Photolabile Precursors of Cyclic Nucleotides with High Aqueous Solubility and Stability

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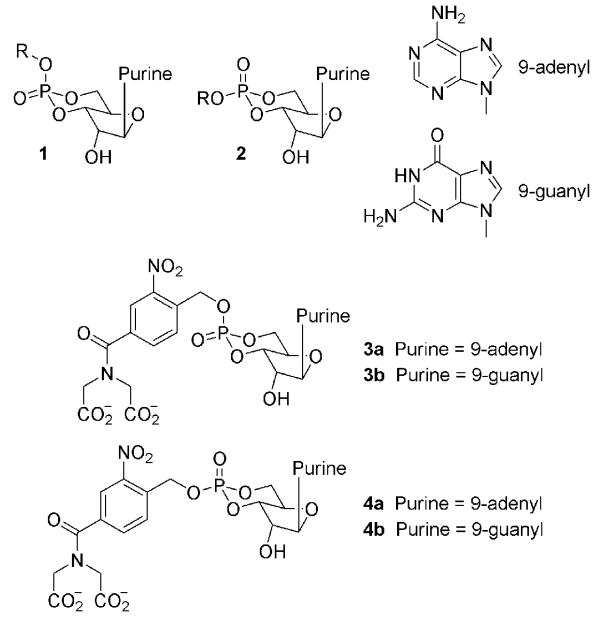
Received January 18, 2002

Photolabile phosphotriester derivatives of cyclic AMP and cyclic GMP are described, where the additional group on the phosphate is a 2-nitrobenzyl that bears an electron-withdrawing and dianionic substituent. This confers high aqueous solubility and excellent resistance to hydrolysis of the phosphotriester ($t_{1/2}$ for hydrolysis at pH 7, 22 °C, is >3 months for the axial isomers **3a** and **4a** and >1 month for the equatorial isomers **3b** and **4b**). The photolysis quantum yields are in the range 0.15−0.24, and the product release rate upon flash photolysis is 1.7 s^{-1} at pH 7.0, 20 °C.

Introduction

Cyclic adenine and guanine nucleotides have important biological functions as second messengers in intracellular signal transduction processes.[1] One approach to studying their role is to use photolabile derivatives that are inert until irradiated by a brief pulse of near-UV light.[2] A number of such "caged" derivatives have been described, in all of which the cyclic phosphate is esterified by a photolabile group, including 2-nitrobenzyl,[3a] 3,4-dimethoxy-2-nitrobenzyl,[3b,c,e,f] 1-(2-nitrophenyl)ethyl,[3c,d] 1-(3,4-dimethoxy-2-nitrophenyl)ethyl,[3c] desyl,[3g] 2-anthraquinonylmethyl,[3h] 2-naphthylmethyl,[3h] 4-(7-methoxycoumarinyl)methyl,[3h,i] and 4-(7-acyloxycoumarinyl)methyl.[3j] Useful generalizations can be drawn from these many derivatives. First, axial esters **1** (Chart 1) are significantly more resistant to hydrolysis than the comparable equatorial esters **2**. Second, stability is decreased by substituents on the caging group at its benzylic position or by electron-donating groups on its aromatic ring. Furthermore, most of the above derivatives suffer from poor aqueous solubility that can limit their utility in biological systems (see discussion in ref 3h). Their hydrophobicity is such that they tend to partition into cell membranes and cannot be assumed to retain a stable intracellular location, even if introduced to a particular cell by microinjection. In an endeavor to address these issues, we now describe new caged cyclic nucleotides **3a,b** and **4a,b** that have good aqueous solubility by virtue of the presence of two anionic centers. As an additional benefit, these compounds have been found to be unexpectedly stable with respect to hydrolysis. While our work was in progress, Hagen et al. described new variants of the 4-coumarinylmethyl caged cyclic nucleotides referenced above,[3h−j] also bearing anionic substituents, that have similarly good resistance to hydrolysis but appear somewhat less soluble than the compounds now described.[3k]

Chart 1



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10.1021/jo020040g CCC: $22.00
Published on Web 04/25/2002

Scheme 1

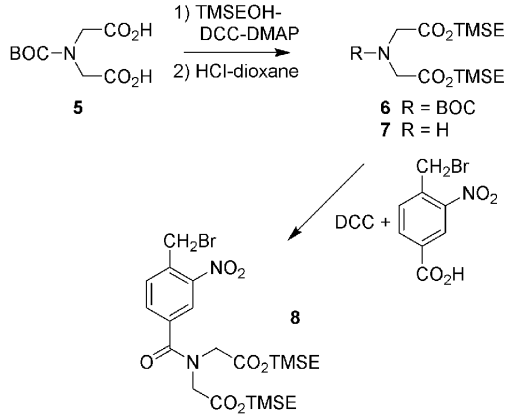
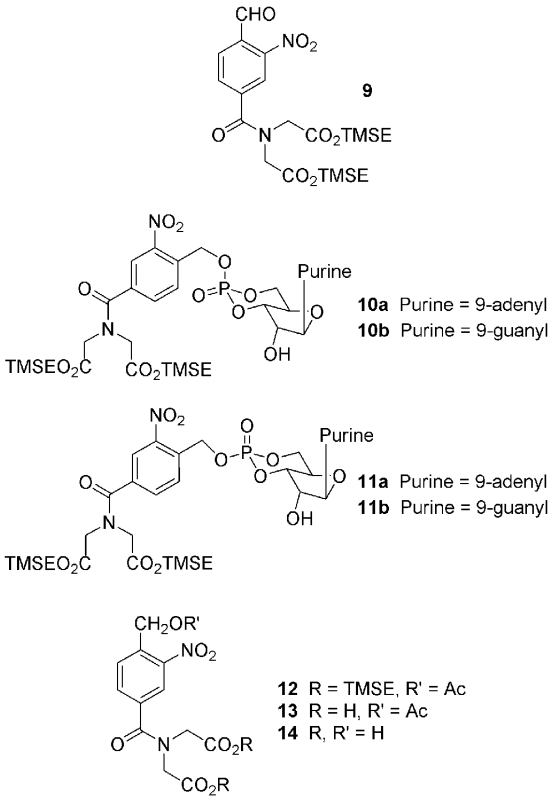


Chart 2



Results and Discussion

Synthesis of the reagent **8** required for coupling with cyclic nucleotides is shown in Scheme 1. *N*-BOC-iminodiacetic acid **5** was esterified (DCC−DMAP) with 2-(trimethylsilyl)ethanol, and the protected diester **6** was converted to amine **7** with anhydrous HCl−dioxane. DCC-mediated coupling of **7** with 4-(bromomethyl)-3-nitrobenzoic acid gave the amide **8**. Furuta et al.[4] have reported formation of the benzyl ester of cAMP by treating the free acid form of the nucleotide with benzyl bromide and Ag_2O in MeCN−DMSO, but under these conditions, the substituted benzyl bromide **8** gave only the nitroaldehyde **9** (Chart 2) in high yield, presumably derived by DMSO-mediated oxidation of **8**.[5] Related examples of such oxidations by DMSO have been reported to be promoted by silver nitrate,[6a] barium hydroxide,[6b] or sodium bicarbonate.[6c] Thus, the presence of Ag_2O under our conditions probably accounts for the efficient oxidation.

An alternate esterification procedure, described by Kataoka et al.,[7] used the tri-*n*-butylammonium salt of cAMP, benzyl bromide, and sodium carbonate in dimethylacetamide. When applied to esterification of cAMP with the substituted benzyl bromide **8**, these conditions successfully gave a mixture of **10a** and its equatorial isomer **11a** (∼3:1 ratio) in overall 25−30% yield. The acetate **12**, prepared for purposes of quantification (see below), was readily obtained from bromide **8** by similar displacement with sodium acetate.

Stereochemical assignments for **10a** and **11a** were made from the ^{31}P NMR spectra by analogy with previous data on cNMP esters, in which the axial isomer has been assigned as having the more upfield ^{31}P resonance.[3a,b] Attempted cleavage of the TMSE esters from **10a** by treatment with (TBA)F caused extensive degradation, but they were cleanly removed from **10a**, **11a**, and **12** by brief exposure to TFA. This effective but little-used protocol allows residues from the protecting group to be removed cleanly during evaporation of TFA at the end of the reaction.[8,9] Final purification of the caged cyclic nucleotides was achieved by reverse-phase HPLC.

The esterification of cyclic GMP gave **10b** and its equatorial isomer **11b** in similar ratio but in much reduced yield, apparently because of poor solubility of the nucleotide. Separation of these isomers was not achieved, and the mixture, after chromatography, was directly subjected to TFA deprotection. The individual isomers **3b** and **4b** were then readily separated by preparative reverse phase HPLC.

Accurate quantification of aqueous solutions of the caged cyclic nucleotides requires the extinction coefficients for the different compounds. These data were estimated using the model compound **13**, obtained as a clean solid after deprotection of **12**, to provide a spectrum for the caging group (calibrated in terms of absorbance coefficients at all wavelengths) that was added to comparable spectra of adenine or guanine nucleotides, thereby giving computed spectra for **3a/4a** and **3b/4b** (see Supporting Information). The calculated extinction coefficients for **3a/4a** and **3b/4b** were 21 300 M^{-1} cm^{-1} (λ_{max} 258 nm) and 20 300 M^{-1} cm^{-1} (λ_{max} 251 nm), respectively. Other important physicochemical properties of caged compounds are their aqueous solubility and stability and the rate and efficiency of release of the bioeffector species upon flash photolysis. The limit of aqueous solubility was not established, in part because of limited amounts of the compounds, but dipotassium salts of all the compounds were easily soluble to at least 55 mM concentration.

The stabilities of **3a,b** and **4a,b** in aqueous buffers were determined by incubating solutions at pH values

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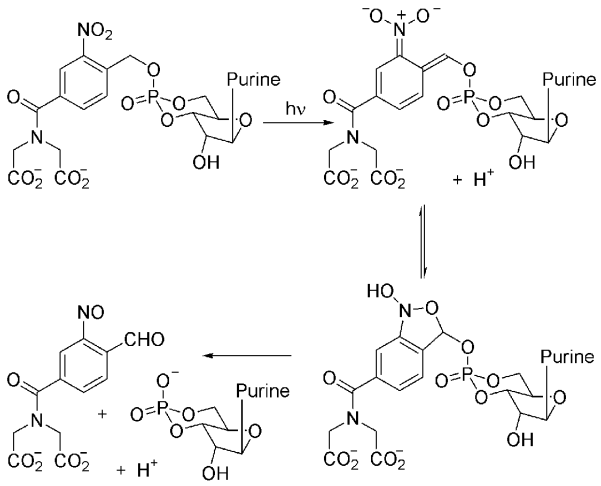
Table 1. Hydrolysis Rates for Caged Cyclic Nucleotides

	pseudo-first-order rate constant, h^{-1}		
compd	pH 7.00	pH 8.01	pH 9.04
3a	2.3×10^{-4}	3.2×10^{-4}	8.0×10^{-4}
3b	2.0×10^{-4}	3.0×10^{-4}	3.4×10^{-4}
4a	6.8×10^{-4}	9.6×10^{-4}	1.3×10^{-3}
4b	7.0×10^{-4}	8.7×10^{-4}	9.4×10^{-4}

Scheme 2. Mechanism for Photorelease of Cyclic Nucleotides from Their Caged Precursors



in the range 7−9 in the dark at 22 °C. Samples were removed at time points up to 4 days and analyzed by anion exchange HPLC. Only peaks corresponding to the appropriate cyclic nucleotide and the expected alcohol **14** were detected. The estimated pseudo-first-order rate constants for hydrolysis are shown in Table 1 and confirm the expected higher stability of the axial isomers, which have half-lives for hydrolysis in the region of 3000 h at pH 7 and ambient temperature. This aqueous stability is at least 5-fold better than for any previously reported caged cyclic nucleotides. The data in Table 1 also show that the hydrolysis rates increase only slowly with increasing pH, presumably because of a combination of spontaneous and base-catalyzed hydrolysis.

The quantum yield for photorelease of cyclic nucleotides from the more stable axial isomers was evaluated by comparison in mixed solution with the well-characterized P^3-1-(2-nitrophenyl)ethyl ester of adenosine 5′-triphosphate (caged ATP).[10] The values determined for **3a,b** were 0.16 and 0.24, respectively. Stoichiometric formation of free cyclic nucleotides upon photolysis was confirmed by RP-HPLC analysis. The rate of product release following flash irradiation was also determined. It is important to recall that the 2-nitrobenzyl photochemistry involves dark reactions after the photochemical event that are rate limiting for release of the bioeffector.[2] Thus, the rate of release of bioactive products from 1-(2-nitrophenyl)ethyl esters of cyclic nucleotides is controlled by the decay rate of the light-induced *aci*-nitro anion intermediate,[3c] which can be monitored by time-resolved absorption spectrophotometry at 406 nm. The expected photolysis pathway, including the *aci*-nitro intermediate, is shown in Scheme 2. This mechanism is based on that previously established for substituted 2-nitrobenzyl esters of other nucleoside phosphates[10] but modified to incorporate findings of recent studies by Wirz and colleagues[11] regarding the mode of decay of *aci*-nitro anions. Laser-initiated photolysis (320 nm) of **3a** or **3b** at 20 °C, pH 7.0, in the presence or absence of 2 mM dithiothreitol (DTT; added to sequester the liberated nitroso byproduct) generated an "instantaneous" rise in 406 nm absorbance, followed by a single-exponential decay of 400 ms half-time, corresponding to a rate of 1.7 s^{-1} that is believed to represent the rate of product release.

Conclusion

The properties of **3a,b** include high solubility, excellent stability, and reasonably efficient photorelease under appropriate conditions for intracellular applications. At physiologically relevant pH values they should remain localized in the cellular compartment to which they are applied, since they will be present as biomembrane-impermeant dianions. They share the relatively slow rates of product release upon flash photolysis that are common to other nitrobenzyl phosphotriesters of cyclic nucleotides,[3c] but for many applications the favorable properties of water solubility and membrane impermeance will likely be more significant than the slow photorelease rate.

Experimental Section

General Methods. Caged ATP was prepared as described previously,[10] and all other reagents were obtained commercially and used without further purification. Silica gel was used for flash chromatography. Analytical reverse phase HPLC used a 125 × 4.6 mm C_{18} column [mobile phase CH_3CN−10 mM K phosphate, pH 5.5 (5:95 v/v)] and anion exchange HPLC was on a 125 × 4.6 mm SAX column [mobile phase CH_3CN−10 mM K phosphate, pH 5.5 (15:85 v/v)]. Flow rates were 1.5 mL min^{-1} and detection was by absorbance at 254 nm. Preparative reverse phase HPLC was on a 120 × 0.78 cm column, with flow rate at 2 mL min^{-1}. The composition of mobile phases for preparative HPLC is described at relevant points below. ^1H and ^{13}C NMR spectra were recorded in deuteriochloroform with TMS as internal reference, unless otherwise specified. ^{31}P NMR spectra were referenced indirectly to trimethyl phosphate, following IUPAC recommendations.[12] On this scale, the chemical shift of 85% H_3PO_4 used as the reference in previous work to assign stereochemistry of cyclic nucleotide triesters[3a,b] is δ −3.52 ppm.[13] Organic extracts were dried over $MgSO_4$, and solvents were removed under reduced pressure. Buffer solutions were prepared from solutions of appropriate acids at the specified molarities and adjusted to the required pH by addition of concentrated KOH.

***N*-BOC-Iminodiacetic Acid Bis[2-(trimethylsilyl)ethyl] Ester (6).** A solution of DCC (1.07 g, 5.19 mmol) in CH_2Cl_2 (10 mL) was added to an ice-cold solution of *N*-(carboxymethyl)-*N*-[(1,1-dimethylethoxy)carbonyl]glycine[14] (**5**) (0.55 g, 2.36 mmol), 2-(trimethylsilyl)ethanol (0.56 g, 4.75 mmol), and DMAP (0.58 g, 4.75 mmol) in CH_2Cl_2 (40 mL). The mixture was stirred at 0 °C for 1 h and then kept at room temperature overnight and filtered. The filtrate was washed with 1 M $NaHSO_4$ (100 mL), saturated $NaHCO_3$ (60 mL), and H_2O (60 mL), dried, and evaporated. Flash chromatography (98:2 CH_2Cl_2−EtOAc) gave **6** as an oil (0.917 g, 90%): ^1H NMR δ 4.13−4.32 (m, 4H), 4.08 (s, 2H), 3.98 (s, 2H), 1.44 (s, 9H), 0.90−1.09

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(m, 4H), 0.04 (s, 18H); ^{13}C NMR δ 170.2, 170.0, 155.3, 81.1, 63.62, 63.58, 49.9, 49.3, 28.4, 17.6, 17.5, −1.30; HRMS (CI) *m*/*z* 432.2238 (M − H), calcd for $C_{19}H_{38}NO_6Si_2$ 432.2230.

Iminodiacetic Acid Bis[2-(Trimethylsilyl)ethyl] Ester (7). A solution of 4 M HCl in anhydrous dioxane (7 mL) was added to **6** (0.34 g, 0.78 mmol), and the mixture was stirred at room temperature for 1 h. The solvent was evaporated, and the residue was dissolved in water (60 mL). The solution was adjusted to pH 8 with 5% aqueous ammonia, saturated with NaCl, and extracted with CH_2Cl_2 (3 × 60 mL). The organic layer was washed with brine (60 mL), dried, and evaporated. The crude product was purified on a short silica gel column (92:8 CH_2Cl_2−EtOAc) to give **7** as an oil (0.25 g, 95%). In subsequent runs the crude material was used without chromatography: ^{1}H NMR δ 4.25−4.13 (m, 4H), 3.41 (s, 4H), 2.24 (s, 1H), 0.86−1.09 (m, 4H), 0.00 (s, 18H); ^{13}C NMR δ 169.4, 60.7, 47.8, 14.9, −3.9; HRMS (CI) *m*/*z* 334.1870 (M + H), calcd for $C_{14}H_{32}NO_4Si_2$ 334.1856.

***N*-(4-(Bromomethyl)-3-nitrobenzoyl)iminodiacetic Acid Bis[2-(trimethylsilyl)ethyl] Ester (8).** A solution of **7** (130 mg, 0.39 mmol) and 4-(bromomethyl)-3-nitrobenzoic acid[15] (101 mg, 0.39 mmol) in anhydrous CH_2Cl_2 (10 mL) at 0 °C was treated with a solution of DCC (88.6 mg, 0.43 mmol) in CH_2Cl_2 (4 mL). The reaction was stirred at 0 °C for 30 min and then left at room temperature overnight and filtered. The evaporated filtrate was flash chromatographed (98:2 CH_2Cl_2−EtOAc) to give **8** as an oil (168 mg, 75%): ^{1}H NMR δ 8.12 (d, *J* = 1.8 Hz, 1H), 7.70 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.62 (d, *J* = 7.8 Hz, 1H), 4.80 (s, 2H), 4.21−4.28 (m, 6H), 4.05 (s, 2H), 0.95−1.05 (m, 4H), 0.03 (s, 9H), 0.01 (s, 9H); ^{13}C NMR δ 166.7, 166.4, 166.2, 145.4, 133.9, 132.2, 130.7, 129.7, 121.8, 62.2, 61.6, 49.4, 45.5, 25.6, 15.0, −3.97; HRMS (CI) *m*/*z* 574.1166, calcd for $C_{22}H_{35}BrN_2O_7Si_2$ 574.1160.

***N*-(4-Formyl-3-nitrobenzoyl)iminodiacetic Acid Bis[2-(trimethylsilyl)ethyl] Ester (9).** A solution of cyclic AMP monohydrate (20 mg, 0.056 mmol) and **8** (32.4 mg, 0.056 mmol) in CH_3CN (2 mL) and DMSO (2 mL) was stirred under N_2, and Ag_2O (26.2 mg, 0.113 mmol) was added. The mixture was stirred at 65 °C in the dark for 16 h and then filtered, and the solids were washed with CH_2Cl_2. The filtrate was evaporated and then mixed with H_2O (20 mL), saturated with NaCl, and extracted with CH_2Cl_2 (3 × 10 mL). The organic extract was washed with brine (3 × 10 mL), dried, and evaporated. Flash chromatography (95:5 CH_2Cl_2−EtOAc) gave **9** as a gum (25.7 mg, 90%): ^{1}H NMR δ 10.41 (s, 1H), 8.21 (d, *J* = 1.6, 1H), 7.98 (d, *J* = 7.5, 1H), 7.84−7.87 (m, 1H), 4.21−4.30 (m, 6H) 4.01 (s, 2H), 0.94−1.06 (m, 4H), 0.05 (s, 9H), 0.02 (s, 9H); ^{13}C NMR δ 187.3, 168.6, 168.6, 168.5, 149.4, 140.2, 132.3, 132.1, 130.3, 123.3, 64.7, 64.1, 51.65, 47.85, 17.4, −1.55; HRMS (CI) *m*/*z* 510.1856, calcd for $C_{22}H_{34}N_2O_8Si_2$ 510.1854.

Adenosine 3′,5′-Cyclic([*R*$_P$]-{4-[*N*,*N*-bis(2-(trimethylsilyl)ethoxycarbonylmethyl)carbamoyl]-2-nitrophenyl}-methyl Phosphate (10a) and [*S*$_P$] Isomer (11a). Cyclic AMP (free acid, 108 mg, 0.33 mmol) was suspended in EtOH (2 mL) and treated with tri-*n*-butylamine (95 μL, 0.4 mmol) in EtOH (2 mL). The mixture was evaporated to dryness, suspended in EtOH (4 mL), and evaporated to dryness again and then dried in vacuo overnight. The solid residue was dissolved in *N*,*N*-dimethylacetamide (10 mL), treated with Na_2CO_3 (69.6 mg, 0.66 mmol), and stirred under N_2 at 80 °C. A solution of **8** (196 mg, 0.33 mmol) in *N*,*N*-dimethylacetamide (1 mL) was added, and after 1 h at 80 °C the solvent was evaporated in vacuo. The residue was suspended in acetonitrile and filtered, and the insoluble material was washed with EtOH. The combined filtrates were evaporated, and the residue was redissolved in MeOH (1 mL), diluted with CH_2Cl_2 (15 mL), and filtered to remove a flocculent precipitate. Flash chromatography of the residue (step gradient from CH_2Cl_2 to 9:1 CH_2Cl_2−MeOH) gave axial isomer **10a** (44 mg) as the faster-migrating product and two mixed fractions that were enriched in the axial or equatorial isomers. These fractions were each chromatographed again to give additional **10a** (9 mg) and pure **11a** (17 mg). Data for **10a**: ^{1}H NMR (9:1 $CDCl_3$−MeOH-d_4, acetone reference) δ 8.19 (d, *J* = 1.5 Hz, 1H), 8.13 (s, 1H), 7.86 (s, 1H), 7.82 (d, *J* = 7.9 Hz, 1H), 7.76 (dd, *J* = 7.9 Hz, 1.5 Hz, 1H), 5.91 (s, 1H), 5.56 (d, $J_{H,P}$ = 7.3 Hz, 2H), 5.27 (dd, *J* = 8.7 Hz, 4.7 Hz, 1H), 4.75 (d, *J* = 4.9 Hz, 1H), 4.59−4.64 (m, 1H), 4.36−4.40 (m, 2H), 4.16−4.22 (m, 4H), 4.00 (s, 2H), 3.37 (s, 2H), 0.97 (t, *J* = 8.7 Hz, 2H), 0.91 (t, *J* = 8.7 Hz, 2H), −0.03 (s, 9H), −0.06 (s, 9H); ^{31}P NMR (9:1 $CDCl_3$−MeOH-d_4, TMP reference) δ −8.65; HRMS (FAB) *m*/*z* 824.2511 (M + 1), calcd for $C_{32}H_{47}N_7O_{13}PSi_2$ 824.2508. Data for **11a**: ^{1}H NMR (9:1 $CDCl_3$−MeOH-d_4, acetone reference) δ 8.19 (d, *J* = 1.4 Hz, 1H), 8.18 (s, 1H), 7.87 (s, 1H), 7.81 (d, *J* = 8.1 Hz, 1H), 7.75 (dd, *J* = 8.1 Hz, 1.4 Hz, 1H), 5.93 (s, 1H), 5.67 (d, $J_{H,P}$ = 7.7 Hz, 2H), 5.31 (dd, *J* = 8.8 Hz, 4.9 Hz, 1H), 4.70 (d, *J* = 4.9 Hz, 1H), 4.63−4.68 (m, 1H), 4.48−4.53 (m, 2H), 4.15−4.22 (m, 4H), 3.99 (s, 2H), 3.37 (s, 2H), 0.97 (t, *J* = 8.6 Hz, 2H), 0.91 (t, *J* = 8.6 Hz, 2H), −0.02 (s, 9H), −0.05 (s, 9H); ^{31}P NMR (9:1 $CDCl_3$−MeOH-d_4, TMP reference) δ −6.89; HRMS (FAB) *m*/*z* 824.2511 (M + 1), calcd for $C_{32}H_{47}N_7O_{13}PSi_2$ 824.2508.

Adenosine 3′,5′-Cyclic([*R*$_P$]-{4-[*N*,*N*-bis(carboxymethyl)carbamoyl]-2-nitrophenyl}methyl Phosphate (3a) and [*S*$_P$] Isomer (4a). TFA (1 mL) was added to **10a** (107 mg, 130 μmol), and the solution was stirred at room temperature for 30 min. The TFA was removed in vacuo, the residue was reevaporated with MeOH (×3), and solid K_2HPO_4 (97.4 mg) was added to provide a 2-fold molar excess relative to carboxylate groups. The material was suspended in water, adjusted to pH 6.7 with dilute KOH, whereupon the solid dissolved. The solution was washed with CH_2Cl_2 and lyophilized. The residue was dissolved in water (2 mL) and applied to the preparative reverse phase HPLC column (preequilibrated with 10 mM K phosphate, pH 5.5) and eluted with the same buffer. After passage of 50 mL, the mobile phase was changed to water. The product began to elute (single peak) after 150 mL of H_2O and was collected in a further 250 mL and lyophilized to give **3a** (113 μmol, 87%) that was redissolved in water and stored at −20 °C. The elution protocol, originally developed for purification of other caged compounds,[16] enables recovery as its potassium salt but free of extraneous ions. Analytical RP-HPLC showed a single peak, t_R 7.7 min, and 10.2 min on anion exchange HPLC: ^{1}H NMR (D_2O) δ 8.37 (d, *J* = 1.6 Hz, 1H), 8.18 (s, 1H), 8.14 (s, 1H), 7.94 (d, *J* = 8.1 Hz, 1H), 7.90 (dd, *J* = 8.1 Hz, 1.6 Hz, 1H), 6.18 (s, 1H), 5.68 (d, $J_{H,P}$ = 7.7 Hz, 2H), 5.08 (ddd, *J* = 9.9 Hz, 5.1 Hz, $J_{H,P}$ = 1.3 Hz, 1H), 4.82 (ddd, J_{gem} = 9.3 Hz, J_{vic} = 5.1 Hz, $J_{H,P}$ = 22.2 Hz, 1H), 4.79 (d, *J* = 5.0 Hz, 1H), 4.62 (t, *J* = 9.8 Hz, 1H), 4.49 (dt, *J* = 10.3 Hz, 4.8 Hz, 1H), 4.12 (s, 2H), 3.87 (s, 2H); ^{31}P NMR (D_2O, TMP reference) δ −7.65; HRMS (FAB) *m*/*z* 624.1092 (M + 1), calcd for $C_{22}H_{23}N_7O_{13}P$ 624.1091.

The equatorial isomer **11a** (36 mg, 44 μmol) was processed similarly to give **4a** (32 μmol, 73%). Its retention time on analytical RP-HPLC was 8.5 min, with 9.8 min on anion exchange HPLC: ^{1}H NMR (D_2O) δ 8.39 (d, *J* = 0.8 Hz, 1H), 8.22 (s, 1H), 8.21 (s, 1H), 7.90−7.94 (m, 2H), 6.20 (s, 1H), 5.70 (d, $J_{H,P}$ = 8.3 Hz, 2H), 5.26 (dd, *J* = 9.0 Hz, 5.1 Hz, 1H), 4.84−4.90 (m, 2H), 4.69 (t, *J* = 10.3 Hz, 1H), 4.64 (dt, *J* = 10.4 Hz, 5.4 Hz, 1H), 4.13 (s, 2H), 3.90 (s, 2H); ^{31}P NMR (D_2O, TMP reference) δ −6.48; HRMS (FAB) *m*/*z* 624.1095 (M + 1), calcd for $C_{22}H_{23}N_7O_{13}P$ 624.1091.

Guanosine 3′,5′-Cyclic([*R*$_P$]-{4-[*N*,*N*-bis(carboxymethyl)carbamoyl]-2-nitrophenyl}methyl Phosphate (3b) and [*S*$_P$] Isomer (4b). Cyclic GMP (Na^+ salt) was converted to its triethylammonium salt by ion-exchange chromatography over DEAE cellulose eluted with a gradient of triethylammonium bicarbonate (10−250 mM). Fractions containing the nucleotide were rotary evaporated under reduced pressure (1 mmHg), and residual triethylammonium bicarbonate was removed by repeated evaporation of methanol. The nucleotide was redissolved in water−methanol (1:9 v/v) and stored at −20 °C until used. An aliquot of this solution containing cGMP (0.49 mmol) was mixed with a solution of tri-*n*-butylamine (0.139 mL, 0.58

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mmol) in EtOH (2.4 mL), evaporated to dryness and reevaporated twice from EtOH (2 × 6 mL), and then kept under vacuum overnight. A solution of the dried residue in dry *N*,*N*-dimethylacetamide (14 mL) was mixed with Na_2CO_3 (80 mg, 0.75 mmol) and stirred under N_2 at 80 °C. A solution of **8** (0.425 g, 0.74 mmol) in *N*,*N*-dimethylacetamide (1.5 mL) was added, the mixture was heated for 30 min and then cooled, and the solvent was evaporated in vacuo. The residue was dissolved in MeOH (6 mL), diluted with $CHCl_3$ (120 mL), washed with water and brine, dried, and evaporated. Flash chromatography (91:9 CH_2Cl_2−MeOH) gave a pale gum (68.6 mg). This contained a mixture of the isomers **10b** and **11b** that was not further characterized but directly deprotected by TFA treatment. The crude product was neutralized as potassium salts, as described for the individual cAMP derivatives. The aqueous solution (pH 6.5−7.0) was applied to the preparative reverse phase HPLC column [equilibrated with CH_3CN−10 mM K phosphate, pH 5.5 (5:95 v/v)] and developed isocratically with the same solvent. The axial isomer **3b** eluted after ∼160 mL in a volume of 110 mL followed, with essentially no overlap, by the equatorial isomer **4b** in a further 380 mL. After pH adjustment to 6.5−7.0 with 1 M KOH, the eluates were lyophilized and the dried residues were each dissolved in water (3.2 mL) and separately desalted on the same preparative HPLC column, as described above for the cyclic AMP derivatives. Recoveries of the purified isomers were 0.021 and 0.013 mmol of the axial **3b** and equatorial **4b** isomers, respectively. The elution patterns of the two isomers under the desalting conditions are essentially identical. Analytical HPLC (anion exchange) showed each isomer as a single peak of almost identical retention time (13.1 and 12.8 min for **3b** and **4b**, respectively) and reverse phase HPLC gave t_R 3.0 and 5.0 min for **3b** and **4b**, respectively.

The axial isomer **3b** had ^{1}H NMR (D_2O; acetone reference) δ 8.37 (d, J = 1.5 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.90 (dd, J = 8.1 Hz, 1.5 Hz, 1H), 7.86 (s, 1H), 6.03 (s, 1H), 5.69 (d of AB quartet, J_{gem} = 13.6 Hz, $J_{H,P}$ = 6.7 Hz, 2H), 5.18 (dd, J = 10.0 Hz, 5.2 Hz, 1H), 4.83 (d, J = 5.5 Hz, 1H), 4.65 (t, J = 10.0 Hz, 1H), 4.45 (dt, J = 10.4 Hz, 4.9 Hz, 1H), 4.13 (s, 2H), 3.89 (s, 2H). Signals for one further proton were obscured by the HOD peak. ^{31}P NMR (D_2O; TMP reference): −7.66. HRMS (FAB): *m*/*z* 662.0885 (M + 2H + Na); calcd for $C_{22}H_{20}N_7O_{14}P$ + 2H + Na, 662.0860. The equatorial isomer **4b** had ^{1}H NMR (D_2O, acetone reference) δ 8.39 (d, J = 1.5 Hz, 1H), 7.93 (d, J = 8.2 Hz, 1H), 7.91 (dd, J = 8.2 Hz, 1.5 Hz, 1H), 7.88 (s, 1H), 6.04 (s, 1H), 5.71 (d, $J_{H,P}$ = 8.5 Hz, 2H), 5.35 (dd, J = 9.9 Hz, 5.2 Hz, 1H), 4.85 (d, J = 5.5 Hz, 1H), 4.83 (ddd, $J_{H,P}$ = 22 Hz, J = 12.1 Hz, 6.5 Hz, 1H), 4.64 (m, 1H), 4.55 (dt, J = 10.1, 5.5 Hz, 1H), 4.12 (s, 2H), 3.89 (s, 2H). ^{31}P NMR (D_2O; TMP reference): −6.24. HRMS (FAB): *m*/*z* 678.0585 (M + 2H + K); calcd for $C_{22}H_{20}N_7O_{14}P$ + 2H + K, 678.0599.

***N*-(4-(Acetoxymethyl)-3-nitrobenzoyl)iminodiacetic Acid Bis[2-(trimethylsilyl)ethyl] Ester (12).** Na_2CO_3 (8.5 mg, 0.08 mmol) was added to a solution of acetic acid (3.8 μL, 0.067 mmol) in DMF (1 mL), followed by a solution of **8** in DMF (0.5 mL), and the mixture was stirred at room temperature overnight. The solution was diluted with brine and extracted with EtOAc (3 × 10 mL). The combined organic solution was washed with brine (×3), dried, and evaporated. Flash chromatography (95:5 CH_2Cl_2−EtOAc) gave **12** as an oil, 35.2 mg (95%): ^{1}H NMR δ 8.20 (d, J = 1.6 Hz, 1H), 7.75 (dd, J = 8.1 Hz, 1.6 Hz, 1H), 7.64 (d, J = 8.1 Hz, 1H), 5.51 (s, 2H), 4.21−4.28 (m, 6H), 4.04 (s, 2H), 2.16 (s, 3H), 0.95−1.05 (m, 4H), 0.04 (s, 9H), 0.01 (s, 9H); ^{13}C NMR δ 170.4, 169.6, 169.1, 168.9, 147.5, 135.8, 134.5, 132.45, 129.7, 124.05, 64.8, 64.3, 62.8, 52.1, 48.2, 21.0, 17.7, −1.3.

***N*-(4-(Acetoxymethyl)-3-nitrobenzoyl)iminodiacetic Acid (13).** The diester **12** (136 mg, 0.24 mmol) was dissolved in TFA (2.25 mL) and kept for 0.5 h at room temperature. TFA was evaporated, and the residue was triturated with Et_2O and stored at −20 °C. The solid material was filtered out, resuspended in boiling Et_2O (10 mL), and filtered out again to give **13** as a colorless solid (70 mg, 82%), mp 56−57 °C: ^{1}H NMR (methanol-d_4) δ 8.25 (d, J = 1.2 Hz, 1H), 7.81 (dd, J = 7.9 Hz, 1.2 Hz, 1H), 7.68 (d, J = 7.9 Hz, 1H), 5.53 (s, 2H), 4.30 (s, 2H), 4.08 (s, 2H), 2.19 (s, 3H); HRMS (FAB) *m*/*z* 355.0755 (M + 1), calcd for $C_{14}H_{15}N_2O_9$ 355.0778. A portion was converted to the alcohol **14** by brief alkaline hydrolysis and isolated by anion-exchange chromatography as the sodium salt. The product had ^{1}H NMR (D_2O; acetone reference) δ 8.28 (d, J = 1.5 Hz, 1H), 7.87 (d, J = 8.0 Hz, 1H), 7.84 (dd, J = 8.0 Hz, 1.5 Hz, 1H), 5.03 (s, 2H), 4.11 (s, 2H), 3.88 (s, 2H). On anion exchange HPLC it had t_R 13.1 min, coincident with the axial isomer **3b** of caged cGMP.

Hydrolytic Stability of Caged Cyclic Nucleotides. Solutions of **3a,b** and **4a,b** (each 50 μM) were prepared in 71 mM aqueous buffers of the following compositions: K phosphate, pH 7.0 and pH 8.0, and K CHES, pH 9.0. The solutions were incubated at 22 °C, and at recorded times, aliquots (5 μL) were withdrawn and analyzed by anion exchange HPLC. Retention time data for the caged cyclic nucleotides and the hydrolysis product **14** are given above: values for cAMP and cGMP were 1.9 and 2.3 min, respectively. The proportions of free and caged nucleotide were estimated from the peak areas, normalized for differences in ϵ_{254}, and the pseudo-first-order rate constants for the disappearance of caged nucleotide were determined by linear regression analysis of ln(% caged) vs time. No peaks for products other than the alcohol **14** and the cyclic nucleotides were detected.

Photolytic Properties of Caged Cyclic Nucleotides. (a) Product Quantum Yield (Q_p). Solutions containing caged ATP and caged cNMP (each ∼40 μM) and 1 mM DTT were prepared in 50 mM K phosphate, pH 7.0. Aliquots (400 μL) were irradiated in a 1-cm path-length quartz cuvette, using light from a 100 W xenon arc lamp that passed through a band-pass filter before illuminating the cell. Portions (50 μL) were removed for analysis at time zero and after total irradiation times of 2, 4, 8, 16, 32, and 64 s for replicate analysis of caged and uncaged cNMP by anion exchange HPLC using the conditions described above and for caged ATP by the same procedure but by employing 100 mM K phosphate rather than 10 mM in the HPLC mobile phase. The Q_p value for the photorelease of cNMP was calculated from the known Q_p (0.63) for caged ATP[10] and the ratio of the slopes of the linear regression plots of ln(% caged nucleotide) vs total time of irradiation.

(b) Stoichiometry of Product Formation. Solutions of **3a** or **3b** (each 0.50 mM) in 10 mM K phosphate, pH 7.0, with DTT (2 mM) were irradiated in 0.1 cm path-length quartz cuvettes in a photochemical reactor (16 × 350 nm lamps) for 3−5 min and analyzed by anion exchange HPLC. Conversions of starting material were ∼30% under these conditions, and the only nucleotide products observed were the respective free cyclic nucleotides. Measured yields of each free cyclic nucleotide were in the range 102−105% of that expected from the amount of starting material consumed.

(c) Rates of Product Release. Solutions of **3a** or **3b** (∼0.3 mM) in 50 mM K phosphate, pH 7.0, ± DTT (2 or 11 mM) were flash irradiated in an absorption spectrophotometer linked to a dye laser (320 nm). Details of the apparatus have been described previously.[10] Absorption transients were monitored at 406 nm.

Acknowledgment. We are grateful to Drs. V. R. N. Munasinghe and G. Kelly for recording NMR spectra, to Ms. Carolyn Coghill for technical assistance, and to Dr. D. R. Trentham for the flash photolysis data. We thank the MRC Biomedical NMR Centre for access to facilities and Drs. K. Welham and S. L. Mullen for HRMS data. This work was supported by the College of Veterinary Medicine of Cornell University, the Medical Research Council in the U.K., and in part by NIH Grant 2P01 HL 19242-24.

Supporting Information Available: ^{1}H NMR spectra for compounds **3a,b**, **4a,b**, **6**−**9**, **10a**, **11a**, and **12**−**14** and computed UV spectra for **3a,b** and **4a,b**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO020040G