Synthesis, Characterization, Mechanism of Decomposition, and Antiproliferative Activity of a Class of PEGylated Benzopolysulfanes Structurally Similar to the Natural Product Varacin

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Benzopolysulfanes, 4-CH₂(OCH₂CH₂)₃NHC(O)-C₆H₄-1,2-Sₓ (x = 3–7 and 9) were synthesized with a PEG group attached through an amide bond and examined for water solubility, antitumor activity, and propensity to equilibrate and desulfurate. LCMS and HPLC data show the PEG pentasulfane ring structure predominates, and the tri-, tetra-, hexa-, hepta-, and nonasulfanes were present at very low concentrations. The presence of the PEG group improved water solubility by 50-fold compared to the unsubstituted benzopolysulfanes, C₆H₄Sₓ (x = 3, 5, and 7), based on intrinsic solubility measurements. Polysulfur linkages in the PEG compounds decomposed in the presence of ethanethiol and hydroxide ion. The PEG pentathiepin desulfurated rapidly, and an S₃ transfer reaction was observed in the presence of norbornene; no S₂ transfer reaction was observed with 2,3-dimethylbutadiene. The antitumor activities of the PEG-substituted benzopolysulfane mixtures were analyzed against four human tumor cell lines PC3 (prostate), DU145 (prostate), MDA-MB-231 (breast), and Jurkat (T-cell leukemia). The PEG-conjugated polysulfanes had IC₅₀ values 1.2–5.8 times lower than the parent “unsubstituted” benzopolysulfanes. Complete cell killing was observed for the PEG polysulfanes at 4 μM for PC3 and DU145 cells and at 12 μM for MDA-MB-231 cells. The results suggest that solubilization of the polysulfur linkage is a key parameter to the success of these compounds as drug leads.

Introduction

Tunicates or their associated microorganisms produce benzopolysulfanes, such as varacin (1), lissoclinotoxin A (2), and N-dimethyl-5-(methylthio)varacin (3) (Scheme 1).1–6 Unnatural benzopolysulfanes have also been synthesized, e.g., 6-(2-aminooethy]l)benzopentathiepin (4)7 and the parent benzopentathiepin (5B)8,9 (alphabetical labels will be given to some polysulfanes, as will be elaborated on below). Benzopolysulfanes represent an attractive target but are an understudied class of
SCHEME 1. Natural and Unnatural Benzopolysulfanes

SCHEME 2. Equilibration Between Tri-, Penta-, and Heptasulfanes

TABLE 1. GC–MS Detection of Parent Benzopolysulfanes 5A–C

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of S Atoms</th>
<th>MS tR (min)</th>
<th>Molecular Weights</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-C6H4S3 5A</td>
<td>3</td>
<td>14.2</td>
<td>172</td>
</tr>
<tr>
<td>o-C6H4S5 5B</td>
<td>5</td>
<td>26.5</td>
<td>236</td>
</tr>
<tr>
<td>o-C6H4S7 5C</td>
<td>7</td>
<td>39.1</td>
<td>300</td>
</tr>
</tbody>
</table>

"Reference 12. See also ref 13. 12 GC–MS retention time. 13 Low-resolution GC–MS. Support for the GC–MS peak assignments came from spectroscopic comparisons of 5A–C, which were independently synthesized and examined shortly after their purification, i.e., before equilibration was pronounced, which took 1–3 days.

Compounds for antitumor drug discovery. High nanomolar and low micromolar antiproliferative IC50 values have been reported for benzopolysulfanes. 1–7 It was suggested that the bioactivity of varacin derives from DNA damage because of an observed difference in toxicity toward the CHO cell line EM9 (chlorodeoxyuridine sensitive) compared to BR1. 1

Benzopolysulfanes have not been studied widely in the context of drug discovery because of the instability of the polysulfur ring. Even determining the number of sulfur atoms in o-benzopolysulfanes has not been a trivial task. 11–13 MS fragmentation patterns can be difficult to interpret; for example, the structure of the natural product lissoclinotoxin A was assigned first as a trisulfane 14 and later revised as a pentasulfane. 11 In 2007, the synthesis and purification of parent o-C6H4S3 5B revealed an equilibration involving elemental sulfur, S0 (Scheme 2). 12 A facile equilibration took place between the pentasulfane and the tri- and heptasulfanes (o-C6H4S5 and o-C6H4S7); the ratio 5A:B:C was 49:45:6 in CH2Cl2 over 1–3 days. In 5, the labels A, B, and C correspond to compounds with 3, 5, and 7 sulfur atoms, respectively. Analysis of GC–MS retention times revealed that 5A and 5B differed by 12.3 min, and the retention times of 5B and 5C differed by 12.6 min (Table 1).

Because natural benzopolysulfanes are in short supply and their stability is difficult to assess, we synthesized benzopolysulfanes with a short-chain PEG, in which the benzene ring and PEG group replaced the naturally occurring dopamine core 1–3. The aims of the present work were to determine (1) whether benzopolysulfanes could be synthesized with a PEG side group, (2) whether the pentasulfur species predominates, (3) whether the polysulfur linkage(s) are unstable to medium effects, (4) whether the PEGylated benzopolysulfanes decompose at different rates and transfer sulfur to norbornene and butadiene traps, (5) the extent the PEG group enhances water solubility, (6) whether the polysulfur ring is essential for bioactivity, and (7) whether enhanced benzopolysulfane water solubility is correlated with an enhanced pharmacological activity against human tumor cells. We synthesized a mixture of PEG-benzopoly- sulfane conjugates 4-(CH2OCH2CH2)2NHC(O)-C6H4-1,2-Sx (x = 3–7 and 9) 6A–F and explored their stability and activity in a variety of tumor cell lines [PC3 (prostate), DU145 (prostate), MDA-MB-231 (breast), and Jurkat (T-cell leukemia)]. In 6, the labels A–E and F correspond to compounds with 3–7 and 9 sulfur atoms, respectively. The octasulfane species was not detected.

Results and Discussion

Synthesis and Characterization. Pentasulfane 6C was synthesized as the major constituent of a mixture of polysulfanes 4-(CH2OCH2CH2)2NHC(O)-C6H4-1,2-Sx (x = 3–7 and 9) in 8 steps and 1.5% overall yield. A procedure developed by Lénard et al. 15 was used for the conversion of 3,4-dihydroxybenzoic acid (7) to 3,4-disulfuranylbenezic acid (11) (steps i–iv, Scheme 3). Dithiastannole-5-carboxylate anion 12 was generated under basic conditions by the reaction of dimethyltin chloride with 11 using a modified procedure by Sato et al. 15 Stannole 12 reacted with p-nitrophenol, DCC, and DMAP to yield 4-nitrophenyl ester stannole 13 in 72% yield. Stannole 13 reacted with disulfur dichloride giving 4-nitrophenyl ester benzopentasulfane (14) in 27% yield. Amino-terminated poly(ethylene glycol) (15) was prepared in 2 steps by the method of Dombi et al. 16 and PEGylated to benzopentasulfane 14 at the 7-position of the benzene ring. It is possible that other cyclic polysulfanes related to benzopentasulfane 14 were formed in ∼1–10% yields, but this was not determined. The resulting mixture was purified by column chromatography to afford 6C in 93% purity. In the 1H NMR, 13C NMR, COSY, HMBC, and HSCQ spectra, the benzene and PEG portions of the structure were confirmed (Supporting Information). For example, in COSY NMR, a strong J correlation was found between C8 = H and C8 = H, and between N = H and C13 = H, and a weak J-W correlation found between C5 = H and C6 = H (Scheme 4). The HMBC and HSCQ NMR data further bolstered the structural assignment of the
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SCHEME 3. Synthesis of PEG Conjugated Benzopentasulfane 6C

![Scheme 3](image)

"Reagents and conditions: (i) HCl (cat.), MeOH, reflux 80 °C, 7 h; (ii) Me₂NC(=S)Cl, DABCO, DMF, rt, 30 min; (iii) Pb₃O, 230 °C, 40 min; (iv) (a) aqueous NaOH, under N₂, 70 °C, 4 h; (b) 1 N HCl; (v) (a) Me₂SnCl₂, KOH, EtOH, water, (b) 1 N HCl; (vii) p-nitrophenol, DCC, DMAP, CH₂Cl₂, 1 d; (viii) H₂N-(CH₂CH₂O)₃-CH₃ THF, rt, 12 h.

SCHEME 4. COSY NMR Couplings of Benzopentasulfane 6C

![Scheme 4](image)

SCHEME 5. Equilibration of Pentasulfane 6C with Minor Amounts of Tri-, Tetra-, Hexa-, Hepta-, and Nonasulfanes in Methanol/Water (1:1 v/v)

![Scheme 5](image)

nonsulfur portion of 6C. LC/MS data indicated that 6C contained five sulfur atoms (HRMS calced for C₁₄H₁₉NO₄S₅ = 424.9918, found 424.9926).

Lability of the Pentasulfur Ring. Polysulfanes are challenging structures to study because of their instability. Few reports on benzopolysulfanes describe the distribution of the polysulfanes or their equilibration and often incorrectly assume the pentathiepin to be the sole compound present. We found that over a 12–24 h period 6C equilibrates S₈ and forms low concentrations of structurally related polysulfanes in aqueous methanol at room temperature (Scheme 5). Table 2 lists the polysulfane masses and retention times to compare the number of sulfur atoms in the compounds. The LCMS spectra of polysulfanes 6A–F contained peaks spaced by ~0.7 min per additional sulfur atom (Figure 1). For example, a minor amount of a sulfur-rich compound was assigned as nonasulfane 6F (HRMS calcd for C₁₄H₁₉NO₄S₉ = 552.8800, found 552.8788). The ratio 6A:B:C:D:E:F was 1.5:1.5:93:0.1:0.3:0.1 by HPLC (methanol/water), and there was ~2% uncharacterized material and ~1.5% of elemental sulfur. The moderate solubility of the elemental sulfur in methanol/water suggested it to be a residue or colloid particles of the orthorhombic cyclo-S₈ form, but neither the monoclinic S₈ ring form (usually found at ~95°C) nor the polymeric, oligomeric, or amorphous forms (insoluble materials) was observed.

<table>
<thead>
<tr>
<th>compound</th>
<th>formula</th>
<th>tᵣa (min)</th>
<th>calculated mass</th>
<th>experimental mass</th>
<th>error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A</td>
<td>C₁₄H₁₉NO₄S₃</td>
<td>4.6</td>
<td>361.0476</td>
<td>361.0478</td>
<td>0.62</td>
</tr>
<tr>
<td>6B</td>
<td>C₁₄H₁₉NO₄S₄</td>
<td>5.6</td>
<td>393.0197</td>
<td>393.0193</td>
<td>1.00</td>
</tr>
<tr>
<td>6C</td>
<td>C₁₄H₁₉NO₄S₅</td>
<td>6.5</td>
<td>424.9918</td>
<td>424.9926</td>
<td>0.99</td>
</tr>
<tr>
<td>6D</td>
<td>C₁₄H₁₉NO₄S₆</td>
<td>7.1</td>
<td>456.9636</td>
<td>456.9636</td>
<td>0.60</td>
</tr>
<tr>
<td>6E</td>
<td>C₁₄H₁₉NO₄S₇</td>
<td>7.8</td>
<td>488.9359</td>
<td>488.9354</td>
<td>1.08</td>
</tr>
<tr>
<td>6F</td>
<td>C₁₄H₁₉NO₄S₉</td>
<td>9.2</td>
<td>552.8800</td>
<td>552.8787</td>
<td>2.34</td>
</tr>
</tbody>
</table>

*aLCMS retention time. Chromatography was performed on a SB-C18 3.5 μm column using water containing 0.1% formic acid and 5 mM ammonium formate (solvent A) and methanol containing 0.1% formic acid and 5 mM ammonium formate (solvent B) at a flow rate 0.5 mL/min. The gradient program was as follows: 15–85% B (0–13 min), 85% B (13–15 min), 85–15% B (1 min). The experimental exact mass is calculated by the subtraction of a proton (H⁺; 1.00728 Da) from the measured m/z value of the [M + H⁺] ion for the molecular formula of interest.

![Table 2](image)


In the even-numbered cases, 13 it may be noted that Nakayama actions, whereas eclipsing lone-pair electron interactions occurred transitively stable with gauche adjacent lone-pair electron interactions. When eluting at 85:15 methanol/water, the ratio $6A:B:C:D:E:F:S_8$ was 5.3:5.7:7.9:0.3:4.1:2.0:1.5:3 (Method 5, Table 3). The HPLC measurements do not ensure that thermodynamic conditions have been reached. Equilibrium at a new condition is established in 1–3 d in CH$_2$Cl$_2$ and probably much longer in methanol/water. Our results are similar to observations of 7-methylbenzopolysulfane equilibrations enhanced in polar solvents with higher solvation compared to nonpolar solvents. 22,23 However, the preference for odd-membered polysulfanes and elemental sulfur $S_8$ also involves equilibration with $S_6$ and $S_7$, which was investigated next.

**Influence of Solvent on Equilibration of Polysulfanes $6A$–F.** To better understand the equilibration between $6A$–F and $S_8$, the HPLC elution methods were modified. When eluting at 1:1 methanol/water, the ratio $6A:B:C:D:E:F:S_8$ was 2.2:1.8:93.5:0.4:0.3:0.2:1.6 (Method 1, Table 3).

Increasing the methanol concentration in water produces more pronounced polysulfane equilibration. When eluting at 85:15 methanol/water, the ratio $6A:B:C:D:E:F:S_8$ was 5.3:5.7:7.9:0.3:4.1:2.0:1.5:3 (Method 5, Table 3). The HPLC measurements do not ensure that thermodynamic conditions have been reached. Equilibrium at a new condition is established in 1–3 d in CH$_2$Cl$_2$ and probably much longer in methanol/water. Our results are similar to observations of 7-methylbenzopolysulfane equilibrations enhanced in polar solvents with higher solvation compared to nonpolar solvents. 22,23 Elemental $S_8$ also involves equilibration with $S_6$ and $S_7$, which was more pronounced in polar than nonpolar solvents, 24 and sulfur-reactive solvents such as pyridine or other amines that dissolve elemental sulfur readily. 25 The enhanced equilibration in methanol compared to that in methanol/water is likely a result of the increase in the solubility of $S_8$, which is pivotal to the reversible addition reactions leading to the ring compounds $6A$–F (Figure 2).

**Desulfuration of $6A$–F in the Presence of Nucleophiles and Trapping Agents.** The PEGylated benzopolysulfanes $6A$–F

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**TABLE 3. Polysulfane Distribution as a Function of HPLC Solvent Elution Condition**

<table>
<thead>
<tr>
<th>Method</th>
<th>MeOH/H$_2$O ratio (v/v)</th>
<th>$6A$</th>
<th>$6B$</th>
<th>$6C$</th>
<th>$6D$</th>
<th>$6E$</th>
<th>$6F$</th>
<th>$S_8$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50/50</td>
<td>2.2</td>
<td>1.8</td>
<td>93.5</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>60/40</td>
<td>2.5</td>
<td>2.1</td>
<td>94.7</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>75/25</td>
<td>4.3</td>
<td>3.2</td>
<td>84.6</td>
<td>1.5</td>
<td>0.9</td>
<td>0.5</td>
<td>3.4</td>
</tr>
<tr>
<td>4</td>
<td>80/20</td>
<td>4.7</td>
<td>4.8</td>
<td>83.6</td>
<td>1.0</td>
<td>0.3</td>
<td>0.4</td>
<td>5.1</td>
</tr>
<tr>
<td>5</td>
<td>85/15</td>
<td>5.3</td>
<td>5.7</td>
<td>79.0</td>
<td>3.4</td>
<td>1.2</td>
<td>0.1</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*HPLC analysis at 254 nm at room temperature with a flow rate of 1 mL/min in water/methanol mixtures. HPLC methods: Method 1 consisted of a gradient of methanol from 10% to 90% over 53 min and maintained for 2 min before reverting to 15% methanol over 1 min. Method 2 consisted of a gradient of methanol from 60% to 95% over 30 min, which was maintained for 10 min before reverting to 15% methanol over 5 min. Method 3 consisted of a gradient of methanol from 60% to 95% over 30 min, which was maintained for 15 min before reverting to 15% methanol over 3 min. Method 4 consisted of a gradient of methanol from 10% to 90% over 53 min and maintained for 5 min before reverting to 15% methanol over 1 min. Method 5 consisted of a gradient of methanol from 60% to 95% over 30 min, which was maintained for 15 min before reverting to 15% methanol over 2 min. Method 6 consisted of water (0.1% formic acid and 5 mM ammonium formate in water) and methanol (0.1% formic acid and 5 mM ammonium formate in MeOH) with a gradient of methanol from 15% to 85% over 13 min, which was maintained for 2 min before reverting to 15% methanol over 1 min.

TABLE 4. Decomposition of PEGylated Benzopolysulfanes 6A–F as a Function of Hydroxide Ion Equivalents Added

<table>
<thead>
<tr>
<th>hydroxide ion equiv</th>
<th>internal standard</th>
<th>polysulfanes and elemental sulfur distributiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>100</td>
<td>6A 6B + X' 6C 6D 6E 6F S8</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>13.2 19.6 289.2 2.1 2.8 3.7 23.1</td>
</tr>
<tr>
<td>0.75</td>
<td>100</td>
<td>14.2 38.1 222.9 1.8 4.7 3.8 26.7</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>14.2 64.9 163.9 1.5 1.6 3.1 32.4</td>
</tr>
<tr>
<td>1.0/2</td>
<td>100</td>
<td>14.9 85.5 113.6 0.7 2.2 3.1</td>
</tr>
</tbody>
</table>

aReaction of benzopolysulfanes 6A–F (2.5 mM) and hydroxide ion in the presence of internal standard acetamide (0.25 mM) in methanol/water (99:9, v/v). Poly sulfane ratios were determined by HPLC monitoring at 254 nm at room temperature with the flow rate 1 mL/min using method 4 (Table 3). HPLC analysis was carried out after 1 h of equilibration between benzopolysulfanes 6A–F and hydroxide ion. X is an unidentified compound that was eluted with 6B. bAnalysis was done after 24 h.

**SCHEME 6.** PEGylated Benzopolysulfanes 6A–F as Sulfur-Transfer Reagents to Norbornene but Not to 2,3-Dimethylbutadiene

Decomposed in the presence of NaOH or ethanethiol (Table 4), which led to an increase in elemental S8 and uncharacterized products, such as oligomers and/or polymers of desulfurated PEGylated benzopolysulfanes. The reaction of hydroxide ion with benzopolysulfanes 6A–F was comparatively slower than that with ethanethiol and could be monitored by HPLC. As can be seen in Table 4, the decomposition rate of pentasulfane 6C was rapid, whereas the concentrations of the other benzopolysulfanes were barely reduced. Determining whether the concentration of tetrathiolane 6B increased or decreased was not possible because it eluted along with an unidentified compound (X in Table 4), and the two peaks could not be deconvoluted. The data suggested that pentasulfane 6C desulfurated more quickly compared to other polysulfanes, which led us to trapping studies to analyze the sulfur-transfer reaction with butadiene and norbornene traps.

The reaction of benzopolysulfanes 6A–F with ethanethiol and norbornene (16) led to the formation of norbornenethithiocarbonate (17) and desulfurated or polymerized PEGylated benzopolysulfanes (Scheme 6). Unlike 6C, the concentrations of 6A, 6B, and 6D–F did not change significantly over the course of the trapping experiment. Thus, we propose that 6C is the most reactive polysulfane and responsible for the S2-transfer to norbornene. Interestingly, the decomposition of benzopolysulfanes 6A–F did not show an S2 transfer reaction. The sulfuration of 2,3-dimethylbutadiene (18) by 6A–F with ethanethiol did not yield the disulfide product 19. A possible mechanism for the desulfuration of pentathiepin 6C is shown in Scheme 7, which begins with an apical attack of ethanethiolate ion to the sulfur atom (S1) adjoining the aryl ring. A previous DFT study showed that attack of the HS− nucleophile at the S1 position of a pentathiepin was preferred to S2.24 The resulting open-chain polysulfide anion (20) has the potential for thiozone (S3) elimination driven by the delocalization of the negative charge in the remaining carbon–sulfur fragment (21)24,25 followed by thiozation of norbornene. Ab initio calculations predict the open C2S, zwitterionic form26 of thiozone S3 to be energetically preferred to the cyclic D3S form.27,28

**Intrinsic Solubilities.** Elemental S8 suffers from poor solubility.19,29,30 We investigated the intrinsic solubilities of elemental S8, 5A−C, and 6A−F (Table 5). Aliquots of methanol or water were added to 1−3 mg sample quantities, and the solutions were vortexed (2 min) and stirred (10−15 min) at room temperature. As expected, the presence of the PEG group at the 7-position of 6A−F significantly increased their solubilities. By comparison to 6A−F, elemental sulfur S8 and 5A−C had ~50-fold lower solubility. Our aqueous solubility measurements are consistent with previous values for elemental sulfur S8 reported in the literature, namely, the solubility of S8 in water was reported to be 0.4 μg/mL.59 The solubility of S8 in ethanol was reported to be 0.51 mg/mL.30

**Antiproliferative Activities of Polysulfanes.** We examined the effects of 1,2-benzenedithiol, o-benzopolysulfanes 5A−C, and PEGylated benzopolysulfanes 6A−F on the proliferation of several cancer cell lines. Their bioactivities are shown in Table 6 and Figure 3. 1,2-Benzenedithiol was nontoxic; it stimulated the growth of PC3 cells and had a minimal effect on the proliferation of several cancer cell lines. However, at concentrations of 10 μM and above, it inhibited the proliferation of DU145 cells by ~40%. In a MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carb-
boxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium in the presence of phenazine methosulfate), 31 1,2-benzenedithiol was also not effective in inhibiting Jurkat cell growth (IC$_{50}$ of 60 μM).

The requirement of the polysulfur ring was also observed by Molinski et al.5 in the natural lissoclinotoxin A system when judged against the corresponding benzenedithiol compound.

### TABLE 5. Experimental Solubility Values

<table>
<thead>
<tr>
<th>Solvent</th>
<th>methanol (mg/mL)</th>
<th>water (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>elemental sulfur, $S_8$</td>
<td>0.33 ± 0.03</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>$\alpha$-benzopolysulfanes 5A–C (97%)</td>
<td>0.37 ± 0.02</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>PEGylated benzopolysulfanes 6A–F (93%)</td>
<td>19.00 ± 0.05</td>
<td>8.70 ± 0.25</td>
</tr>
</tbody>
</table>

Equilibrium time of 24 h was used in the solubility study. Measurements were conducted three times, and the solubility value was averaged.

### TABLE 6. Inhibition of Cancer Cell Growth by 1,2-Benzenedithiol, $\alpha$-Benzopolysulfanes 5A–C, and PEGylated Benzopolysulfanes 6A–F

<table>
<thead>
<tr>
<th>compound</th>
<th>PC3</th>
<th>DU145</th>
<th>MDA-MB-231</th>
<th>Jurkat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-benzenedithiol</td>
<td>no effect</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>60</td>
</tr>
<tr>
<td>$\alpha$-benzopolysulfanes 5A–C (97% purity)</td>
<td>10.5 (20)$^b$</td>
<td>4.9 (6)$^b$</td>
<td>27</td>
<td>0.5</td>
</tr>
<tr>
<td>PEGylated benzopolysulfanes 6A–F (~98% total purity)</td>
<td>1.8 (4)$^b$</td>
<td>3 (4)$^b$</td>
<td>5.5 (12)$^b$</td>
<td>0.4</td>
</tr>
</tbody>
</table>

$^a$IC$_{50}$ values were obtained for 72 h incubations with Jurkat cells and 48 h for PC3, DU145 and MDA-MB-231 cells. $^b$Concentrations that resulted in 100% cell kill are shown in parentheses. Experiments were conducted in 96-well plates with 8 replicates for each concentration (0–30 μM) of the indicated compounds. Experiments were conducted in 96-well plates with 8 replicates for each concentration (0–30 μM) of the indicated compounds.

also not effective in inhibiting Jurkat cell growth (IC$_{50}$ of 60 μM). The requirement of the polysulfur ring was also observed by Molinski et al.5 in the natural lissoclinotoxin A system when judged against the corresponding benzenedithiol compound.

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Parent polysulfanes $5A-6C$ had a moderate antiproliferative effect on MDA-MB-231 cells, inhibiting the proliferation with an $IC_{50}$ value of 28 $\mu M$. PC3 cell proliferation was inhibited with an $IC_{50}$ of 11 $\mu M$ with complete cell killing at 20 $\mu M$. DU145 and Jurkat cells were sensitive to parent polysulfanes $5A-C$; 6 $\mu M$ killed 100% of the DU145 cells, and the $IC_{50}$ was 0.5 $\mu M$ for Jurkat cells. PEGylated benzopolysulfanes $6A-F$ were the most potent of the three compound classes for the four cell lines examined and were toxic for all four cell lines. Complete cell killing was observed for $6A-F$ at 4 $\mu M$ for PC3 and DU145 cells and at 12 $\mu M$ for MDA-MB-231 cells. The PEG compounds $6A-F$ were cytotoxic in Jurkat cells with an $IC_{50}$ of 0.4 $\mu M$.

The data in Table 6 indicate that the polysulfur ring is significant in the enhancement of antiproliferative activity. The data also demonstrate that the PEG group leads to an increase in the antiproliferative effect. The PEGylated benzopolysulfanes $6A-F$ have higher water solubility compared to that of the parent polysulfanes $5A-C$ and produced the highest growth inhibition. It was previously reported that lissoclinotoxin A 2 was cytotoxic against L1210 leukemia cells ($IC_{50}$ of 3.1 $\mu M$) and N-Dimethylvaracin 3 was cytotoxic against MDA-MB-231 cells ($IC_{50}$ of 3.6 $\mu M$).

Interestingly, varacin 1 showed greater cytotoxicity in HCT-116 colon cancer cells ($IC_{50}$ of 0.15 $\mu M$).

**Potential of Benzopolysulfanes as Drug Candidates.** Even though high nanomolar and low micromolar antiproliferative $IC_{50}$ values for benzopolysulfanes were mentioned in the Introduction and the above results for $6A-F$ appear promising, in vivo stability and deliverability studies are needed to evaluate benzopolysulfane lability to glutathione, protein thiols, etc. Like some other drugs, benzopolysulfanes are reductively activated. Our in vitro studies showed that ethanethiol-induced desulfuration of $6C$ in aqueous methanol took $\sim 2$ min but in benzene took 4 h, suggesting that greater thiol ionization/nucleophilicity increases the polysulfane lability. Thus, hydrophobic or mild acidic conditions that maintain the less reactive thiol form will preserve $6C$, but in the presence of thiolate ion $6C$ decomposes rapidly. In the absence of thiol and thiolate ion, the solvent effect is in the opposite direction; benzopolysulfane equilibria in organic solvents such as CH$_2$Cl$_2$ are more facile than in methanol or methanol/water, driven by solvation of elemental sulfur and the benzopolysulfanes (vide supra). Lastly, although a DNA cleaving study of 7-methylbenzopentathiepin suggested a metal- and oxygen-dependent Fenton pathway, more work is needed to evaluate a potential anaerobic $S_2$ transfer pathway that may underlie the antitumor activity.

**Conclusion**

The following conclusions can be made: (1) The synthesis of benzopolysulfanes $6A-F$ was accomplished with attached PEG groups of 160 Da molecular weight via an amide linkage in 1.5% overall yield. (2) Pentathiepin $6C$ was the main product, but even after its purification, benzopolysulfanes $6A, 6B, 6D-F$ formed in very low concentrations. (3) The pentasulfur linkage of $6C$ was sensitive to the solvent composition in the HPLC experiments. Methanol-rich elution conditions reduced the ratio of $6C$ relative to the other polysulfanes. (4) Thiol-initiated reactions of $6A-F$ led to an $S_2$ transfer to norbornene, but no $S_2$ transfer was observed to 2,3-dimethylbutadiene. (5) While the cause of the poor solubility of benzopolysulfanes is the polysulfur linkage itself, a key issue in the present study was the enhanced water solubility the PEG group provided, which did not reduce the prevalence of the pentasulfur ring species. (6) The results confirm the requirement of the polysulfur ring for low micromolar antiproliferative $IC_{50}$ values; 1,2-benzenedithiol showed little or no antiproliferative activity. (7) The PEG polysulfanes $6A-F$ were more water soluble and more active against four cancer cell lines than the parent polysulfanes $5A-C$, suggesting that enhanced solubilization of benzopolysulfanes holds promise for advancing these compounds as drug candidates.

**Experimental Section**

3,4-Dihydroxybenzoic acid, $N,N$-dimethylthiocarbamoyl chloride, DABCO, sodium hydroxide, potassium hydroxide, ethane-thiol, acetanilide, formic acid, ammonium formate, dimethylthion chloride, $p$-nitrophenol, DCC, S$_8$, triethylene glycol mono-methyl ether, phthalimide, triphenylphosphine, DIAD, hydrazine monohydrate, elemental sulfur ($S_0$), norbornene, 2,3-dimethylbutadiene, DMAP, sodium sulfate (anhydrous), magnesium sulfate (anhydrous), NaCl, DMF, Na$_2$CO$_3$, NaHCO$_3$, THF, CHCl$_3$, CH$_2$Cl$_2$, methanol, ethanol, ethyl acetate, hydrochloric acid (12 M), diphenyl ether, benzene, acetone-$d_6$, CDCl$_3$, CD$_3$CN, CD$_3$OD, and hexanes were used as received without further purification. Purification of product mixtures was carried out by column chromatography using silica gel with 40–60 $\mu$ particle size. TLC was carried out using silica gel 60F$_{254}$ TLC plates. Proton NMR data were acquired at 400 MHz, and $^{13}$C NMR data were acquired at 100.6 MHz. HRMS, GC–MS, HPLC, and melting point data were collected.

**HPLC Instrumentation and Analysis.** The HPLC instrument was equipped with an autosampler and diode array detector. The C18 column was 150 mm × 3.9 mm in size. The flow rate was 1 mL/min, and the injection volume was 50 $\mu$L. The mobile phase consisted of methanol and water. Compounds were detected by UV at 254 nm at rt.

**LCMS Instrumentation and Analysis.** The LCMS system consisted of a high-resolution TOF mass spectrometer attached to an HPLC equipped with an autosampler, diode array detector, and binary pump. The chromatography was conducted with a 2.1 mm × 30 mm SB-C18 3.5 $\mu$m column using water containing 0.1% formic acid and 5 mM ammonium formate (solvent A) and methanol containing 0.1% formic acid and 5 mM ammonium formate (solvent B) at a flow rate of 0.5 mL/min. The gradient program was as follows: 15–85% B (0–13 min), 85% B (2 min), 85–15% B (1 min). The mass spectra were collected over a range of 100–1600 $m/z$. The reference masses used were purine with (M + H$^+$) ion at 121.05087 $m/z$ and HP-922 with ion at 922.00980 $m/z$. They were infused into the spray chamber using a calibration delivery system.

**GC–MS Instrumentation and Analysis.** GC–MS samples were ionized using the EI auto mode. The capillary column was a VF-5 ms 30 m × 0.25 mm × 0.25 $\mu$m (where ID = 0.25 mm, DF = 0.25 $\mu$m). The solvent delay was set to 3 min. Temperature program was as follows: 80 °C (0–5 min), 80–250 °C (5–22 min, at a rate 10 °C/min), 250 °C (22–28 min). Total run was 28 min. The instrument parameters were set as follows: injector temperature 200 °C, column flow rate 1 mL/min. Data were collected with the instrument set to mass range 40–650 $m/z$.

**Equilibration and Solubility Determinations.** To determine the extent of polysulfane equilibration, a 6.4 mg sample of dry $6A-F$ was dissolved in 3 mL of methanol or methanol/water mixtures by stirring for 2 min at rt. Then 0.2 mL was placed in a vial and diluted to 1 mL with methanol. The solution was stirred.

for 1 min, in which the concentration of each sample was 0.42 mg/mL. Finally, 50 µL of the solution was injected into the HPLC autosampler and quantitated by the absorption signal at 254 nm. The solubilities of elemental S₈, 5A–C, or 6A–F were determined by adding 10–50 µL aliquots of methanol or water to 1–3 mg of the compounds. The solutions were vortexed for 2 min and then stirred for ~15 min at rt. Polysulfane decompositions were also conducted in the presence of sodium hydroxide. To a solution of polysulfanes 6A–F (2.5 mM) and internal standard, acetanilide (0.25 mM), was added NaOH (0.625, 1.25, 1.875, or 2.5 mM) in 0.1 mL of methanol. After certain periods of time, the reaction was analyzed by HPLC by injecting 15 µL of the sample.

**Sulfur Transfer and Trapping Studies.** Trapping studies were carried out in 0.2 mL of benzene solution. To a solution of PEGylated benzopentathiole-6A–F (2.3 mM) and norbornene or 2,3-dimethylbutadiene (2.3 mM) was added ethanethiol (2.3 mM). The reaction mixture was vortexed for 2 min and analyzed by GC–MS by injecting 1 µL of the sample. Both scan and single ion mode (SIM) analyses were conducted on the sample.

**Cell Proliferation Assays.** The cell lines were grown from fresh stocks or obtained from the Type Culture Collection. The prostate cancer cell lines PC3 and DU145 cells were grown in F12K and DMEM, respectively. MDA-MB-231 breast cancer cells were grown in DMEM. All the media were supplemented with 10% FBS and penicillin/streptomycin (100 U/mL and 100 µg/mL). Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and warmed to rt and followed by the addition of the penicillin-streptomycin (100 U/mL and 100 µg/mL) solution. The cytotoxicity assay was conducted as follows: Cells were treated with different concentrations of the tested compound for 24 h. After 24 h, cells were treated with different concentrations of the sulfur-containing compounds (0–30 µM). After 48 h incubation, the media was removed, and the plates were frozen at −80 °C. The assay was performed by warming the plates to rt and followed by the addition of the reagent to the wells. The fluorescence was measured with a plate reader. (excitation/emission, 485/530 nm).

**Quantification of growth medium containing the sulfur-containing compounds** 361.0476, found 361.0478.

- **The cytotoxicity assay was conducted as follows:** Cells were plated at 100,000 cells/well in 96-well plates and cultured in DMEM. All the media were supplemented with 10% FBS and penicillin/streptomycin (100 U/mL and 100 µg/mL). Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and warmed to rt and followed by the addition of the penicillin-streptomycin (100 U/mL and 100 µg/mL) solution. The cytotoxicity assay was conducted as follows: Cells were treated with different concentrations of the tested compound for 24 h. After 24 h, cells were treated with different concentrations of the sulfur-containing compounds (0–30 µM). After 48 h incubation, the media was removed, and the plates were frozen at −80 °C. The assay was performed by warming the plates to rt and followed by the addition of the reagent to the wells. The fluorescence was measured with a plate reader (excitation/emission, 485/530 nm).

The cytotoxicity assay was conducted as follows: Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively) and maintained at a 37 °C humidified 5% CO₂ incubator. On the day before the drug treatment, cells were plated onto each well of the 96-well plate at 2,000 cells/well (200 µL of the medium per well). After 24 h, cells were treated with different concentrations of the sulfur-containing compounds and incubated for 72 h. After the incubation, cell growth was evaluated using a 96-well solution cell proliferation assay. UV absorption (490 nm) of each well was quantified with a microplate reader.
hexane (2.98 to 40:60) to afford 79 mg (40%) of 10 as a light brown solid. $R_f = 0.75$ (EtOAc/hexane 1:2), mp 140–142 °C. 1H NMR (CDCl$_3$) δ 3.95 (3H, 1H), 7.57 (d, $J = 8.5$ Hz, 1H), 7.98 (dd, $J = 8.5$, 2.0 Hz, 1H), 8.18 (d, $J = 2.0$ Hz, 1H), 8.15, 8.18, 8.03 (d, $J = 8.1$, 1.6 Hz, 1H), 7.39 (d, $J = 8.3$ Hz, 2H), 8.36 (d, $J = 8.1$ Hz, 1H), 1.09 (s, 3H); 13C NMR (CDCl$_3$) δ 162.4, 155.2, 146.5, 139.9, 129.7, 129.0, 125.1, 124.1, 4.76; HRMS (+ESI) calcd for C$_{15}$H$_{13}$NO$_4$S$_2$Sn = 446.9334, found 446.9324.

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**Supporting Information Available:** Spectroscopic data for compounds 6A–F and 8–17. This material is available free of charge via the Internet at http://pubs.acs.org.