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A New Nonhydrolyzable Reactive cAMP Analog, (Sp)-Adenosine-3′,5′-cyclic-S-(4-bromo-2,3-dioxobutyl)monophosphorothioate Irreversibly Inactivates Human Platelet cGMP-Inhibited cAMP Phosphodiesterase

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Levels of cAMP that control critical platelet functions are regulated by cGMP-inhibited cAMP phosphodiesterase (PDE3A). We previously showed that millimolar concentrations of the hydrolyzable 8-[(4-bromo-2,3-dioxobutyl)thioadenosine 3′,5′-cyclic monophosphate (8-BDB-TcAMP) inactivate PDE3A. We have now synthesized a nonhydrolyzable affinity label to probe the active site of PDE3A. The nonhydrolyzable adenosine 3′,5′-cyclic monophosphorothioates, Sp-cAMPS and Rp-cAMPS, function as competitive inhibitors of PDE3A with K_i = 47.6 and 4400 μM, respectively. We therefore coupled Sp-cAMPS with 1,4-dibromobutanedione to yield (Sp)-adenosine-3′,5′-cyclic-*S*-(4-bromo-2,3-dioxobutyl)monophosphorothioate, [Sp-cAMPS-(BDB)]. Sp-cAMPS-(BDB) inactivates PDE3A in a time-dependent, irreversible reaction with k_{max} = 0.0116 min^{-1} and K_I = 10.1 μM. The order of effectiveness of protectants in decreasing the rate of inactivation (with K_d in μM) is: Sp-cAMPS (24) > Rp-cGMPS (1360), Sp-cGMPS (1460) > GMP (4250), AMP (10600), Rp-cAMPS (22170). These results suggest that the inactivation of PDE3A by Sp-cAMPS-(BDB) is a consequence of reaction at the overlap of the cAMP and cGMP binding regions in the active site.

Key Words: PDE3A; cAMP; cGMP; affinity labeling; adenosine 3′,5′-cyclic monophosphorothioate; 1,4-dibromobutanedione; platelets; cyclic nucleotide phosphodiesterases.

Abbreviations used: 8-BDB-TcAMP, 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 3′,5′-cyclic monophosphate; Sp-cAMPS and Rp-cAMPS, Sp and Rp isomers of the nonhydrolyzable adenosine 3′,5′-cyclic monophosphorothioate; Sp-cAMPS-(BDB), (Sp)-adenosine-3′,5′-cyclic-S-(4-bromo-2,3-dioxobutyl) monophosphorothioate; Sp-cGMPS and Rp-cGMPS, Sp and Rp isomers of the nonhydrolyzable guanosine 3′,5′-cyclic monophosphorothioate; NO, nitric oxide; PDEs, human cyclic nucleotide phosphodiesterases; cAMP, cyclic AMP; HEL, human erythroleukemia cell line; DBBD, dibromobutanedione; FBS, fetal bovine serum; PIC III, protease inhibitor cocktail set III (100X); PBSG, lysis/binding buffer (20 mM Na_2HPO_4/NaH_2PO_4, 0.5 M NaCl, 20% glycerol, pH 7.8).

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INTRODUCTION

Human cyclic nucleotide phosphodiesterases (PDEs) have been divided into 11 families based on sequence homology, substrate specificity, kinetic characteristics, and inhibitor responses as well as physical, regulatory, and immunological properties (*1*). These enzymes are the products of 25 to 30 genes with splice and initiation site variations producing 55–60 subtypes. The catalytic domains of PDEs, comprising the carboxyl terminal end of the molecules, consist of ~250 conserved amino acids, which are conserved with identities of 30–35% among and 60–65% within families. In contrast, the amino terminal portions of the PDEs function as regulatory domains and are highly variable among families.

Intracellular levels of the second messenger cyclic AMP (cAMP) regulate critical platelet functions such as shape change, aggregation, adhesion, and secretion (*2*). PDE3A is the most abundant cAMP PDE in platelets, has a low K_m for cAMP, and is competitively inhibited by cGMP. Nitric oxide (NO) stimulates soluble guanylate cyclase, thereby raising cGMP levels. Thus the physiologically important inhibition of platelet functions by NO is probably due to cGMP inhibition of PDE3A, leading to elevated intracellular cAMP levels.

We have reported the cloning of the gene for the catalytic domain of platelet PDE3A from HEL cell line (*3*) and the production of functional protein utilizing a baculovirus-transfected Sf9 insect cell expression system to study the active site of PDE3A (*4*). Site-directed mutagenesis has been performed on multiple highly conserved amino acids within the active site (*3–5*). However, this approach cannot elucidate the function of nonconserved amino acids unique to PDE3A.

Affinity labeling using reactive purine nucleotide analogs is useful for probing nucleotide binding sites (*6,7*). We have previously described the use of the cAMP affinity analog 8-[(4-bromo-2,3-dioxobutyl)thio]-adenosine 3′,5′-cyclic monophosphate (8-BDB-TcAMP) in studies to identify amino acids within the active site of PDEs. 8-BDB-TcAMP irreversibly inactivates cAMP-hydrolyzing PDEs and has also been used to probe the active site of PDE2A (*8*), PDE3A (*9*), and PDE4A (*10*). In the case of PDE4A, a peptide containing the residue modified by 8-BDB-TcAMP was isolated and the amino acid sequence identified. However, the utility of 8-BDB-TcAMP is limited because it only inactivates PDEs at millimolar concentrations, probably because of continuous hydrolysis to the 5′-AMP derivative by the enzymes under investigation.

The Sp isomer of the monophosphorothioate analog of cAMP is an activator of cAMP dependent protein kinase I and II, which mimics the effect of cAMP and is resistant to PDEs (*11–13*). We now report the synthesis of a new nonhydrolyzable reactive cAMP derivative (Sp)-adenosine-3′,5′-cyclic-S-(4-bromo-2,3-dioxobutyl) phosphorothioate, Sp-cAMPS-(BDB), which contains both reactive bromoketo and dioxo groups (Fig. 1). The bromoketo group can form covalent bonds with the nucleophilic side chains of many amino acids, including cysteine, aspartate, glutamate, histidine, tyrosine, and lysine (*6,7,14,15*), while the dioxo provides the ability to react with arginine residues (*16,17*). We here demonstrate the use of this novel nonhydrolyzable cAMP reagent as an effective affinity label of the catalytic site of PDE3A. A preliminary abstract of this work has been presented (*18*).

EXPERIMENTAL PROCEDURES

Materials

1,4-Dibromobutanedione (DBBD), thiophosphoryl chloride, and ammonium bicarbonate were purchased from Aldrich (Milwaukee, WI). The Sp isomer of adenosine 3′,5′-cyclic phosphorothioate [Sp-cAMPS], used as standard during synthesis, was obtained from Research Biochemicals International (Natick, MA). Adenosine was purchased from Sigma (St. Louis, MO) and used without further purification. HPLC grade acetonitrile, methanol and triethylamine were obtained from Fisher Scientific (Pittsburgh, PA). DE-52 was purchased from Whatman (England). Silica plates (Silica gel 60, 0.2-mm thickness) used for thin layer chromatography were obtained from E. Merck (Darmstadt, Germany).

Sf9 insect cell lines, MaxBac Transfection and Expression System, the Xpress Protein Purification System, NuPage Protein Gel Electrophoresis, Xcell II module, Chromogenic WesternBreeze System, and Anti-Xpress Antibody were purchased from Invitrogen (Carlsbad, CA). Hink's TNM-FH media and FBS were purchased from Cellgro (Mediatech, Inc., Herndon, VA). PIC III was purchased from Calbiochem (San Diego, CA). Microcon YM-30 centrifugal filter devices were purchased from Millipore (Bedford, MA). Coomassie Plus Protein Assay Reagent kit and GelCode Blue Stain Reagent were purchased from Pierce (Rockford, IL). Gentamicin sulfate, cAMP, Sp-cGMPS, Rp-cGMPS, 5′-AMP, 5′-GMP, and NAD^+ were purchased from Sigma. Sp-cAMPS and Rp-cAMPS used in protection studies were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Adenosine 3′,5′-cyclic phosphate ammonium salt [2,8-^{3}H]-cAMP was purchased from NEN Life Science Products Inc. (Boston, MA). Biodegradable counting cocktail, Bio-Safe II, was purchased from Research Products International Corp (Mount Prospect, IL).

Synthesis of Sp-cAMPS-(BDB)

Synthesis of Sp-cAMPS. Sp-cAMPS was synthesized starting from adenosine according to the previously reported procedures (*19*). Typically, 1 mmol of adenosine was dissolved in 5 ml of freshly dried trimethyl phosphate by heating. The mixture was cooled to room temperature and 200 μl freshly distilled thiophosphoryl chloride were added in four portions over 30 min. The mixture was stirred for 5 h and the resulting solution was then rapidly dropped into a refluxing solution of 120 ml of 0.08 M KOH containing 60% acetonitrile. The reaction mixture was neutralized quickly (within 1 min) with HCl and concentrated to 5 ml by evaporation under reduced pressure. The solution was extracted with diethyl ether to remove trimethyl phosphate and the solid residue was suspended in 100 ml methanol to precipitate insoluble salts.

After the removal of salts, the filtrate (which contained the crude cAMPS), was evaporated to dryness and redissolved in 15 ml water. The pH of the solution was adjusted to 7.8 with ammonium bicarbonate and the solution was applied to a DE-52 column (30 $\times$ 1.5 cm) equilibrated with water (pH 6.9). Initially, the column was washed with 270 ml water and a gradient was then started from water (500 ml) to 50 mM ammonium bicarbonate, pH 7.8 (500 ml), and fractions (7.5 ml) were collected.

The fractions were monitored at 260 nm, and were analyzed by thin layer chromatography on silica plates (Silica gel 60 with fluorescent indicator) using as solvent butanol:acetic acid:water (75:10:15). While adenosine shows an R_f value of 0.8, cAMPS has an R_f value of 0.6 under these conditions. cAMPS eluted between fractions 90–110.

Fractions containing the desired product were pooled, lyophilized, and subjected to further purification by HPLC using a Varian Model 5000 HPLC system equipped with a reverse-phase Micropak MCH-10 column (50 cm × 8 mm) equilibrated with 0.1% trifluoroacetic acid (TFA) in water (solvent A). A solution of 0.1% TFA in acetonitrile was used as solvent B. A flow rate of 2 ml/min was maintained throughout the experiment. After injection of the sample (1 ml), the column was eluted with solvent A for 15 min. A gradient was then started to 5% solvent B over a time period of 5 min. A slower gradient was then run to 20% solvent B over a time period of 160 min, within which two diastereomers Sp-cAMPS and Rp-cAMPS eluted with retention times of 62 min (9% Solvent B) and 71 min (9.8% solvent B), respectively. This separation was followed by a faster gradient to 100% solvent B over a time period of 30 min. Fractions of 2 ml were collected and monitored for A_{260nm}. The desired fractions were pooled, lyophilized, and stored at −80°C. The diastereomeric purity and assignment of peaks during the HPLC purification of the synthetic sample were made by comparison with the HPLC elution position of the commercial Sp-cAMPS on an analytical Vydac C_{18} column (0.46 × 25 cm) equilibrated with 0.1% TFA/water (solvent A). A solution of 0.1% TFA/acetonitrile was used as solvent B. After injection of the sample, the column was washed with solvent A for 15 min at a flow rate of 1 ml/min. A gradient was then started to 10% solvent B over a time period of 80 min, followed by a steeper gradient to 100% solvent B over a time period of 30 min. Throughout the experiment, a flow rate of 1 ml/min was maintained. The Sp-cAMPS elutes earlier than the Rp-cAMPS. The samples pooled from the preparative HPLC run showed 100% diastereomeric purity. TLC analysis indicated an R_f value of 0.6.

The proton NMR spectrum of our synthesized Sp-cAMP in dimethylsulfoxide had peaks centered at δ 8.35 (H_8), 8.27 (H_2), 5.93 (H_1'), 4.83 (H_3'), 4.80 (H_2'), 4.59 (H_5'), and 4.21 (H_4'). Assignments of the ribose protons were made by comparison with those in the literature (*20*). For comparison, the commercial Sp-cAMPS in dimethylsulfoxide exhibited peaks centered at δ 8.22 (H_8), 8.16 (H_2), 5.92 (H_1'), 4.80 (H_3'), 4.78 (H_2'), 4.58 (H_5'), and 4.16 (H_4').

Synthesis of Sp-cAMPS-(BDB). As shown in Fig. 1, Sp-cAMPS-(BDB) was prepared by reaction of Sp-cAMPS with DBBD. Freshly recrystallized DBBD (165 mg, 675 μmol) was dissolved in 0.5 ml of methanol contained in a 5 ml round bottom flask and cooled to 4°C on an ice bath. Sp-cAMPS (7.75 mg, 22.5 μmol) was dissolved in 0.5 ml of methanol by the addition of sufficient amount of triethylamine. The pH of the solution was maintained between 5.3 and 5.6. This solution was added in two portions to the vigorously stirred solution of DBBD in methanol over 1 min. The reaction was allowed to proceed for 3 min. After the completion of the reaction, the total volume of the reaction mixture was brought down to 0.5 ml by bubbling nitrogen gas through the solution at 4°C. Cold diethyl ether (13 ml) was added and the solution was cooled in an ice bath for 15 min. The precipitated products were collected by centrifugation. The crude product was washed three times with 6 ml of cold diethyl

ether to remove any traces of unreacted DBBD. The pale yellow product was dried in a jet of nitrogen and stored at −80°C. The yield is 6.6 mg (58%).

Analytical Procedures

UV-VIS spectra were recorded using a Hewlett-Packard model Vectra XA spectrophotometer. The ^{1}H and ^{31}P spectra were recorded on a Bruker model DRX-400 instrument. Determination of hydrolyzable bromine was performed according to a previously reported procedure (*21*).

Enzyme Expression and Purification

Expression of PDE3A using a baculovirus insect cell system and preparation of Sf9 cell lysates have been previously described (*4*). In short, the DNA fragment encoding the catalytic domain of platelet PDE3A (nt 679 to 1141) isolated from a HEL cell cDNA library was subcloned into the hexahistidine-tag (HIS-tag)-containing baculovirus vector pBlueBacHis2B (pBBH3031). The PDE3A recombinant virus was produced by cotransfecting Sf9 cells with pBBH3031 and linearized AcMNPV DNA (Invitrogen) using lipofection methods. Sf9 cells grown at 27°C in Hink's-TNM-FH media, containing 10% FBS, and 10 μg/ml gentamicin, were infected with recombinant virus. After 72 h cells were harvested, mixed with protease inhibitor, and frozen as pellets at −80°C.

All protein purification procedures were carried at 4°C (*4*). Initially, cell pellets were thawed, suspended in lysis/binding buffer PBSG, and retreated with PIC III (10 μl in 1 ml of lysis buffer), and sonicated three times using Sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) at output control 5 and pulsed duty cycle 50% for 1 min at a time with 30-s rest intervals. Cell debris was removed by centrifugation at 12,000*g* for 20 min using a Sorvall refrigerated centrifuge (Rotor SS34, Dupont, Wilmington, DE). The supernatant was either immediately purified or flash-frozen in liquid nitrogen and stored at −80°C until further use. HIS-tag PDE3A was isolated using the ProBond Nickel resin column contained in the Xpress Protein Purification System. Two milliliter of lysis/binding buffer and 2 ml of resin were added to each 1 ml of cell lysate supernatant. The mixture was rocked for 20 min. The resin-protein complex was washed two times with PBSG, pH 6.0, 25 mM imidazole, once with PBSG, pH 6.5, 75 mM imidazole, and once with PBSG, pH 6.7, 150 mM imidazole to remove non HIS-tag-containing impurities. The PDE3A was eluted in 10 fractions of 0.5 ml using PBSG, pH 6.7, 250 mM imidazole, concentrated, and exchanged into 50 mM Tris–HCl buffer, pH 7.8, containing 10 mM $MgCl_2$ and 20% glycerol using Microcon YM-30 centrifugal filter devices. Each individual fraction was then assayed for catalytic activity and protein identity using NuPAGE Protein Gel Electrophoresis System. Active fractions were pooled and stored at −80°C prior to further characterization.

Protein Concentration Determination

Protein concentrations were determined with the Coomassie Plus Protein Assay Reagent, using bovine serum albumin as the protein standard. The absorbance at 595 nm was measured using a Bio-Tek automatic microplate reader.

Protein Gel Electrophoresis and Western Blot Analysis of PDE3A

PDE3A-containing protein solutions and protein standards were subjected to electrophoresis in 10% Bis-Tris Gel with MOPS running buffer using NuPAGE Electrophoresis System. Gels were either stained with GelCode Blue Stain Reagent or transferred to a PVDF membrane using the Xcell II module at constant voltage of 25 V for 1 h at room temperature for Western blotting. Transferred membranes were processed using the Chromogenic WesternBreeze System and probed with Anti-Xpress Ab (1:5000 dilution) to detect HIS-tag PDE3A.

Enzyme Activity Assay

PDE3A activity was measured as previously described (*22*). Enzyme containing solutions were added to buffer containing 50 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, and 0.8 μM [^{3}H]cAMP (10,000 cpm/assay) to make a final volume of 100 μl. Reaction mixtures containing experimental samples or no enzyme were incubated at 30°C for 15 min. Catalysis was terminated by serial addition of 0.2 ml of 0.2 M of $ZnSO_4$ and 0.2 ml of 0.2 M $Ba(OH)_2$. Samples were vortexed and centrifuged at 10,000*g* for 5 min. The pellets containing $BaSO_4$ precipitated [^{3}H]-5′-AMP were discarded. Aliquots of supernatants containing unreacted [^{3}H]cAMP were removed and counted in a Beckman KS1800 liquid scintillation counter. Enzyme activity was measured by comparing the amount of cAMP hydrolyzed in PDE3A containing samples to no enzyme controls. This data was then used to calculate enzyme specific activity in nmoles of cAMP hydrolyzed per mg of protein per minute.

Inactivation of PDE3A by Sp-cAMPS-(BDB)

Purified PDE3A (77 μg/ml) was incubated at 25°C with Sp-cAMP-(BDB) at concentrations of 12.5 to 100 μM in a reaction buffer containing 45 mM Hepes, pH 7.2, 20 mM $MgCl_2$, 4 mM MES. At timed intervals, 5-μl aliquots of the reaction mixture were withdrawn, diluted 20-fold in buffer containing 47.5 mM Hepes, pH 7.04, 20 mM $MgCl_2$, 4 mM MES, and assayed in duplicate for residual PDE3A activity. Control samples were incubated under identical conditions except for the absence of the Sp-cAMPS-(BDB).

Protection by Ligands Against Sp-cAMPS-(BDB) Inactivation of PDE3A

The effect of the Sp-cAMPS, Sp-cGMPS, Rp-cGMPS, GMP, AMP, and NAD on the rate of inactivation of PDE3A were evaluated by incubation of the purified enzyme with each compound for 2 min prior to the addition of Sp-cAMPS-(BDB). Five microliters of the aliquots of each final reaction mixture were removed at time intervals, diluted 20-fold in buffer containing 47.5 mM Hepes, pH 7.04, 20 mM $MgCl_2$, 4 mM MES, and assayed in duplicate for residual PDE3A activity.

Docking of Sp-cAMPS and Rp-cAMPS to PDE3A

We used the previously constructed PDE3A-cAMP model (*23*) based on the crystal structure of the catalytic domain of PDE4B (*24*) as the target for docking the ligands, Sp-cAMPS and Rp-cAMPS. Briefly, after side chain replacement and loop grafting the final PDE3A structure was subjected to four cycles of dynamics (200 steps) and

50 steps of minimization. The minimization was done by the conjugate gradient method, using Kollman all-atom force field and Gasteiger–Hückle charges at the dielectric constant of 4 and nonbonded cut-off of 12 Å. Each of the ligands, Sp-cAMPs and Rp-cAMPS, was docked into the PDE3A model, which had contained cAMP using Flexidock in the Biopolymer module of SYBYL Molecular Modeling Environment (Tripos Inc., St. Louis, MO). This program incorporates the van der Waals, electrostatic, torsional, distance, and angle constraint energy terms of the Tripos force field as defined in Sybyl 6.6 (Force Field Manual, p. 81). The default conditions of Flexidock differ from Tripos force field only in three respects. The hydrogen van der Waals radius is 1.0 Å, the hydrogen van der Waals epsilon is 0.03, and the van der Waals cut off distance is 16 Å between fragment centroids. The protein is fixed in space and the ligand is mobile. The flexible side chain bonds of both ligand and protein are adjusted by the program. Only nonring single bonds are rotatable and backbone atoms are considered fixed. A genetic algorithm is used to determine the optimum ligand geometry. Charges are computed using the Kollman all-atom method for the protein and Gasteiger–Hückle method for the ligand as described in Sybyl 6.6 (Receptor-Based Design Manual, p. 39). First, the active site pocket was defined to consist of amino acid residues (N845, E866, E971, F972, and F100) that exhibited interaction with cAMP from our previous studies (*23*), which utilized site-directed mutagenesis, enzyme kinetics, and molecular modeling. We next extracted cAMP from the target PDE3A. The water molecules were removed from both of Sp-cAMPS/Rp-cAMPS and PDE3A. The atom types were checked and the hydrogens and charges were added. The ligand, Sp-cAMPS or Rp-cAMPS, was prepositioned in the cavity so that the adenosine moiety coincided with the position of adenosine of cAMP in the model (*23*). The flexible docking between the target enzyme and ligand was then performed. The energy of the Sp-cAMPS or Rp-cAMPS docking to PDE3A was obtained from the log file after the completion of each docking process. Each of the Sp-cAMPS and Rp-cAMPS was docked at least three times to the target enzyme. The close up views of the docking models between Sp-cAMPS or Rp-cAMPS and PDE3A were graphed utilizing the Z-clipping plane features of the Sybyl Molecular Modeling Program with a width of 4 and 12 Å.

RESULTS

Analogs of cAMP and cGMP Tested as Competitive Inhibitors of PDE3A

We evaluated the nonhydrolyzable analogs of the substrate cAMP (Sp-cAMPS and Rp-cAMPS) as reversible inhibitors of PDE3A. Sp-cAMPS is a competitive inhibitor of PDE3A with a K_i value of 47.6 $\pm$ 6.2 μM, indicating a weaker affinity when compared with the substrate cAMP (K_m = 0.46 $\pm$ 0.20 μM). The other isomer, Rp-cAMPS, inhibits PDE3A much more weakly (K_i = 4400 $\pm$ 2600 μM), demonstrating stereospecificity in the binding of the cyclic phosphorothioates (Table 1). The nonhydrolyzable analogs of the competitive inhibitor cGMP (Sp-cGMPS and Rp-cGMPS) were also competitive inhibitors of PDE3A. The K_i values for Sp-cGMPS and Rp-cGMPS are 305 $\pm$ 54 and 210 $\pm$ 33 μM, respectively, indicating that the enzyme binds these cyclic thiophosphates less well than it binds the competitive inhibitor cGMP (K_i = 0.76 $\pm$ 0.11 μM). However, the enzyme exhibits the weakest affinity

TABLE 1

K_i Values of cAMP/cGMP Analogs Tested as Competitive Inhibitors

cAMP and cGMP analogs	K_i (μM)
Sp-cAMPS	47.6 ± 6.2
Rp-cAMPS	4,400 ± 2,600
AMP	9,600 ± 3,060
cGMP	0.76 ± 0.11
Sp-cGMPS	305 ± 54
Rp-cGMPS	210 ± 33
GMP	5,600 ± 3,110

Note. The K_m of cAMP is 0.46 ± 0.20 μM.

for the noncyclic 5′-monophosphates, 5′-AMP (K_i = 9.6 ± 3.0 mM) and 5′-GMP (K_i = 5.6 ± 3.1 mM). Since Sp-cAMPS exhibited the strongest affinity for PDE3A, we coupled the nonhydrolyzable cAMP analog, Sp-cAMPS with DBBD to synthesize the affinity label reagent, Sp-cAMPS-(BDB).

Characterization of Sp-cAMPS-(BDB)

The ultraviolet absorption spectrum of Sp-cAMPS-(BDB) in water at pH 6.4 showed a single peak at 259 nm (ε = 15,400 $M^{-1}cm^{-1}$) with a shoulder at 304 nm (ε = 1,924 $M^{-1}cm^{-1}$), which is similar to that reported for adenosine 5′-O-[S-(4-bromo-2,3-dioxobutyl]thiophosphate (*25*). Determination of the hydrolyzable bromine shows the presence of 1.04 mol of bromine per mole of the compound. While the ^{31}P NMR spectrum of Sp-cAMPS recorded in DMSO(D_6) gives a signal with a chemical shift of 50.0 ppm (both the commercial compound and the Sp-cAMPS we synthesized and purified), that of Sp-cAMPS-(BDB) under identical conditions shows a single peak centered at 17.2 ppm, indicative of the alkyl substitution at sulfur of Sp-cAMPS by DBBD. For comparison, the ^{31}P NMR spectrum of (Sp) *S*-methyl cAMPS has been reported to exhibit a single signal centered at 24.1 ppm (*26*), while those of adenosine 5′-O-[*S*-(3-bromo-2-oxopropyl)]thiophosphate and adenosine 5′-O-[*S*-(4-bromo-2,3-dioxobutyl)]-thiophosphate were at 19.2 and 20.5 ppm, respectively (*25*). In contrast, the proton-decoupled ^{31}P NMR spectrum of cAMP exhibits a single resonance at 1.58 ppm (*27*). These results are consistent with the structure for Sp-cAMPS-(BDB) shown in Fig. 1.

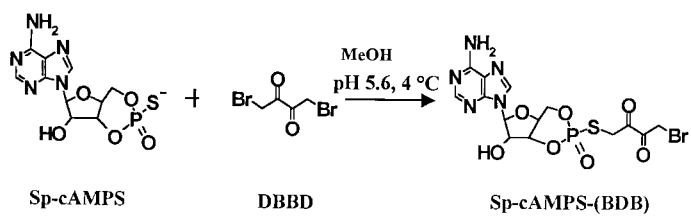

FIG. 1. Synthesis of Sp-cAMP-(BDB).