Novel N7- and N1-Substituted cGMP Derivatives Are Potent Activators of Cyclic Nucleotide-Gated Channels

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Cyclic nucleotide-gated (CNG) channels, key players in olfactory and visual signal transduction, generate electrical responses to odorant- and light-induced changes in cyclic nucleotide concentration. Previous work suggests that substitutions are tolerated solely at the C8 position on the purine ring of cGMP. Our studies with C8, 2′-OH, and 2-NH_2-modified cGMP derivatives support this assertion. To gain further insight into determinants important for CNG channel binding and activation, we targeted previously unexplored positions. Modifications at N7 of 8-SH-cGMP (**6**) are well tolerated by olfactory and retinal rod CNG channels. Toleration of a very large substituent, a 3400 molecular weight PEG, at either N7 or C8 argues for broad accommodation at these positions in the binding site. Modification at N1 of cGMP reduces the apparent affinity for the channel; however, when combined with 8-parachlorophenylthio derivatization, the resulting cGMP analogue is more potent than cGMP itself. These studies establish the N7 and N1 positions of cGMP as targets for modification in the design of novel CNG channel agonists.

Introduction

The essential role played by CNG[a] channels in olfactory sensory neurons and retinal photoreceptor cells is well established; odorant- and light-induced changes in cyclic nucleotide concentrations gate these nonselective cation channels, thus generating electrical signals that are transmitted to the brain (reviewed in refs 1−6). CNG channels are members of the voltage-gated K^+ channel superfamily; however, despite global structural similarities, CNG channels are endowed with unique functional properties. They are voltage-independent, gated solely by cyclic nucleotide binding, and nonselective, with a significant fraction of the cationic current carried by Ca^{2+}.[2,4] Acting as a cyclic nucleotide-activated Ca^{2+} source, CNG channels are uniquely situated at the intersection of two second messenger signaling systems. Indeed, in both vision and olfaction, Ca^{2+} entry through CNG channels is crucial for sensory adaptation. Beyond these sensory systems, CNG channels have been detected in many other tissues including brain, heart, kidney, testis, liver, lung, skeletal muscle, adrenal gland, pancreas, and colon; yet their physiological roles are poorly understood.[7−9] Development of selective pharmacological agents will significantly accelerate the elucidation of CNG channel participation in cellular signaling. While progress has been made in generating potent activators and inhibitors, development of highly selective probes remains an important, yet elusive, goal.[10]

Native CNG channels are composed of different combinations of six pore-forming subunits, including both α (CNGA1−4) and β (CNGB1,3) subunits (reviewed in refs 2, 4, 6). Many of the α subunits can be functionally expressed as homomultimeric channels, but coexpression of the β subunits confers distinct functional properties in terms of ion permeation, ligand sensitivity, gating mechanisms, and regulation. Rod channels are composed of two types of subunits: CNGA1 and CNGB1.

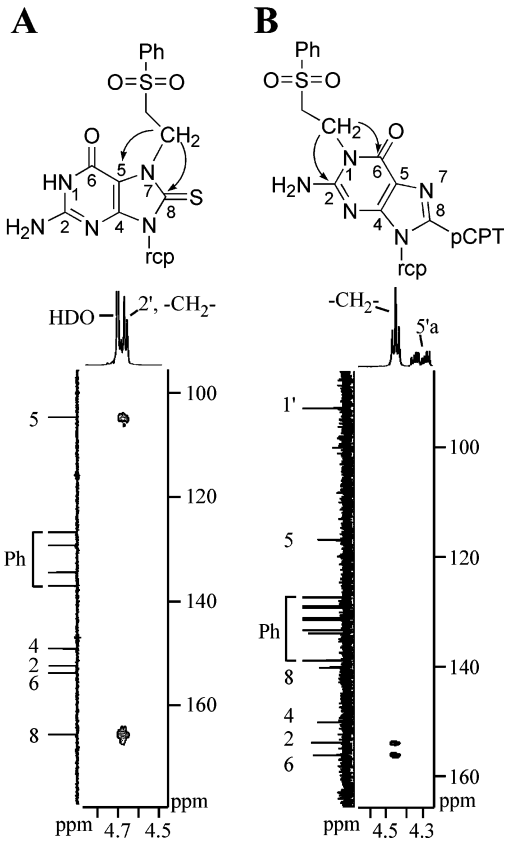

Figure 1. HMBC spectroscopy of compound **10** in D_2O (A) and compound **14** in CD_3OD (B). A select region from each 2D spectrum is shown to highlight the relevant signals. The ^{13}C 1D projections are on the left, while the ^1H 1D projections are at the top in panels A and B. An abbreviated structure of each derivative is shown above with arrows indicating the three-bond $^1H/^{13}C$ couplings for the methylene protons of interest. In panel A the 2′ and $-CH_2-$ ^1H resonances are overlapping, and in panel B the 5′a ^1H resonance is labeled for clarity.

Recent evidence suggests that the stoichiometry of the native rod photoreceptor channel is 3 CNGA1 to 1 CNGB1. The CNG

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[a] Abbreviations: COSY, correlation spectroscopy; CNG, cyclic nucleotide-gated; HMBC, heteronuclear multiple bond coherence; HSQC, heteronuclear single quantum correlation; pCPT, parachlorophenylthio.

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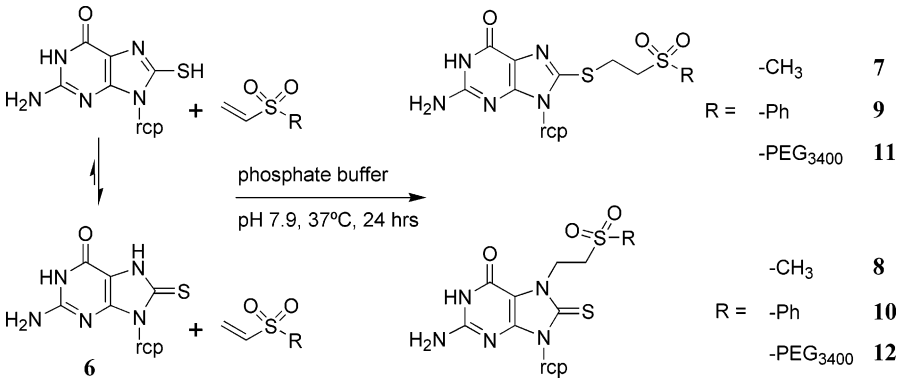
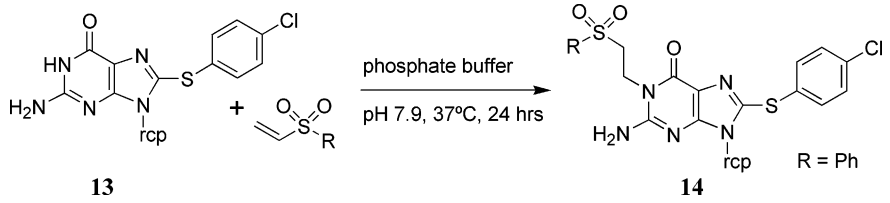
Scheme 1. Vinyl Sulfone Reaction with 8-SH-cGMP[a]



[a] The ribose and cyclic phosphate rings are abbreviated as rcp.

Scheme 2. Vinyl Sulfone Reaction with 8-pCPT-cGMP[a]



[a] The ribose and cyclic phosphate rings are abbreviated as rcp.

channel in cone photoreceptors is composed of CNGA3 and CNGB3 subunits, and in contrast to rod channels, functional evidence suggests that the stoichiometry of the cone channel is 2:2. The native olfactory channel contains three subunit types, including CNGA2, CNGA4, and CNGB1b, in a ratio of 2:1:1.

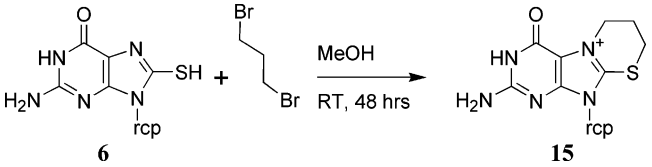
The study of cyclic nucleotide analogues provides insight into how these second messengers bind and activate CNG channels, revealing a template for the development of selective agonists and antagonists. The earliest report of electrical recordings of a cGMP-stimulated cationic conductance in rod photoreceptors employed cyclic nucleotide derivatives; neither 5′-GMP nor 2′, 3′-cGMP was active, demonstrating the importance of the cyclic phosphate.[11] Another early study employing a Ca^{2+}-sensitive dye to report cGMP-stimulated cation flux in rod outer segment membranes revealed that 250 μM 2′-deoxy-cGMP, 2′-*O*-butyryl-cGMP, and N2-butyryl cGMP were ineffectual.[12] In contrast, 250 μM cGMP, 8-Br-cGMP, and 7-deaza-cGMP triggered identical rates of Ca^{2+} release. Further reports reinforced the importance of the 2′-OH for binding and showed that C8 is amenable to a wide range of substituents.[13−15] Brown et al. systematically studied the effect of C8 derivatives on native rod CNG channel activation, demonstrating an unfavorable impact of positive charge and a broad tolerance for apolar and negatively charged substituents.[16] Although many of the C8-substituted cGMP derivatives are more potent than cGMP itself, including the membrane permeant and widely used 8-parachlorophenylthio(pCPT)-cGMP, these compounds are also high-affinity activators of cGMP-dependent protein kinase.[17,18] Some advances have been made in generating cyclic nucleotide-based channel antagonists by combining modifications at several positions on cGMP. Simultaneous derivatization of both 2-NH_2 and N1 positions of cGMP blocks the ability to activate native retinal CNG channels. However, in combination with C8 and phosphorothioate modifications, the resulting derivatives act as competitive antagonists,[10,19,20] albeit with low affinity and uncertain specificity.

Here, we have taken advantage of the enhanced reactivity of 8-thio-substituted cGMP analogues toward vinyl sulfones to examine the effect of modifications at N1 and N7 on heteromeric rod and olfactory CNG channel activation. These novel cGMP derivatives target positions previously unexplored with regard to activation of CNG channels. Additionally, we have investigated the impact of modifications at 2′-OH, 2-NH_2, and C8 relative to cGMP on heteromeric olfactory CNG channel activation. These studies provide new insight into the cyclic nucleotide determinants that are important for activation of CNG channels and identify two novel sites on the cyclic nucleotide that are amenable to modification.

Chemistry

8-SH-cGMP (**6**) is a useful starting material for the synthesis of a plethora of C8-modified cGMP derivatives.[16,18,21] We investigated the reaction of vinyl sulfones, regarded as thiol-selective electrophiles, with **6**, expecting a single C8-S adduct. Surprisingly, we observed the formation of approximately equivalent amounts of two distinct products upon reaction at pH 7.9 (Scheme 1). To further explore this unanticipated reactivity, we tested reaction of phenyl vinyl sulfone with 8-pCPT-cGMP (**13**), an 8-thio-cGMP derivative lacking a free thiol, and observed the formation of a single main product (Scheme 2). While vinyl sulfones are highly selective for reaction with thiols, given elevated pH (>9) and long reaction time (days), significant reaction with amines can be achieved.[22] In order to definitively determine the site of reaction of vinyl sulfones with **6** and **13**, we employed heteronuclear multiple bond correlation (HMBC) spectroscopy, a 2D NMR technique that yields cross-peaks for $^1H/^{13}C$ pairs connected by two and three bonds. The ^1H resonances were assigned by their chemical shifts, $^1H/^1H$ COSY experiments, multiplicities, and comparison with published spectra.[23] Assignment of ^{13}C resonances was aided by heteronuclear single quantum coherence (HSQC) spectroscopy, HMBC, and comparison with published spectra.[24] Figure 1 displays selected regions of HMBC spectra of phenyl vinyl sulfone derivatives, showing that **6** (Figure 1A) forms an N7 adduct (**10**) while reaction with **13** (Figure 1B) yields an N1 adduct (**14**) (Schemes 1 and 2, respectively). Modification of 8-thioguanosine at N7 has been achieved by reaction with

Scheme 3. 1,3-Dibromopropane Reaction with 8-SH-cGMP[a]



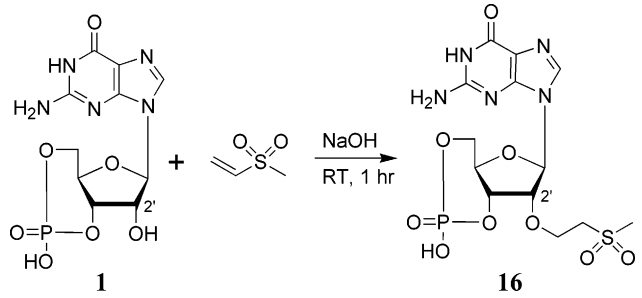
[a] The ribose and cyclic phosphate rings are abbreviated as rcp.

allyl bromide to form a C8-S adduct, which is then transformed to the 7-allyl, 8-thioxo product via a [3,3] sigmatropic rearrangement.[25] More recently, an acyclic 8-thioguanosine derivative was reported to react with *n*-butyl vinyl ether directly at N7.[26]

The equivalent reactivity of the C8 thio group and N7 of compound **6** is indicative of a thioketo−thioenol tautomerization that favors the thioketo form (Scheme 1). In fact, ^{15}N NMR experiments show that 8-thioguanosine exists as the thioketo form.[27] The N7 vinyl sulfone derivatives **8**, **10** and **12** also adopt the thioketo form (not the charged tautomer) based on several lines of evidence. First, the ^{13}C chemical shift for the carbon at the 8-position is sensitive to whether C8-S is a single or a double bond. Compound **6** and the N7 phenyl vinyl sulfone derivative, **10**, give ^{13}C shifts of 167.9 and 166.2 ppm, respectively, while the ^{13}C chemical shifts for compounds with a single bond at C8-S, **7**, **9**, **14**, and **15**, are on average >20 ppm upfield: 140.9, 143.8, 140.2, and 147.8 ppm, respectively. The presence of a positive charge at N7, as in compound **15**, has a relatively small impact on the ^{13}C chemical shift at C8. Second, the UV−vis spectra of **6** and the N7 derivative, compound **8**, share a characteristic two-peak shape: λ_{max} = 208/302 and 208/304, respectively. The slight red shift for **8** relative to **6** is reminiscent of the red shift observed for 7-ethylguanosine relative to guanosine.[28] In contrast, the λ_{max} for C8-S alkyl derivatives such as compound **7** at 276 nm is significantly blue-shifted from the parent, **6**. Third, N7 derivatives that impose a positive charge at N7 such as 7-ethylguanosine[28] and compound **15** are brightly fluorescent; none of the other compounds described here are. Fourth, the N7 derivatives of **6** elute after the analogous C8-S derivatives. If these N7 derivatives adopted the charged tautomeric form at N7-C8-S, we would expect them to display significantly reduced retention times on RP-HPLC. Last, **10** does not participate in a second round of Michael addition even when the vinyl sulfone reactant is present in excess. Dual alkylation of C8-S and N7 can be achieved by reaction of **6** with 1,3 dibromopropane (Scheme 3). The reaction proceeds via a rapid alkylation of C8-S, followed by a slower intramolecular reaction at N7 to form a six-membered ring and impart a permanent positive charge to the purine ring system.

The presence of sulfur at C8 activates the purine ring for reaction with vinyl sulfones. In contrast to the 8-thio derivatives (**6** and **13**), cGMP is resistant to reaction, requiring a strong base and a large excess of vinyl sulfone to yield a 2′-O adduct (compound **16**) in relatively low yield (Scheme 4). Similar conditions have been employed in the methylation of cyclic nucleotides; reaction with iodomethane in the presence of NaOH has been reported to result in methylation of the 2′-O (cAMP) when DMF was included in the reaction or at N1 (cGMP) when DMF was omitted.[29,30] Our interest in an alternative route to N1-modified cGMP derivatives compelled us to try both methods. In our hands, reaction of cGMP with iodomethane in the presence of NaOH with or without DMF yields predominantly 2′-*O*-methyl-cGMP (**3**) or 1,2′-*O*-dimethyl-cGMP (**4**), depending on the temperature and reaction time. The 1-methyl-cGMP derivative is a minor product that is difficult to isolate

Scheme 4. Vinyl Sulfone Reaction with cGMP



in purity greater than 90% and was therefore not tested further. The source of the discrepancy between our results and those of Miller et al. is unclear but may be from the different purification methods employed.[30] We used HSQC and HMBC NMR analysis in conjunction with mass spectrometry to ensure correct structural characterization. Full HSQC and HMBC spectra for key compounds (**3**, **4**, **9**, **10**, **14**, and **15**) are provided as Supporting Information.

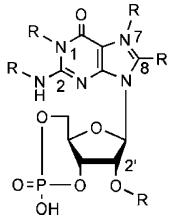
Results

The unexpected reactivity of vinyl sulfones with 8-thio-substituted cGMP derivatives allowed us to study the effects of modifications at N1 and N7 on the activation of CNG channels. Our investigation of cGMP reactivity led us to synthesize additional derivatives at the C8 and 2′-O positions and to examine N2- and 2′-*O*-butyryl derivatives. We have included these in our functional analysis for comparison (Table 1). We initially focused our efforts on heteromeric olfactory CNG channels because they are more sensitive to cGMP than retinal channels, thus facilitating the study of derivatives that bind poorly. We coexpressed CNGA2, CNGA4, and CNGB1b in HEK-293 cells and measured the sensitivity of the resulting channels to cAMP, as this property is a major indicator of subunit composition. Bonigk et al. showed that CNG channels produced by coexpression of CNGA2 with CNGA4 and CNGB1b were as sensitive to cAMP as native channels from the olfactory sensory cilium; the $K_{1/2}$ values of the heterologously expressed and native channels are 4.8 and 4.1 μM, respectively.[31] In contrast coexpression of CNGA2 with either CNGA4 or CNGB1b individually results in channels with markedly decreased cAMP sensitivity. We observed an average $K_{1/2}$ for activation by cAMP of 3.8 μM, a strong indication that the channels are indeed heteromeric.

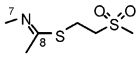
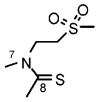
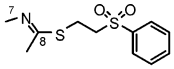
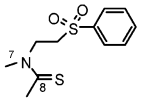
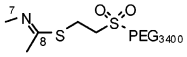
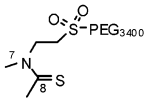
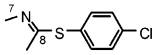
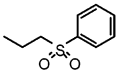
The commercially available monobutyryl cGMP derivatives, N2-butyryl-cGMP (**2**) and 2′-*O*-butyryl-cGMP (**5**), were full activators of the olfactory CNG channel, albeit with $K_{1/2}$ values 20- and 80-fold higher than cGMP, respectively (Table 1). Increasing the size of the substituent at 2′-O leads to decreasing apparent affinity for the channel; 2′-*O*-methyl (**3**) and 2′-*O*-butyryl (**5**) are 32- and 80-fold less potent than cGMP, respectively (Table 1). Preliminary experiments with 2′-*O*-methylsulfonylethyl-cGMP (**16**) showed it to be a very poor activator with a $K_{1/2}$ at least 2.5-fold higher than **5** (data not shown). We also tested the impact of 2′-OH modification of cAMP by comparing the activation of olfactory CNG channels by 8-pCPT-cAMP (**18**) and 2′-*O*-methyl-8-pCPT-cAMP (**19**). Compound **19** was ∼40-fold less potent than **18**, a decrease in apparent affinity similar to that observed with compound **3** (Table 2). The parachlorophenylthio group at the 8-position partially rescues the loss in affinity, leaving compound **19** with a $K_{1/2}$ of 22 μM, only ∼6-fold higher than cAMP (Table 2).

Consistent with prior work, our experiments with 8-substituted cGMP derivatives show that a wide range of substituents are

Table 1. Activation of Olfactory CNG Channels by cGMP Derivatives[a]



compd	R_1	R_2	$R_{2'}$	$K_{1/2}$ (μM)	n	
1	—H	—H	—H	1.1 ± 0.3	2.2 ± 0.3	(7)
2	—H		—H	22 ± 5.3	2.2 ± 0.2	(7)
3	—H	—H	—CH_3	35 ± 8.1	2.0 ± 0.3	(8)
4	—CH_3	—H	—CH_3	290 ± 77[b]	ND	(7)
5	—H	—H		88 ± 28	2.6 ± 0.2	(6)

compd	R_1	$R_{7/8}$[c]	$K_{1/2}$	n	
6	—H		0.18 ± 0.04	1.8 ± 0.3	(8)
7	—H		0.37 ± 0.08	2.1 ± 0.1	(9)
8	—H		0.11 ± 0.03	1.5 ± 0.3	(5)
9	—H		0.21 ± 0.04	2.5 ± 0.5	(9)
10	—H		0.082 ± 0.024	2.2 ± 0.3	(8)
11	—H		1.9 ± 0.3	1.9 ± 0.2	(6)
12	—H		1.3 ± 0.5	2.2 ± 0.3	(5)
13	—H		0.073 ± 0.021	2.3 ± 0.6	(10)
14			0.38 ± 0.16	1.7 ± 0.5	(10)
15	—H		10 ± 2	2.1 ± 0.2	(8)

[a] Excised patch recordings from HEK-293 cells expressing heteromeric olfactory CNG channels (CNGA2, CNGA4, and CNGB1b). $K_{1/2}$ and n (Hill equation coefficients) are defined in the Experimental Section, and the values shown are the mean ± the standard deviation. The number of experiments is given in parentheses in the right-hand column. ND = not determined. [b] The $K_{1/2}$ was estimated assuming **5** to be a full agonist. The average I/I_{max} for 242 μM **5** is 0.44. [c] N7 and C8 are shown in addition to the substituent(s) for clarity.

Table 2. Activation of Olfactory CNG Channels by cAMP Derivatives[a]

	compd	$K_{1/2}$ (μM)	n
17	cAMP	3.8 ± 1.2	2.0 ± 0.4 (75)
18	8-pCPT-cAMP	0.55 ± 0.13	2.4 ± 0.6 (7)
19	8-pCPT-2′-O−Me-cAMP	22 ± 4	2.4 ± 0.7 (8)

[a] Excised patch recordings from HEK-293 cells expressing heteromeric olfactory CNG channels (CNGA2, CNGA4 and CNGB1b). $K_{1/2}$ and n (Hill equation coefficients) are defined in the Experimental Section, and the values shown are the mean ± the standard deviation. The number of experiments is given in parentheses in the right-hand column.

well-tolerated.[10,13−16,32] Addition of sulfur to cGMP at the 8-position (compound **6**) enhances the apparent affinity for the channel ∼6-fold. Derivatization of the thio group of **6** has two consequences: addition of a new substituent and a shifting of the tautomeric form of the N7-C8-S system from the predominantly thioketo to the thioenol form (Scheme 1). Among the C8-S-substituted derivatives, the $K_{1/2}$ varies somewhat depending on the substituent. 8-pCPT-cGMP (**13**), widely used as a membrane-permeant cGMP analogue, is ∼2.5-fold more potent than the precursor (**6**). The methyl vinyl sulfone derivative (**7**) is ∼2-fold less potent than **6**, whereas the phenyl vinyl sulfone derivative (**9**) is essentially equipotent with **6**.

In contrast to C8-S derivatives, N7 derivatives of **6** adopt the thioketo form. While the contribution of the thioketo−thioenol tautomerization at N7-C8-S to activation is difficult to assess, we find that N7 derivatives of **6** are also well tolerated (Figure 2A). In fact, the methyl and phenyl vinyl sulfone derivatives at N7 (compounds **8** and **10**, respectively) are on average 3-fold more potent than the analogous C8-S derivatives

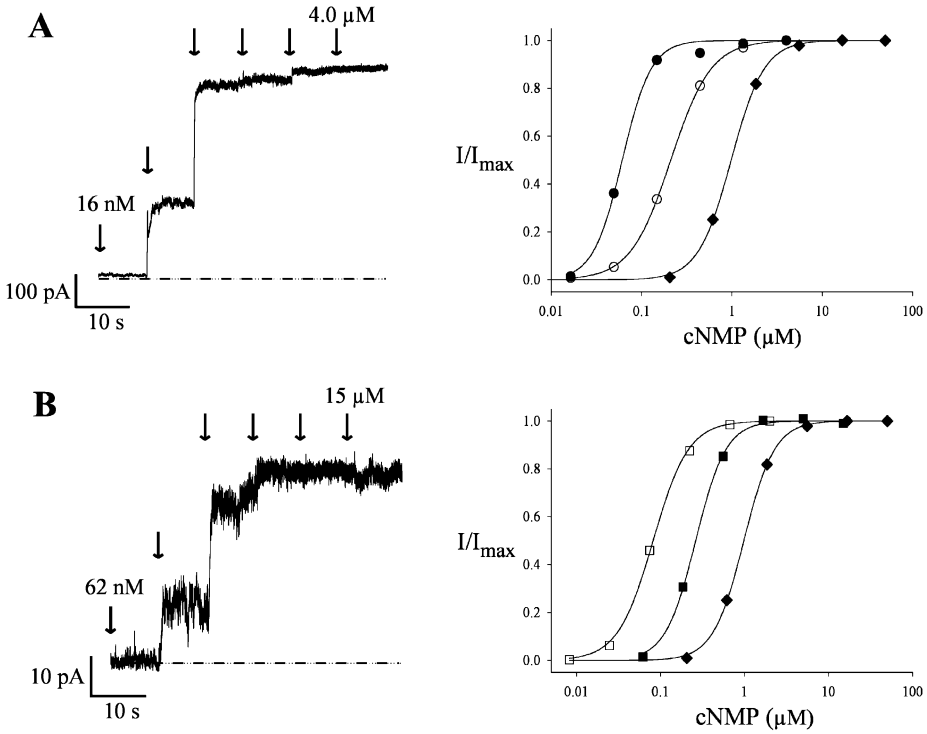


Figure 2. Inside-out patch recording. Activation of heteromeric olfactory CNG channels by compound **10** (A) and compound **14** (B). Traces on the left were recorded at a holding potential of +60 mV with 3-fold increasing concentrations of each compound applied to the patch as indicated by arrows above. The dose response curve is shown to the right: (A) **10** (filled circles), **6** (open circles), and **1** (diamonds); (B) **13** (open squares), **14** (filled squares), and **1** (diamonds). The dashed line indicates the zero current level.

(compounds **7** and **9**) (Table 1). Compound **10** is a potent activator of olfactory CNG channels, equivalent to **13**. We extended these studies to test the impact of very large substituents at N7; a 3400 molecular weight (MW) polyethylene glycol N7 derivative (**12**) remains a full agonist of heteromeric olfactory CNG channels and is at least as potent as the analogous C8-S derivative (**11**) (Table 1). We were able to generate a N7, C8-S doubly alkylated compound (**15**) by reaction of **6** with 1,3-dibromopropane (Scheme 3). Compound **15** is a poor activator of the olfactory CNG channel, >50-fold less potent than **6** and 9-fold less potent than cGMP.

While **6** is primed for reaction with vinyl sulfones at N7, **13** reacts with phenyl vinyl sulfone at N1 to give compound **14** (Figure 1). In contrast to the N7 derivatives (**8** and **10**), **14** is less potent than its parent compound (**13**), requiring ~5-fold higher concentrations to yield similar activation (Table 1, Figure 2B). We also examined the effect of N1-methylation of cGMP for CNG channel activation. While 2′-*O*-methyl-cGMP (**3**) has a $K_{1/2}$ for activation of olfactory CNG channels of 35 μM, 1,2′-*O*-dimethyl-cGMP (**4**) has a $K_{1/2}$ estimated to be 290 μM (Table 1). This estimate was made with the assumption that **4** is a full agonist. Since the highest concentration of **4** tested was 242 μM, it is unclear whether this assumption is correct. If, in the extreme case, 242 μM **4** is saturating, we have overestimated the $K_{1/2}$ by ~2-fold. Thus, methylation of N1 reduces the apparent affinity for the channel 4- to 8-fold on a background of 2′-O-methylation (Table 1).

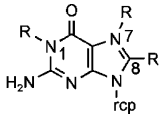
Numerous C8-S substituted cGMP derivatives have been tested for activation of retinal CNG channels.[10,12−16,32−35] These studies demonstrate a broad tolerance for negatively charged and hydrophobic substituents at C8. To determine the effect of N1 and N7 modifications of cGMP on activation of retinal rod CNG channels, we coexpressed CNGA1 and CNGB1 in *Xenopus* oocytes and measured activation of the heteromeric channels by a subset of cGMP derivatives. As with olfactory channels, derivatization of cGMP with sulfur at C8 (compound **6**) enhances the apparent affinity ~3-fold for the rod CNG channel (Table 2). Also, derivatization of **6** at N7 had a similar effect on activation of the rod CNG channel as on the olfactory channel. The phenyl vinyl sulfone derivative of **6** (compound **10**) was 3-fold more potent than the parent compound (Table 3). Modification of N1 was detrimental to activation; the loss of apparent affinity for the retinal channel was larger than observed for the olfactory channel. Compound **14** was ~12-fold less potent than the precursor **13** (Table 3).

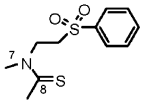
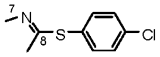
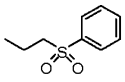
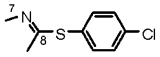
Discussion

We were able to determine the impact of modification at N1 and N7 on CNG channel activation because of our serendipitous discovery of enhanced reactivity of 8-thio-substituted cGMP derivatives toward vinyl sulfones. Furthermore, examining the activation of heteromeric olfactory CNG channels by new mono- and dimethylated cGMP derivatives (**3** and **4**), as well as the commercially available monobutyryl derivatives (**2** and **5**), allows us to assess the relative impact of derivatization at 2′-O and 2-NH positions of cGMP. In previous studies of native retinal CNG channels, cGMP derivatives modified at 2′-OH and 2-NH_2 were inactive.[12,15] Here, we show that for olfactory channels the N2-butyryl derivative of cGMP (**2**), while 20-fold less potent than cGMP, is still 4-fold more potent than the 2′-*O*-butyryl derivative (**5**). We further demonstrate a trend toward lower apparent affinity for the channel as the size of the 2′-O substituent is increased.

Our experiments with a 2′-*O*-methyl derivative of cAMP are noteworthy because compound **19** has found increasing utility as a membrane-permeant and selective activator of the guanine nucleotide exchange factor Epac.[36−38] Compound **19** is a very poor activator of the cAMP sensitive protein kinases, PKAI and PKAII, but is a potent Epac1 activator with a $K_{1/2}$ of 2.2 μM.[36,39] We find that the $K_{1/2}$ for activation of olfactory CNG channels

Table 3. Activation of Retinal Rod CNG Channels by cGMP Derivatives[a]



comp	R_1	$R_{7/8}$[b]	$K_{1/2}$ (μM)	n	
1	—H		41 ± 8	2.2 ± 0.5	(6)
6	—H		14 ± 4	1.9 ± 0.3	(4)
10	—H		4.1 ± 0.7	1.9 ± 0.4	(6)
13	—H		1.0 ± 0.2	1.7 ± 0.22	(5)
14			12 ± 2	2.1 ± 0.2	(6)

[a] Excised patch recordings from *Xenopus* oocytes expressing heteromeric retinal rod CNG channels (CNGA1 and CNGB1). $K_{1/2}$ and n (Hill equation coefficients) are defined in the Experimental Section, and the values shown are the mean ± the standard deviation. The number of experiments is given in parentheses in the right-hand column. [b] N7 and C8 are shown in addition to the substituent(s) for clarity.

by **19** is 22 μM. This relatively modest difference in $K_{1/2}$ for Epac1 and CNG channel activation, and uncertainties regarding membrane permeability, highlight the need for improved activators of all cyclic nucleotide-binding proteins.

The N7 derivatives of **6** (compounds **8**, **10**, and **12**) combine N7 derivatization with a thioketone at C8 and represent a novel type of cyclic nucleotide derivative. Previously reported modification at N7 of cGMP is limited to the 7-deaza derivative in which N7 is replaced with C. While 7-deaza-cGMP was shown to activate cation efflux from lysed rod outer segments, its potency relative to cGMP is unclear, as both were employed at a concentration that elicited maximal Ca^{2+} release kinetics.[12] Interestingly, while 7-deaza-cGMP displayed 16-fold lower affinity relative to cGMP for the rapidly exchanging site of cGMP-dependent protein kinase, the opposite was true of the type II cAMP-dependent protein kinase, where 7-deaza-cGMP bound with 50-fold higher affinity than cGMP.[17,40]

Our results show that N7 modification of **6** is well tolerated by both heteromeric olfactory and retinal rod CNG channels and can increase the apparent affinity for the channel, as with **10**. While N7 derivatives are slightly more potent, N7 modification is broadly similar to C8 modification, as very large substituents, such as a 3400 MW PEG, can be accommodated at either position. This argues that, when bound to the channel, this face of the nucleotide is largely exposed. While dual alkylation of **6** at N7 and C8-S is not generally practical, we were able to accomplish this using 1,3-dibromopropane, where intramolecular reaction during the second alkylation step at N7 forms a six-membered ring. The product (**15**) is a very poor activator of the olfactory channel relative to **6** and is ~10-fold less potent than cGMP. This is likely a result of the formation of a positive charge in the purine ring system and not steric clash due to the presence of the propyl group linking N7 and C8-S.

The finding that phenyl vinyl sulfone reacts with **13** to give an N1 adduct (**14**) led us to pursue alternative routes to modification of N1. While reaction of iodomethane with cGMP in NaOH was reported to yield 1-methyl-cGMP in 44% yield,[30] we found 1-Me-cGMP to be a minor product compared with **3** and **4**. Comparing the activation of olfactory CNG channels by **3** and **4** indicates that methylation of N1 causes a 4- to 8-fold loss of affinity. This is similar to the 5-fold reduction in apparent affinity observed for phenylsulfonylethyl modification at N1 (compare compounds **13** and **14**). A similar decrease in potency for activation of cGMP-dependent protein kinase PKGI was reported for 1-methyl-cGMP relative to cGMP.[41] That substituents of such disparate size at N1, as in **4** and **14**, yield similar changes in apparent affinity for the olfactory CNG channel is in contrast with the size dependence observed at 2′-O with **3**, **5**, and **16**. This could indicate available space in this region of the binding site or that the N1 substituent of **14** makes favorable contacts with the protein that compensate for steric clashes. Despite the loss of affinity due to the N1 substituent of **14**, it remains ~3-fold more potent for activation of both heteromeric olfactory and retinal rod CNG channels than cGMP.

These studies introduce a new method for N7 and N1 modification of cGMP, enhance our understanding of the interaction between cGMP and CNG channels, and point to two additional sites on cGMP that are amenable to modification. Such modifications can be exploited for increasing membrane permeability, attachment of polymers and/or fluorescent probes, and future development of agonists selective for CNG channels as well as other cyclic nucleotide binding proteins.

Experimental Section

Channel Expression and Electrophysiology. Inside-out excised patch recordings were made at room temperature using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). A RSC-100 rapid solution changer (Molecular Kinetics, Pullman, WA) was used to control the solution bathing the patch. Electrodes pulled from borosilicate glass and heat-polished had resistances between 1.5 and 3.2 MΩ. Currents were low-pass-filtered at 1 kHz with an eight-pole Bessel filter and digitized at 4 kHz. pCLAMP-8 software (Axon Instruments) was used for data acquisition and analysis, and currents in the absence of cGMP were subtracted from all traces. All dose−response relations were fit with the Hill equation,

$$\frac{I}{I_{max}} = \frac{1}{1 + \left(\frac{K_{1/2}}{[\text{cyclic nucleotide}]}\right)^n}$$

where I/I_{max} is the ratio of current elicited by a particular concentration of a cyclic nucleotide derivative divided by the current

elicited by saturating cAMP or cGMP, *n* is the Hill coefficient, and $K_{1/2}$ is the concentration of cyclic nucleotide that gives half-maximal current.

Heteromeric retinal rod CNG channels were produced by coexpression of CNGA1 and CNGB1 in *Xenopus* oocytes. pGEM-HE expression plasmids containing CNGA1 or CNGB1 cDNA were linearized with NheI and used as a template for production of CNG channel cRNA using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). *Xenopus* oocytes were microinjected with 5−25 ng of cRNA (2:1 ratio of CNGB1 to CNGA1) and incubated at 18 °C. Recordings were made within 1−6 days after injection. Pipet and bath solutions were identical and consisted of the following (mM): 130 NaCl, 2 HEPES, 1 EGTA, and 0.02 EDTA, pH 7.6. Formation of heteromeric channels was confirmed by testing for sensitivity to block by *l*-*cis*-diltiazem and by measuring the fractional current elicited by 8 mM cAMP, as described previously.[42] Heteromeric olfactory CNG channels were expressed by transient transfection of HEK-293 cells with CNGA2, CNGA4, and CNGB1b cDNAs in the mammalian expression plasmid, pcDNA3. Mirus *Trans*IT-293 (Mirus Corporation, Madison, WI) was used for transfection according to the manufacturer's instructions, employing a ratio of 1:1.75:1.75 CNGA2/CNGA4/CNGB1b. A CD4 expression plasmid was included ($^1/_{10}$ total DNA) to aid in the identification of transfected cells. Cells were incubated with anti-CD4 antibody-coated beads (Invitrogen, Carlsbad, CA) for 2 min prior to recording. Recordings were made 1−3 days after transfection. The extracellular solution consisted of the following (mM): 140 NaCl, 5 KCl, 10 Hepes, 1 EGTA, pH to 7.4 with NaOH. The intracellular solution consisted of the following (mM): 140 KCl, 5 NaCl, 10 Hepes, 1 EGTA, pH to 7.4 with KOH. The production of heteromeric channels was confirmed by measuring the $K_{1/2}$ for activation by cAMP and current rectification under these bi-ionic conditions as described previously.[31]

Chemistry. All small-molecule reagents, including N2-butyryl-cGMP (**2**), 2′-*O*-butyryl-cGMP (**5**), 8-pCPT-cGMP (**13**), and 8-pCPT-cAMP (**18**), were obtained from Sigma-Aldrich, and solvents were from Fisher Scientific. NHS-PEG_{3400}-VS was obtained from Shearwater Polymers (Huntsville, AL). 2′-*O*-Methyl-8-pCPT-cAMP (**19**) was from Biolog (Bremen, Germany). 8-SH-cGMP (**6**) was synthesized as described previously.[16] Reactions were followed by analytical reverse-phase (RP) HPLC (Xterra RP8, 4.6 mm × 250 mm, 5 μm column, Waters, Milford, MA) or by thin layer chromatography (TLC) with visualization by UV shadowing. Preparative RP-HPLC was performed using an Xterra Prep RP8, 19 mm × 100 mm, 5 μm column (Waters, Milford, MA) with a water−methanol gradient (5 mM ammonium acetate, pH 5). Preparative anion exchange (AE) HPLC was performed using an RSIL ANION 10U, 22 mm × 250 mm column (Alltech, Deerfield, IL). All NMR spectra were recorded on a 400 MHz Bruker Avance spectrometer. The chemical shifts for ^{1}H and ^{13}C are reported in parts per million relative to external TMS via reference to signals from residual protons in the deuterated solvent. The purity of all compounds (except for those commercially available) was analyzed using two modes of analytical HPLC and is presented in the Supporting Information.

General Method for Synthesis of Methylated cGMP Derivatives. This method follows closely the method of Tazawa et al. for preparation of 2′-*O*-methyl-cAMP with some modifications.[29] Briefly, 100 mg of cGMP (**1**) (272 μmol) was dissolved in 4 mL of 4 N NaOH, and 10 mL of DMF was added. To this mixture was added 2 mL of iodomethane (32 mmol) with vigorous stirring. Rapid addition of iodomethane results in the evolution of heat and leads primarily to 1,2′-*O*-dimethyl-cGMP (**4**), whereas slow dropwise addition of iodomethane yields primarily 2′-*O*-methyl-cGMP (**5**). The mixture was diluted to 1 L with H_2O, and 5 g of charcoal was added. The charcoal was washed with H_2O on a Buchner funnel, and the products were eluted with 2% NH_4OH in 50% ethanol. Alternatively, the method of Miller et al. was employed without modification.[30] The products were purified on a DEAE-cellulose (DE-52) column, eluting with a gradient of 0 to 1 N ammonium acetate, pH 7, followed by preparative RP-HPLC.

2-Amino-9-[(4a*R*,6*R*,7*R*,7a*R*)-2-hydroxy-7-methoxy-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl]-1,9-dihydro-6*H*-purin-6-one (2′-*O*-Methylguanosine Cyclic 3′,5′-Phosphate) (3). White solid, yield 10%. ^{1}H NMR (400 MHz, D_2O): δ 3.53 (s, 3H, $-CH_3$), 4.16 (td, *J* = 10.3, 4.4 Hz, 1H, H4′), 4.25 (td, *J* = 10.0, 1.8 Hz, 1H, H5′b), 4.41−4.49 (m, 2H, H2′, H5′a), 5.02 (ddd, *J* = 9.9, 5.5, 2.3 Hz, 1H, H3′), 6.04 (s, 1H, H1′), 7.87 (s, 1H, H8). ^{13}C NMR (100 MHz, D_2O): δ 58.9, 67.9 (d, $^2J_{C5',P}$ = 6.9 Hz), 72.6 (d, $^3J_{C4',P}$ = 4.0 Hz), 77.6 (d, $^2J_{C3',P}$ = 4.3 Hz), 81.4 (d, $^3J_{C2',P}$ = 7.9 Hz), 90.4, 117.2, 138.8, 151.8, 154.5, 159.6. MS (ESI) *m*/*z* 358.2 $(MH)^+$.

2-Amino-9-[(4a*R*,6*R*,7*R*,7a*R*)-2-hydroxy-7-methoxy-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl]-1-methyl-1,9-dihydro-6*H*-purin-6-one (1„2′-*O*-Dimethylguanosine Cyclic 3′,5′-Phosphate) (4). White solid, yield 36%. ^{1}H NMR (400 MHz, D_2O): δ 3.44 (s, 3H, $-CH_3$), 3.54 (s, 3H, $-CH_3$), 4.18 (td, *J* = 10.0, 4.4 Hz, 1H, H4′), 4.26 (td, *J* = 9.9, 1.6 Hz, 1H, H5′b), 4.42−4.50 (m, 2H, H2′, H5′a), 4.98−5.02 (ddd, *J* = 9.8, 5.5, 2.4 Hz, 1H, H3′), 6.03 (s, 1H, H1′), 7.95 (s, 1H, H8). ^{13}C NMR (100 MHz, D_2O): δ 29.21, 58.91, 67.9 (d, $^2J_{C5',P}$ = 7.0 Hz), 72.7 (d, $^3J_{C4',P}$ = 4.0 Hz), 77.6 (d, $^2J_{C3',P}$ = 4.4 Hz), 81.3 (d, $^3J_{C2',P}$ = 7.9 Hz), 90.5, 116.0, 138.7, 149.6, 155.6, 159.0.

Synthesis of Methyl Vinyl Sulfone Derivatives of 6. An amount of 8.4 mg (21.4 μmol) of 8-SH-cGMP (**7**) was dissolved in 700 μL of 100 mM sodium phosphate, pH 7.9, and 1.89 μL (21.5 μmol) of methyl vinyl sulfone was added. The reaction was stirred at 37 °C for 24 h. Products were purified by RP-HPLC; residual **6** eluted first followed by 8-methylsulfonylethylthio-GMP (**7**) and 7-methylsulfonylethyl-8-thio-cGMP (**8**).

2-Amino-9-[(4a*R*,6*R*,7*R*,7a*S*)-2,7-dihydroxy-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl]-8-{[2-(methylsulfonyl)ethyl]thio}-1,9-dihydro-6*H*-purin-6-one (8-[2-(Methylsulfonyl)-ethyl]thioguanosine Cyclic 3′,5′-Phosphate) (7). White solid, yield 24%. ^{1}H NMR (400 MHz, CD_3OD): δ 2.85 (s, 3H, $-CH_3$), 3.91 (td, *J* = 10.1, 4.8 Hz, 1H, H4′), 4.02 (m, 1H, H5′b), 4.12 (ddd, *J* = 21.5, 9.2, 4.7 Hz, 1H, H5′a), 4.64 (d, *J* = 5.3 Hz, 1H, H2′), 5.18 (ddd, *J* = 9.9, 5.3, 2.3 Hz, 1H, H3′), 5.63 (s, 1H, H1′). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.0, 40.5, 53.3, 65.3, 70.6, 72.3, 76.7, 92.2, 116.9, 140.9, 152.5, 153.6, 155.6. MS (ESI) *m*/*z* 482.1 $(MH)^+$.

2-Amino-9-[(4a*R*,6*R*,7*R*,7a*S*)-2,7-dihydroxy-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl]-7-[2-(methylsulfonyl-)ethyl]-8-thioxo-1,7,8,9-tetrahydro-6*H*-purin-6-one (7-[2-(Methylsulfonyl)ethyl]-8-thioxoguanosine Cyclic 3′,5′-Phosphate) (8). White solid, yield 14%. ^{1}H NMR (400 MHz, CD_3OD): δ 2.99 (s, 3H, $-CH_3$), 3.53 (td, *J* = 7.3, 2.4 Hz, 2H, $-CH_2-$), 3.92 (td, *J* = 10.2, 4.6 Hz, 1H, H4′), 4.06 (td, *J* = 9.8, 1.6 Hz, 1H, H5′b), 4.16 (ddd, *J* = 21.8, 9.0, 4.8 Hz, 1H, H5′a), 4.59 (d, *J* = 5.56 Hz, 1H, H2′), 4.70 (t, *J* = 7.5 Hz, 2H, $-CH_2-$), 5.21 (ddd, *J* = 9.9, 5.4, 2.2 Hz, 1H, H3′), 6.39 (s, 1H, H1′). MS (ESI) *m*/*z* 482.1 $(MH)^+$.

Synthesis of Phenyl Vinyl Sulfone Derivatives of 6. An amount of 14.2 mg (35.9 μmol) of 8-SH-cGMP (**6**) was dissolved in 100 mM sodium phosphate, pH 7.9, and 13.4 mg (79.7 μmol) of phenyl vinyl sulfone was dissolved in methanol. The two solutions were combined (final solvent composition: 38% methanol and 63 mM sodium phosphate) and stirred at 37 °C for 24 h. Products were purified by RP-HPLC; residual **6** eluted first followed by 8-phenylsulfonylethylthio-GMP (**9**) and 7-phenylsulfonylethyl-8-thio-cGMP (**10**).

2-Amino-9-[(4a*R*,6*R*,7*R*,7a*S*)-2,7-dihydroxy-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl]-8-{[2-(phenylsulfonyl)ethyl]thio}-1,9-dihydro-6*H*-purin-6-one (8-[2-(Phenylsulfonyl)-ethyl]thioguanosine Cyclic 3′,5′-Phosphate) (9). White solid, yield 45%. ^{1}H NMR (400 MHz, CD_3OD): δ 3.23 (m, 2H, $-CH_2-$), 3.59 (m, 2H, $-CH_2-$), 3.98 (td, *J* = 10.0, 4.9 Hz, 1H, H4′), 4.11 (m, 1H, H5′b), 4.20 (ddd, *J* = 21.4, 9.2, 4.8 Hz, 1H, H5′a), 4.69 (d, *J* = 5.6 Hz, 1H, H2′), 5.26 (ddd, *J* = 9.9, 5.3, 2.3 Hz, 1H, H3′), 5.71 (s, 1H, H1′), 7.52 (t, *J* = 7.6 Hz, 2H, phenyl), 7.62 (tt, *J* = 7.5, 1.4 Hz, 1H, phenyl), 7.81 (m, 2H, phenyl). ^{13}C NMR (100 MHz, CD_3OD): δ 28.0, 56.6, 68.5 (d, $^2J_{C5',P}$ = 6.9 Hz), 73.1 (d, $^3J_{C2',P}$ = 8.3 Hz), 73.6 (d, $^3J_{C4',P}$ = 3.9 Hz), 78.9 (d, $^2J_{C3',P}$ = 4.2

Hz), 94.0, 118.8, 129.4, 130.8, 135.4, 140.2, 143.8, 154.4, 155.4, 158.8. MS (ESI) *m*/*z* 544.1 $(MH)^+$.

2-Amino-9-[(4a*R*,6*R*,7*R*,7a*S*)-2,7-dihydroxy-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl]-7-[2-(phenylsulfonyl-)ethyl]-8-thioxo-1,7,8,9-tetrahydro-6*H*-purin-6-one (7-[2-(Phenylsulfonyl)ethyl]-8-thioxoguanosine Cyclic 3′,5′-Phosphate) (10). White solid, yield 40%. ^{1}H NMR (400 MHz, D_2O): δ 4.08 (td, J = 10.2, 4.7 Hz, 1H, H4′), 4.16−4.41 (m, 3H, H5′b, $-CH_2-$), 4.45 (ddd, J = 21.6, 9.4, 4.7 Hz, 1H, H5′a), 4.70−4.73 (m, 3H, H2′, $-CH_2-$), 5.11 (ddd, J = 9.9, 5.6, 2.2 Hz, 1H, H3′), 6.29 (s, 1H, H1′), 7.42 (t, J = 8.0 Hz, 2H, phenyl), 7.60 (t, J = 7.6 Hz, 1H, phenyl) 7.68 (dd, J = 8.5, 1.1 Hz, 2H, phenyl). ^{13}C NMR (100 MHz, D_2O): δ 41.2, 53.0, 68.1 (d, $^2J_{C5',P}$ = 7.0 Hz), 71.3 (d, $^3J_{C2',P}$ = 8.3 Hz), 72.2 (d, $^3J_{C4',P}$ = 4.2 Hz), 77.7 (d, $^2J_{C3',P}$ = 4.2 Hz), 92.7, 105.4, 127.5, 130.0, 135.2, 138.8, 149.8, 153.2, 154.5, 166.2. MS (ESI) *m*/*z* 544.1 $(MH)^+$.

Synthesis of PEG Vinyl Sulfone Derivatives of 6. An amount of 100 mg (28.3 μmol) of a 3400 MW heterobifunctional PEG derivative, NHS-PEG_{3400}-VS, (*N*-hydroxysuccinimide-PEG_{3400}-vinyl sulfone), was dissolved in 1.85 mL of 100 mM sodium phosphate, pH 7.4, and 10 mg (22.7 μmol) of the tetracaine derivative, (3-amino-*N*-(2-ethyl)-*N*,*N*-dimethylpropan-1-aminium acetate) was added.[43] Reaction of the primary amine of the tetracaine derivative with the NHS ester was complete within 60 min. An amount of 60 mg (15.4 μmol) of this intermediate product was isolated by RP-HPLC and mixed with 11 mg (27.7 μmol) of **6** in 300 μL of 100 mM sodium phosphate, pH 7.9. The mixture was stirred for 24 h at 37 °C, and the products were purified by AE-HPLC.

8-[2-(PEG_{3400}sulfonyl)ethyl]thioguanosine Cyclic 3′,5′-Phosphate (11). White solid, 19% yield. For simplicity, only the cyclic nucleotide and aromatic tetracaine ^{1}H NMR resonances are listed. ^{1}H NMR (400 MHz, CD_3OD): δ 3.95 (td, J = 10.2, 4.8 Hz, 1H, H4′), 4.07 (td, J = 9.9, 1.5 Hz, 1H, H5′b), 4.18 (ddd, J = 21.1, 9.4, 4.7 Hz, 1H, H5′a), 4.68 (d, J = 5.1 Hz, 1H, H2′), 5.23 (ddd, J = 9.9, 5.3, 2.0 Hz, 1H, H3′), 5.68 (s, 1H, H1′), 6.45 (d, J = 8.8 Hz, 2H, H-tetracaine), 7.64 (d, J = 8.8 Hz, 2H, H-tetracaine). PEG derivatives are polydisperse, and mass spectrometry gives a Gaussian distribution of peaks with 44 mass unit spacing. The mass listed is for the central mass peak. MS (MALDI) *m*/*z* 4413.25 $(MH)^+$.

7-[2-(PEG_{3400}sulfonyl)ethyl]-8-thioxoguanosine Cyclic 3′,5′-Phosphate (12): White solid, 26% yield. For simplicity, only the cyclic nucleotide and aromatic tetracaine ^{1}H NMR resonances are listed. ^{1}H NMR (400 MHz, CD_3OD): δ 3.95 (td, J = 10.0, 4.9 Hz, 1H, H4′), 4.10 (t, J = 9.6 Hz, 1H, H5′b), 4.21 (ddd, J = 21.6, 9.0, 4.8 Hz, 1H, H5′a), 5.24 (ddd, J = 9.7, 5.3, 1.9 Hz, 1H, H3′), 6.42 (s, 1H, H1′) 6.49 (d, J = 9.1 Hz, 2H, H-tetracaine), 7.68 (d, J = 8.8 Hz, 2H, H-tetracaine). PEG derivatives are polydisperse, and mass spectrometry gives a Gaussian distribution of peaks with 44 mass unit spacing. The mass listed is for the central mass peak. MS (MALDI) *m*/*z* 4413.24 $(MH)^+$.

2-Amino-9-[(4a*R*,6*R*,7*R*,7a*S*)-2,7-dihydroxy-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl]-8-[(4-chlorophenyl)-thio]-1-[2-(phenylsulfonyl)ethyl]-1,9-dihydro-6*H*-purin-6-one (8-(4-Chlorophenyl)thio-1-[2-(phenylsulfonyl)ethyl]guanosine Cyclic 3′,5′-Phosphate) (14). An amount of 24 mg (47 μmol) of 8-pCPT-cGMP (**13**) was dissolved in 1.6 mL of 37.5% methanol, 62.5 mM sodium phosphate, pH 7.9, and 46.3 mg (276 μmol) phenyl vinyl sulfone was added. The mixture was stirred at 37 °C for 20 h. Excess phenyl vinyl sulfone was removed by chromatography on DEAE-cellulose (DE-52). **14** was isolated on RP-HPLC. White solid, 61% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 3.68 (td, J = 6.7, 1.5 Hz, 2H, $-CH_2-$), 3.98 (td, J = 10.1, 4.8 Hz, 1H, H4′), 4.14 (td, J = 9.8, 1.6 Hz, 1H, H5′b), 4.24 (ddd, J = 21.5, 9.1, 4.8 Hz, 1H, H5′a), 4.37 (t, J = 6.7 Hz, 2H, $-CH_2-$), 4.64 (d, J = 5.6 Hz, 1H, H2′), 5.22 (ddd, J = 9.8, 5.4, 2.0 Hz, 1H, H3′), 5.98 (s, 1H, H1′), 7.30 (m, 4H, pCPT), 7.41 (m, 3H, phenyl), 7.82 (m, 2H, phenyl). ^{13}C NMR (100 MHz, CD_3OD): δ 37.3, 53.1, 68.7 (d, $^2J_{C5',P}$ = 7.3 Hz), 73.0 (d, $^3J_{C2',P}$ = 8.1 Hz), 73.5 (d, $^3J_{C4',P}$ = 3.9 Hz), 79.0 (d, $^2J_{C3',P}$ = 4.2 Hz), 94.3, 118.4, 128.9, 130.6, 130.8, 132.8, 133.0, 135.0, 135.5, 140.5, 141.8, 151.9, 155.6, 157.8. MS (ESI) 654.1 $(MH)^+$.

2-Amino-10-[(4a*R*,6*R*,7*R*,7a*S*)-2,7-dihydroxy-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl]-4-oxo-3,4,6,7,8,10-hexahydro[1,3]thiazino[2,3-*f*]purin-5-ium (2-Amino-10-(*β*-D-ribofuranosyl)-4-oxo-3,4,6,7,8,10-hexahydro[1,3]thiazino[2,3-*f*]purin-5-ium Cyclic 3′,5′ Phosphate) (15). An amount of 11.7 mg (29.6 μmol) of 8-SH-cGMP (**6**) was dissolved in 1.6 mL of methanol, and 90.5 μL of 1,3-dibromopropane (888 μmol) was added. The mixture was stirred at room temperature for 48 h. The solvent and excess 1,3-dibromopropane were removed in vacuo, and the resulting residue was dissolved in H_2O. The product was purified by RP-HPLC. White solid, 91% yield. ^{1}H NMR (400 MHz, D_2O): δ 2.39 (bm, 2H, $-CH_2-$), 3.40 (t, J = 5.6 Hz, 2H, $-CH_2-$), 4.13 (td, J = 10.2, 4.7 Hz, 1H, H4′), 4.26 (td, J = 10.0, 1.5 Hz, 1H, H5′b), 4.36−4.50 (m, 3H, H5′a, $-CH_2-$), 4.78 (d, J = 5.5 Hz, 1H, H2′) 5.02 (ddd, J = 9.9, 5.5, 2.3 Hz, 1H, H3′), 5.81 (s, 1H, H1′). ^{13}C NMR (100 MHz, D_2O): δ 21.5, 26.8, 46.5, 68.0 (d, $^2J_{C5',P}$ = 7.2 Hz), 72.6 (d, $^3J_{C2',P}$ = 8.3 Hz), 73.5 (d, $^3J_{C4',P}$ = 4.1 Hz), 77.7 (d, $^2J_{C3',P}$ = 4.1 Hz), 93.4, 109.4, 147.8, 150.8, 155.2, 156.1. MS (ESI) *m*/*z* 418.1 $(MH)^+$.

2-Amino-9-{(4a*R*,6*R*,7*R*,7a*R*)-2-hydroxy-7-[2-(methylsulfonyl)-ethoxy]-2-oxotetrahydro-4*H*-furo[3,2-*d*][1,3,2]dioxaphosphinin-6-yl}-1,9-dihydro-6*H*-purin-6-one (2′-*O*-[2-(Methylsulfonyl)-ethyl]guanosine Cyclic 3′,5′-Phosphate) (16). An amount of 110 mg (300 μmol) of cGMP (**2**) was dissolved in 200 μL of 4 N NaOH, and 72 μL (800 μmol) of methyl vinyl sulfone was added. The mixture was stirred vigorously at room temperature for 1 h, diluted 7-fold with H_2O, and neutralized with HCl. The major product (**17**) was separated from unreacted **2** by RP-HPLC. White solid, yield 10%. ^{1}H NMR (400 MHz, D_2O): δ 3.13 (s, 3H, $-CH_3$), 3.44−3.57 (m, 2H, $-CH_2-$), 4.16 (m, 4H, H4′, H5′b, $-CH_2-$), 4.39 (ddd, J = 21.4, 8.7, 4.0 Hz, 1H, H5′a), 4.55 (d, J = 5.3 Hz, 1H, H2′), 4.96 (ddd, J = 9.2, 5.22, 2.0 Hz, 1H, H3′), 6.01 (s, 1H, H1′), 7.82 (s, 1H, H8). ^{13}C NMR (100 MHz, D_2O): δ 42.8, 54.9, 65.0, 67.8 (d, $^2J_{C5',P}$ = 6.9 Hz), 72.8 (d, $^3J_{C4',P}$ = 4.2 Hz), 77.8 (d, $^2J_{C3',P}$ = 4.3 Hz), 80.4 (d, $^3J_{C2',P}$ = 8.0 Hz), 90.7, 117.1, 138.8, 151.9, 154.6, 159.6. MS (ESI) *m*/*z* 450.2 $(MH)^+$.

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Supporting Information Available: Table of HPLC analysis results for compounds **3**, **4**, **6**−**12**, **14**, and **15** and complete HSQC and HMBC spectra of compounds **3**, **4**, **9**, **10**, **14**, and **15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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