure used for the synthesis of the 5′-*O*-(*tert*-butyldimethylsilyl) analogue of compound (8),[26] starting first with the preparation of [5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-(*H*-phosphonate)]$^-$ $(CH_3CH_2)_3NH^+$: under a nitrogen atmosphere, a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (1.0326 g, 5.0985 mmol) dissolved in 5 mL of 1,4-dioxane was added dropwise over ~3 minutes to a rapidly stirred solution of 5′-*O*-(4,4′-dimethoxytrityl)thymidine[37] (2.0107 g, 3.6921 mmol) and pyridine (0.8080 g, 10.21 mmol) in 40 mL of 1,4-dioxane. The flask was rinsed with 5 mL of 1,4-dioxane and transferred to the reaction solution. The solution was stirred at room temperature for ~20 minutes, then 0.5 mL of water was added, and the mixture was diluted with 60 mL of CH_2Cl_2/Et_3N (99/1 v/v). The solution was washed with 25 mL of 2 M TEAB (*vide infra*). The organic layer was separated and the aqueous layer was back extracted with 2 × 25 mL CH_2Cl_2. The combined organic layers were dried with anhydrous $MgSO_4$, filtered, and the solvent was removed on a rotary evaporator to give an orange gum. The product was purified by silica gel chromatography using a 0%–9% gradient of CH_3OH in 0.5% Et_3N/CH_2Cl_2 (50 g silica gel in a 50 mm diameter column). All fractions with $R_f = 0.24$ (10% MeOH/CH_2Cl_2) were combined and evaporated on a vacuum line to afford the product as a white foam (2.01 g, 77% yield). ^1H, ^{13}C, and ^{31}P NMR spectra matched those taken on an authentic sample purchased from ChemGene.

Under a nitrogen atmosphere, a sample of the above *H*-phosphonate (0.8339 g, 1.175 mmol) and 3′-*O*-(*tert*-butyldimethylsilyl)thymidine[40] (0.4114 g, 1.154 mmol) was co-evaporated with 12 mL of pyridine to remove water and then redissolved in 10 mL of pyridine. With stirring, pivaloyl chloride (0.3290 g, 2.729 mmol) was added dropwise over ~1 minute. The reaction solution was stirred at room temperature for 1 hour, and then evaporated on a vacuum line to give a white foam. The foam was dissolved in THF and then eluted with THF through a short column of silica gel (~10 g silica gel in a 60 mL coarse frit). The UV-active material that came through with the solvent front was collected and evaporated on a vacuum line to give the crude product as a white foam. The compound was purified by silica gel chromatography using a 0%–6% gradient of CH_3OH in CH_2Cl_2 (60 g silica gel in a 50 mm diameter column). The appropriate fractions with $R_f = 0.6$ (10% MeOH/CH_2Cl_2) were combined, and the solvent was evaporated on a vacuum line to afford *H*-phosphonate (8) as a white foam (0.76 g, 70%). ^1H and ^{31}P NMR spectra matched those reported in the literature[27]: ^{31}P NMR (161 MHz, DMSO-d_6, two diastereomers): δ 9.44 (S_P), 8.76 (R_P), 44.4:55.6.

Synthesis of [5′-*O*-(4,4′-Dimethoxytrityl)Thymidin-3′-yl 3′-*O*-(Tertbutyldimethylsilyl) Thymidin-5′-yl-Phosphorothioate]$^{-}$[$(CH_3CH_2)_3NH]^{+}$ (14)

Under a nitrogen atmosphere, BSA (20 μL, 0.082 mmol) was added to a solution of (8) (15 mg, 0.016 mmol) in 0.3 mL CH_3CN. After stirring for 20 minutes, sulfur (6.1 mg, 0.19 mmol S or 0.024 mmol S_8) was added. After stirring the suspension (with the addition of 1 mL more CH_3CN) for 2 hours, 2 M TEAB (*vide infra*) was added (50 μL, 0.10 mmol), and the volatiles were removed using a vacuum pump. The white and brown solid was taken up in chloroform and filtered, and then chromatographed on a 0.5 × 4 cm silica column using 10% CH_3OH in ethyl acetate with ~0.5% Et_3N added and then with 1:1 CH_3OH/ethyl acetate once the product began to elute. Fractions with $R_f \approx 0.15$ (4:1 ethyl acetate:CH_3OH with ~0.5% Et_3N) were combined. While the ^{31}P NMR only exhibited peaks due to (14), the ^{1}H NMR exhibited impurities near 0 ppm in particular, so the chromatography was repeated. The product was treated with 50 μL TEAB in CH_3CN, and the solvent was removed to give (14) as a white, crystalline solid (10.9 mg, 63% yield). The ^{31}P and ^{1}H NMR spectra matched those reported[31]; ^{31}P NMR data follow for the chromatography sample, where some fractionation occurred, so the ratios cannot be compared with other samples of (14), and the chemical shifts were found to be sensitive to purity: (CD_3CN) δ 56.555 (S_{PS}), 56.519 (R_{PS}), ~61:39, HPLC 55.13:44.87 S_{PS}:R_{PS}; (CDCl3) δ 57.41 (R_{PS}), 57.28 (S_{PS}), 44.5:55.5.

Preparation of 2 M Aqueous TEAB

The following was adapted from a literature method using CO_2 gas[26]: 56 mL of Et_3N was added to 144 mL of deionized H_2O, resulting in a two-layer solution. With vigorous stirring, pieces of dry ice (CO_2) were added to the solution until a homogenous solution of pH ~ 8 was attained, as determined with Alkacid test paper. The slightly yellow solution obtained was stored at room temperature.

Preparation of 0.1 M TEAA Buffer for HPLC Analysis

At room temperature, 13.9 mL of Et_3N was added to a vigorously stirred solution of 950 mL HPLC grade H_2O and 5.6 mL of acetic acid. After ~20 minutes, acetic acid was added dropwise to the solution until the pH was 7.0 as determined using an Accumet Basic pH meter (Fisher Scientific). The solution was stored in a sealed bottle and kept at room temperature and protected from ambient light.

Chiral Sulfurization Procedure

Typically up to eight reactions were run at once, in sets of four phosphite triesters and two enantiomers of a sulfurizing reagent, all in the inert atmosphere glove box under nitrogen. For each reaction, a ~0.05 M stock solution of (3), (4), or (8) in CH_3CN was prepared, and 100 μL of this solution [giving 0.0054–0.0060 mmol, ~5 mg each of (3), (4), or (8)] was used for each sulfurization. For (5), BSA was added to the solution of (8) directly (7 μL $\approx$ 5.3 equivalents), while for (6) and (7), CH_3CN solutions of TBDMSCl (100 μL of a 0.27 M solution, ~5.1 equivalents) or Ph_3SiCl (200 μL of a 0.14 M solution, ~5.3 equivalents) were added along with Et_3N (8 μL, ~10.7 equivalents). Each solution was then diluted with either 100 or 200 μL of CH_3CN as appropriate to give ~300 μL of solution (giving concentrations ranging from ~0.017 to 0.019 M in phosphite). After allowing the silylations to proceed at room temperature for 2 hours, the reactions were cooled in the glove box freezer at −32°C for ~20 minutes, and then the sulfurizing reagents (0.25 equivalents) were added as CH_3CN solutions [volumes ranged from 16 to 65 μL for 0.02–0.09 M solutions, ~0.0013–0.0015 mmol, ~0.3–0.8 mg of (1) or (2)]. The solutions were magnetically stirred in the glove box freezer by placing a stirrer below the freezer, for 1 hour, and kept at −32°C for a total of 20 hours. The solutions were then quenched by the addition of 10 μL of a ~3.3 M solution of anhydrous TBHP in toluene[32] in order to oxidize the unreacted phosphite.[26,33] For (4), deprotection with DBU (3.5 equivalents) over 45 minutes followed by the addition of 20 μL of 2 M TEAB gave the triethylammonium phosphorothioate salt (14). For (5)–(7), hydrolysis of the siloxy sulfide and oxide to phosphorothioate (14) and the phosphodiester was carried out by the addition of 10 μL of 2 M TEAB.

Analysis of samples was carried out by HPLC by the injection of 3 μL of solution into a Dionex Acclaim PolarAdvantage II C18 column (5 μm particle size, 120 Å pore size, 4.6 × 150 mm) for (9) and into a Dionex Acclaim 120 C8 column (5 μm, 120 Å, 4.6 × 250 mm) for (14). In all cases, reactions were analyzed at 30°C with a flow rate of 1.0 mL/minute and monitored at 254 nm, using 65:35 CH_3CN:H_2O for (9) and 55:45 or 52:48 CH_3CN:TEAA for (14). Elution times for (9) were 15.75 (R_{PS}) and 16.79 (S_{PS}) minutes, and for the phosphate triester diastereomers, 7.87 and 8.16 minutes; elution times and integrations gave virtually identical results for repeat injections. Elution times for (14) were 16.12 ± 0.09 (R_{PS}) and 20.28 ± 0.13 (S_{PS}) minutes, and 9.86 minutes for the phosphate diester for 55:45 CH_3CN:TEAA, and 23.98 ± 0.18 (R_{PS}) and 31.08 ± 0.28 (S_{PS}) minutes, and 13.81 minutes for the phosphate diester for 52:48 CH_3CN:TEAA, and integration reproducibility for these repeat injections was 0.2%; for repeated experiments (a total of 31 for 14 experiments), integration reproducibility was 0.9%. Peak homogeneity was assessed using a diode array detector capable of

monitoring the UV spectrum at 190–400 nm at any point on a selected peak, and periodic checks showed the integrated peaks to have identical UV spectra near the beginning, middle, and end of each peak.

Analysis of samples by ^{31}P NMR was carried out by preparation as described above for (5)–(7) and (9)–(13), but for (9)–(13), excess sulfur [(MEDITH for (10)] was used; HPLC ratios for (10)–(13) are reported for the hydrolysis product (14): (5): δ 127.76 (R_P), 127.67 (S_P), 55.9:44.1; (6): δ 127.87 (R_P), 127.31 (S_P), 56.3:43.8; (7): δ 128.34 (R_P), 126.89 (S_P), 55.7:44.3; (9): δ 67.04 (R_{PS}), 66.98 (S_{PS}), 50.3:49.7, HPLC 50.59:49.41 R_{PS}:S_{PS}; (10): δ 58.93 (R_{PS}), 58.58 (S_{PS}), 45.5:54.5, HPLC 45.66:54.34 R_{PS}:S_{PS}; (11): δ 55.38 (R_{PS}), 55.11 (S_{PS}), 44.4:55.6, HPLC 43.44:56.56 R_{PS}:S_{PS}; (12): δ 55.55 (S_{PS}), 55.49 (R_{PS}), 56.8:43.2, HPLC 56.62:43.38 S_{PS}:R_{PS}; (13): δ 55.16 (S_{PS}), 55.05 (R_{PS}), 55.4:44.6, HPLC 56.64:43.36 S_{PS}:R_{PS}.

CONCLUSION

For screening the chiral analogues of PADS and MEDITH, in addition to the dithymidine phosphite triesters prepared by the phosphoramidite method, phosphite triesters with different degrees of steric hindrance around the phosphorus were successfully prepared via the *H*-phosphonate method, using BSA, TBDMSCl/Et_3N, and Ph_3SiCl/Et_3N. Because silylation occurred at the H–P=O group and on the thymine bases, it was necessary to use $\geq$5 equivalents of the silylating reagents to give completely silylated phosphite triesters. After sulfurization, analysis of the R_{PS}:S_{PS} diastereomeric ratios of the resulting phosphite sulfides or phosphorothioates was successfully determined by reverse-phase HPLC; ^{31}P NMR did not give sufficient resolution of peaks or accuracy of peak integration to be useful.

A numerical procedure was developed to express the diastereoselectivity ($ds_{R(PS)}$ and $ds_{S(PS)}$) of the sulfurization reactions, and the de (de = $ds_{S(PS)}$ – $ds_{R(PS)}$) was used to compare selectivities of the reactions. The analogues of MEDITH were found to be generally more selective than those of PADS, despite the fact that in many cases, racemization of the MEDITH analogues occurred. A possible reason for the poor selectivity of the PADS analogues could be the known decomposition of PADS itself into a more active sulfurizing reagent that might consist of sulfur oligomers, when it is "aged" in 3-picoline solution.[41] If sulfur oligomers were the primary active reagent, then the chirality of the intact disulfides would have no effect. Fresh solutions of PADS are in fact known to sulfurize phosphites in a 1:1 stoichiometry,[41] so our use of fresh acetonitrile solutions of the PADS analogues and the observation of any selectivity for the chiral analogues suggest that we are in fact observing direct sulfurization by the intact disulfides.

Since the best actual selectivities were achieved with MEDITH analogues (*S*)-(2d) (the naproxen derivative of MEDITH) and (*S*)-(2e) (an ibuprofen

derivative of MEDITH), and the best difference in the diastereomer ratio was achieved with MEDITH analogue (*S*)-(2c), which contained an isopropyl group, a direction for future work will be chiral (and achiral) disulfides with larger groups adjacent to the disulfide. The approach of MEDITH toward the phosphite triester will necessarily be different from that of PADS, and these results suggest that it will be a better choice for selectivity. Development of a practical method for syntheses of P-stereogenic phosphorothioates will still require a means to epimerize the triester, but the use of the five triesters in this study still provides a viable route to screening of new disulfides for diastereoselectivity.

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