Novel Sulfoxides Facilitate GC-Rich Template Amplification

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ABSTRACT

Certain organic solvents, such as DMSO and betaine, have been reported to enhance PCR amplification, particularly for hard-to-amplify high-GC templates. As a result of extensive structure-activity studies between two groups of compounds—amides and sulfones—we have recently discovered several other potent PCR enhancers. Here we describe the effects of a series of different sulfoxides on GC-rich template amplification and report several of these to be exceptionally effective, often outperforming DMSO. We introduce them as novel PCR enhancers. We identify tetramethylene sulfoxide as the most potent sulfur-oxygen compound in the enhancement of PCR amplification and as one of the most potent PCR enhancers currently known.

INTRODUCTION

Since its inception in 1988 (13), PCR has become one of the central techniques in modern molecular biology. However, its application is often compromised by insufficient amplification of the target DNA sequence and the accompanying amplification of undesired nonspecific bands (9,12). These problems, especially low yield, can be particularly severe for templates with high GC contents (8,15). The addition of certain organic additives, such as DMSO, betaine, polyethylene glycol, glycerol, and formamide to the reaction mixture can often provide improvements in yield and specificity (1,10,14,16,17). Despite their general applicability, the performance of these compounds in the case of GC-rich targets is unpredictable; any given compound often fails to provide adequate improvement over the control (2).

Recently, we thoroughly investigated the effects of low molecular weight amides and low molecular weight sulfones on the amplification of GC-rich targets and found 2-pyrrolidone and sulfolane to be especially effective (3,4). Given the effectiveness of DMSO in PCR optimization, we decided to investigate several other water-soluble sulfoxides to determine whether these compounds are also effective. The study incorporated three GC-rich templates and examined the following compounds: DMSO, methyl *sec*-butyl sulfoxide, *n*-propyl sulfoxide, *n*-butyl sulfoxide, and tetramethylene sulfoxide.

Here we identify a few of these compounds as effective enhancers of high-GC template amplification, compare their effectiveness to those of the related sulfones, and consider structure-activity correlations in the enhancement of PCR by sulfur-oxygen compounds. We introduce tetramethylene sulfoxide as an exceptionally potent PCR enhancer.

MATERIALS AND METHODS

PCR was conducted as previously described (4) on a RoboCycler® Gradient 96 thermal cycler (Stratagene, La Jolla, CA, USA) using 50- μ L solutions in 200- μ L thin-walled tubes. Before the introduction of *Taq* DNA polymerase, a hotstart protocol was followed that consisted of an initial cycle at 95°C for 5 min (6) to ensure complete first-strand separation, followed by a cycle at 54°C for 5 min. Amplifications were run for 30 cycles under the following conditions: 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 0.2 μ M primers, 0.06 ng/ μ L template, 0.2 mM each dNTP, 0.04 U/ μ L *Taq* DNA polymerase. To facilitate the comparison with the related sulfone additives, the templates that we studied were identical to those previously described (4): bovine brain glycolipid transfer protein (GTP) cDNA (660 bp), a 996-bp segment of human myeloid leukocyte *c-jun* cDNA, and a 511-bp segment of human prostate-specific membrane (PSM) antigen cDNA.

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The sequences of the primers were as follows:

GTP primer g1: d(GAATTCGAAATGGCGCTGCTGG);

primer g2: d(CTCGAGGTCCA-GAGTACCCGCTGTG);

c-jun primer j1: d(ATGACTGCAAA-GATGGAAACG);

primer j2: d(TCAAAATGTTT-CAACTGCTGCG);

PSM primer p1: d(AAACACTGCTG-TGGTGGGA);

primer p2: d(TAGCTCAACAG-AATCCAGGC).

Primer T_m s were calculated using an oligonucleotide calculator program (Sigma-Genosys, The Woodlands, TX, USA). Calculated T_m s of the primers were as follows: g1, 73.3°C; g2, 74.4°C; j1, 63.9°C; j2, 70.8°C; p1, 60.3°C; and p2, 61.9°C.

Denaturation for PSM and GTP was conducted for 1 min at 92°C, the minimum temperature to afford discernable amplification of these targets in the presence of additives, and at either 92°C or 95°C for *c-jun*. Extension was carried out at 72°C for 1 min. Annealing was done either at fixed concentrations of additives using a temperature gradient of 48°C–62°C or at varying concentrations of additives using fixed temperatures. These temperatures were 50°C, 58°C, or 60°C (depending on the additive) for GTP, 48°C or 52°C (depending on the additive) for PSM, and 50°C for *c-jun*.

The *Taq* DNA polymerase and dNTPs were acquired from Stratagene, and the primers were obtained from Sigma-Genosys. Additive compounds were acquired from the following vendors: *n*-propyl sulfoxide, *n*-butyl sulfoxide, and tetramethylene sulfoxide were obtained from Acros Organics (Morris Plains, NJ, USA), and methyl *sec*-butyl sulfoxide was obtained from Lancaster Synthesis (Clariant). DMSO was purchased from Fluka (Buchs, Switzerland).

The analysis of the amplification products was carried out on 0.8% agarose gels. Before casting, ethidium bromide was added to the gels at a concentration of 5 µg/mL. The quantitation of the products was conducted using an ImageScanner™ densitometer and ImageMaster™ Total Lab software from Amersham Biosciences (Piscataway, NJ, USA).

RESULTS

Figure 1 displays the structures of the sulfoxides that were studied in this investigation. All known water-soluble symmetrical *n*-alkyl sulfoxides were tested, with the exception of ethyl sulfoxide, which was not readily available from known chemical sources. Instead,

we used methyl *sec*-butyl sulfoxide, which has a total number of carbons between those of ethyl and propyl sulfoxides. This was the only asymmetrical sulfoxide studied in this work. Tetramethylene sulfoxide, a simple cyclic sulfoxide, was also included in the study to examine the effects of the ring structure on amplification.

Figure 1. Structures of the additives tested.

Figure 2. Enhancement of GTP amplification by additives. Target band volume is normalized to the potency of DMSO. Interpolation was carried out by applying cubic splines to the data (11).

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In the case of each of the templates studied, the effects of the various additives on PCR amplification were assessed through the assignment of two densitometric quantities to each of the compounds. The first quantity, termed potency, is defined as the maximum densitometric volume of the target band observed at any concentration of an additive. The second quantity, specificity, is defined as the ratio of target band volume to the total volume of all bands, including nonspecific bands, expressed as a percent. In addition to potency and specificity, a third descriptive value, the effective range, was associated with each compound for each target. The effective range of an additive is defined as the range of concentrations over which the volume of target band amplification is greater than or equal to 50% of its maximal value.

Three high-GC templates were used in this study to determine the effects of sulfoxide additives on PCR amplification. Of these, bovine brain GTP cDNA (660 bp; 58% GC), was chosen as the primary target. The various additives were first tested with GTP at a few disparate concentrations over an annealing temperature gradient of 48°C–62°C,

Table 1. Potency^a, Specificity^b, and Effective Range^c of Additives (GTP)

Additive	Potency	Specificity (%)	Effective range (M)
Tetramethylene sulfoxide	3.68	100	0.28–1.00
Propyl sulfoxide	2.82	100	0.15–0.36
Methyl <i>sec</i> -butyl sulfoxide	3.33	100	0.03–0.31
Butyl sulfoxide	0.06	53	NA
DMSO	1.00	89	0.75–1.25
Control	0.06	53	NA

NA, not applicable.
^aNormalized maximum densitometric volume of target band: DMSO = 1.
^bBest specificity (densitometric volume of target as percent of total volume) over the effective range.
^cConcentration interval over which densitometric volume of target is at least 50% of the maximum volume.

employing a 92°C denaturing temperature. Butyl sulfoxide did not yield any detectable amplification and was not included in subsequent studies. The remaining additives were then tested over molar concentration gradients at their optimal annealing temperatures (60°C

for tetramethylene sulfoxide and propyl sulfoxide, 58°C for methyl *sec*-butyl sulfoxide, and 50°C for DMSO). The concentrations were selected partly on the basis of which concentrations were effective in the temperature gradient experiments. These concentrations

Figure 3. Enhancement of PCR amplification of bovine brain glycolipid transfer protein cDNA (660 bp) by sulfoxides. PCR products were analyzed using 0.8% agarose gel electrophoresis. Lane 1, 0.7 M tetramethylene sulfoxide; lane 2, 0.3 M propyl sulfoxide; lane 3, 0.1 M methyl *sec*-butyl sulfoxide; lane 4, DMSO; lane 5, control (no additive); lane 6, 100-bp DNA ladder (Invitrogen, Carlsbad, CA, USA).

Figure 4. Enhancement of PSM amplification by tetramethylene sulfoxide and DMSO. Target band volume is normalized to the potency of DMSO. Interpolation was carried out by fitting cubic splines to the data (11).

Table 2. Potency and Effective Range of Select Additives (*c-jun*)

Additive	92°C	95°C	Effective Range ^c (M)
	Denaturing Potency ^a	Denaturing Potency ^b	
Tetramethylene sulfoxide	1.00	1.18	0.19–0.55
Propyl sulfoxide	0.00	0.25	0.08–0.22
DMSO	0.00	1.00	0.36–1.53
Control	0.00	0.00	NA

^aNormalized maximum densitometric volume of target band: tetramethylene sulfoxide = 1.
^bNormalized maximum densitometric volume of target band: DMSO = 1.
^cConcentration interval over which densitometric volume of target is at least 50% of the maximum volume.

Table 3. Potency^a, Specificity^b, and Effective Range^c of Select Additives (PSM)

Additive	Potency	Specificity	Effective Range (M)
		(%)	
Tetramethylene sulfoxide	1.74	93%	0.33–0.68
Propyl sulfoxide	0.21	34%	NA
DMSO	1.00	88%	0.73–1.35
Control	0.21	34%	NA

^aNormalized maximum densitometric volume of target band: DMSO = 1.
^bBest specificity (densitometric volume of target as percent of total volume) over the effective range.
^cConcentration interval over which densitometric volume of target is at least 50% of maximum volume.

were: DMSO, 0.2, 0.3, 0.5, 0.7, 0.9, 1.2, 1.4 M; methyl *sec*-butyl sulfoxide, 0.1, 0.2, 0.25, 0.3, 0.35, 0.4 M; propyl sulfoxide, 0.1, 0.2, 0.3, 0.4 M; and tetramethylene sulfoxide, 0.1, 0.2, 0.3, 0.5, 0.7, 0.9, 1.2 M. Table 1 shows the potencies, best specificities, and effective ranges displayed by the additives in the GTP studies. Variation of GTP amplification with concentration is displayed graphically for each additive in Figure 2. Figure 3 shows the best results obtained with each compound in the case of this template.

The symmetrical sulfoxide series—DMSO, propyl sulfoxide, and tetramethylene sulfoxide—was then tested with two additional GC-rich targets: a 996-bp region of human myeloid leukocyte *c-jun* cDNA (64% GC) and a 511-bp segment of human PSM cDNA (52% GC with a 158-bp 73% GC region). The asymmetrical compound methyl *sec*-butyl sulfoxide was not test-

ed with these targets. In the absence of additives, the amplification of *c-jun* is impossible; amplification of PSM is weak and accompanied by the amplification of nonspecific bands (3).

As described earlier, the compounds were first tested with these templates at a few different concentrations over a 48°C–62°C annealing temperature gradient, using a conventional 92°C denaturing temperature. In the case of *c-jun*, only tetramethylene sulfoxide gave discernable amplification at this denaturing temperature. To study the abilities of the other compounds to enhance *c-jun* amplification under less stringent conditions, the *c-jun* screenings were repeated by employing a 95°C denaturing temperature, and all three additives were found to be effective. With PSM, both DMSO and tetramethylene sulfoxide were effective in enhancing amplification under 92°C denaturing conditions. Using a denaturing temperature of 92°C

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for *c-jun* and 95°C for PSM, the effective additives were then tested at various concentrations at their best annealing temperatures, 50°C for all additives with *c-jun* and 52°C and 48°C for tetramethylene sulfoxide and DMSO, respectively, with PSM. Tables 2 and 3 list the potencies, best specificities, and effective ranges of the additives for *c-jun* and PSM. The variation of potency with respect to additive concentration is plotted for PSM in Figure 4.

DISCUSSION

This investigation had two primary objectives. First, we sought to identify novel enhancers of high-GC target amplification that have the potential to function as well as or better than the current state-of-the-art technology. Second, we were interested in studying structure-activity correlations in PCR enhancement by the class of low molecular weight sulfoxides. The potency, specificity, and effective range data collected in Tables 1, 2, and 3 provide the basis for a detailed examination of both of these issues.

Consideration of the potencies of the additives tested with GTP (Table 1) reveals that in the case of this template, tetramethylene sulfoxide is the most effective additive. Methyl *sec*-butyl sulfoxide is somewhat less potent, followed by propyl sulfoxide. Except for butyl sulfoxide, which failed to function, DMSO displays the lowest level of potency for this particular template. At the concentrations at which tetramethylene sulfoxide, propyl sulfoxide, and methyl *sec*-butyl sulfoxide produce their highest yields, they also exhibit perfect specificity. This is not the case with DMSO, which exhibits only 89% specificity at its most effective concentration.

Tetramethylene sulfoxide performs best with *c-jun* and PSM as well (Tables 2 and 3). In the case of *c-jun*, it performs somewhat better than DMSO and considerably better than propyl sulfoxide at a 95°C denaturing temperature; at a 92°C denaturing temperature, it is the sole effective additive. It is also the most potent and specific additive in the case of PSM. DMSO ranks second in order of potency and specificity for these targets, followed by propyl sulfoxide.

Table 4. Representative Results from Sulfoxide and Sulfone PCR Enhancement Studies

Additive	I. GTP		II. <i>c-jun</i> 95°C	III. <i>c-jun</i> 92°C
	Potency ^a	Range ^b (M)	Denaturing Potency ^a	Denaturing Potency ^c
DMSO	1.00	0.75–1.25	1.00	0.00
Methyl sulfone ^d	1.06	0.65–1.20	0.85	0.00
Methyl <i>sec</i> -butyl sulfoxide	3.33	0.03–0.31	NA	0.00
Ethyl sulfone ^d	NA	NA	0.20	0.00
Propyl sulfoxide	2.82	0.15–0.36	0.25	0.00
Propyl sulfone ^d	NA	NA	0.00	0.00
Butyl sulfoxide	0.00	NA	0.00	0.00
Tetramethylene sulfoxide	3.68	0.28–1.00	1.18	3.02
Sulfolane ^d	3.30	0.18–0.55	1.10	1.00
Betaine ^d	NA	NA	0.52	0.00

^aNormalized maximum densitometric volume of target band: DMSO = 1.
^bConcentration interval over which densitometric volume of target is at least 50% of maximum volume.
^cNormalized maximum densitometric volume of target band: sulfolane = 1.
^dData taken from Reference 4.

Based on the collective results of the GTP, *c-jun*, and PSM studies, we identify tetramethylene sulfoxide as the most effective sulfoxide in the enhancement of GC-rich template amplification.

In recent work, we studied the effects of low molecular weight sulfones on the PCR amplification of GC-rich targets (4) using the same templates examined here. The current work on sulfoxides completes the analysis of the effects of sulfur-oxygen compounds on GC-rich template amplification. Table 4 displays the representative results from the two studies. As can be seen, while methyl sulfone is more potent than DMSO, the higher sulfoxides are more potent PCR enhancers than the corresponding sulfones. The commonly used GC-rich PCR enhancer, betaine, is included for the sake of comparison.

It is known that many organic solvents that enhance PCR amplification can also unwind the DNA double helix (7). Several investigators have accordingly proposed that organic additives such as DMSO enhance PCR by hydrogen bonding to the major and minor grooves of the template DNA and destabilizing the double helix (5,15). Our

discovery that tetramethylene sulfoxide is the most effective low molecular weight sulfoxide in the enhancement of PCR bears a strong resemblance to our recent findings that 2-pyrrolidone and tetramethylene sulfone (sulfolane) are the most effective low molecular weight amide and sulfone enhancers, respectively, of PCR (3,4). In those cases, we proposed that the particular effectiveness of the cyclic compounds may be because the ring structure constrains the molecule in a conformation that is optimal for complementary hydrogen bonding with donor and acceptor moieties in the major and minor grooves and perhaps also minimizes steric repulsion with neighboring groups on the DNA backbone by restricting free rotation. Similar effects may also be responsible for the outstanding performance of tetramethylene sulfoxide.

The extra H-bond in GC compared to AT base pairs faces the minor groove. The significant geometric constraints of the minor groove might make it difficult for longer straight-chain molecules to bond there. However, the cyclic structure of tetramethylene sulfoxide and sulfolane may fit

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perfectly into the minor groove architecture and remain there stably because of van der Waals interactions with neighboring groups on the DNA backbone. This would be expected to position the oxygen molecules on the compounds in orientations that are highly conducive to effective hydrogen bonding. This model predicts that, as the template GC content increases, the effectiveness of the additives that are incapable of hydrogen bonding in the minor grooves should decrease. Indeed, as described earlier, propyl sulfoxide is completely ineffective with PSM, the most GC-rich template used (73% GC 158-bp region), and is only marginally effective with *c-jun* (64% GC). However, Table 4, section I, shows that both propyl and methyl *sec*-butyl sulfoxides are more effective than the smaller additives DMSO and methyl sulfone for the lower GC content template GTP (58% GC). The higher acyclic sulfones and sulfoxides may destabilize AT base pairs as well as, if not better than,

DMSO and methyl sulfone because the H-bond donors in AT base pairs all face the major groove, where the higher sulfones and sulfoxides may engage in efficient hydrophobic packing. Of course, detailed comprehension of the manner in which these additives exert their differential effects in PCR amplification will require further structural and mechanistic studies.

In conclusion, we have shown that a variety of low molecular weight sulfoxides are effective in enhancing the amplification of GC-rich templates. Propyl sulfoxide and methyl *sec*-butyl sulfoxide were shown to be very effective with moderately high GC content targets. Tetramethylene sulfoxide was identified as the most effective sulfur-oxygen compound in the enhancement of PCR, displaying greater potency than other PCR enhancers for all the targets studied.

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