Site-Specific Modification of Epstein – Barr Virus-Encoded RNA 1 with N^2-Benzylguanosine Limits the Binding Sites Occupied by PKR

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Protein kinase dependent on RNA (PKR) is a part of the human intracellular antiviral response.[1–3] Viral RNAs with double-helical segments longer than ~20 base pairs bind and activate PKR, a process that includes dimerization and autophosphorylation.[4, 5] Once activated, the enzyme phosphorylates the α subunit of the translation initiation factor eIF-2; this stops translation.[6] Viruses have developed numerous ways to circumvent the translation-inhibition effects of PKR.[7–9] One viral strategy is to synthesize decoy RNAs that bind the enzyme and prevent its association with activating RNA molecules.[7] This paper describes a chemical approach to examine and control the interaction between PKR and one of these viral inhibiting RNAs, EBER1 from Epstein – Barr virus.

Epstein – Barr virus (EBV) is a human B-cell lymphotropic herpes virus that causes infectious mononucleosis. The virus has also been associated with several malignant diseases, such as Burkitt's lymphoma.[10] In these diseases, EBV establishes a persistent infection and constitutively expresses several gene products, including EBER1 RNA.[11] EBER1 is 167-nucleotide-long RNA with extensive secondary structure whose physiological function is controversial.[11–13] However, data suggest that at least one of EBER1's roles is to inhibit the activity of PKR. EBER1 was found to bind PKR in vitro.[13] Also, using an in vitro reticulocyte lysate system, it was shown that EBER1 prevented inhibition of protein synthesis caused by dsRNA's activation of PKR.[14]

Human PKR weighs 68 kDa, consisting of a 20 kDa N-terminal double-stranded RNA-binding domain (dsRBD) and a C-terminal kinase domain.[1] The dsRBD is composed of two copies of the dsRNA-binding motif (dsRBM), an ~70 amino acid motif found in many dsRNA-binding proteins.[15] The mode of dsRBMs' binding to dsRNA is reported to be largely RNA sequence-independent, although selective binding by these proteins has been observed.[16, 17] The solution structure of the PKR dsRBD was determined by NMR and shows that both dsRBMs have the characteristic α-β-β-β-α fold of this protein family.[18] A crystal structure of dsRBMII from *Xenopus laevis* RNA-binding protein A (Xlrbpa) bound to dsRNA revealed that the dsRBM spans a major groove and contacts two flanking minor grooves (~16 bp).[19]

In a previous study, we used PKR dsRBD mutants modified with a hydroxyl radical-generating EDTA·Fe group to cleave EBER1 RNA.[20] These experiments located the PKR binding site on EBER1 in stem-loop IV and indicated the orientation of dsRBMI on this structure. A short RNA comprising only the nucleotides of stem-loop IV (mEBER for "minimal" EBER1) bound the dsRBMI of PKR in a manner similar to the full-length RNA. Interestingly, the data suggested that dsRBMI bound mEBER in two different locations. We proposed models for these complexes (**A** and **B**, Figure 1) based on our cleavage results and the Xlrbpa – RNA crystal structure.

Since our models for PKR – RNA complexes predict minor-groove sites that are in contact with the protein, introducing a steric block specifically at that site should prevent formation of the complex. Thus, we prepared a phosphoramidite of N^2-benzylguanosine and used this compound to create a modified mEBER RNA that has a sterically occluded minor groove site (Figure 1). Verdine and colleagues had shown previously that N^2-benzylation of guanosine does not significantly affect the stability of a simple RNA duplex.[21] For the synthesis of the phosphoramidite, we used a new strategy we had developed to prepare N^2-modified guanosine derivatives.[22] N^2-$O^{2'}$,$O^{3'}$,$O^{5'}$-Tetraacetyl-6-bromopurine ribonucleoside (**1**) was alkylated at the N^2-position with benzyl bromide in the presence of LiOH (Scheme 1).[23] The alkylated product was converted to the guanosine derivative without prior purification by treatment with $LiOAc \cdot 2H_2O$ in DMF at 55 °C. Under these conditions, reaction occurs only at C6; this allows for the isolation of tetraacetyl derivative (**2**) by normal silica gel chromatography in 80% yield over the two steps. Deacylation and protection at the 5′-hydroxyl to give (**3**), followed by formation of the 2′-*t*-butyldimethylsilyl (TBDMS) derivative (**4**) and phosphoramidite (**5**) were then carried out under standard conditions.

We used phosphoramidite (**5**) to synthesize derivatives of the mEBER RNA described above. As previously noted, dsRBMI of PKR modified with EDTA·Fe at position 29 cleaves this RNA at two separate sites. Our interpretation of this result is that dsRBMI binds at two distinct locations, forming two different complexes (**A** and **B**, Figure 1). Models for these complexes predict that base pairs formed by nucleotides 6 – 8 and 31 – 33 form the minor-groove binding site for α-helix 1 of dsRBMI in complex **A** (Figure 1). In contrast, it is the base pairs formed by nucleotides 10 – 12 and 27 – 29 that are suggested to contact α-helix 1 in complex **B** (Figure 1). We replaced the guanosine at position 6 with N^2-benzylguanosine and monitored the binding of PKR with the E29C-EDTA·Fe-modified dsRBD. mEBER is ideal for this experiment since there are two binding sites for the protein. Thus, the cleavage efficiency at one site can be compared to that at the other to determine the extent to which any observed effects are site specific. We previously used relative cleavage efficiencies at multiple sites in a similar manner to ascertain the importance of individual protein domains in the formation of specific complexes.[24]

We find that PKR dsRBD, modified with EDTA·Fe at amino acid position 29, cleaves G6-benzyl mEBER significantly less efficiently at nucleotides corresponding to complex **A** as compared to the unmodified mEBER RNA (Figure 2). However, the complex **B** cleavage site is unaffected by the benzyl modification at G6 (Figure 2). Thus, blocking access to the minor groove near G6 disrupts complex **A**, but not complex **B**, as predicted by our

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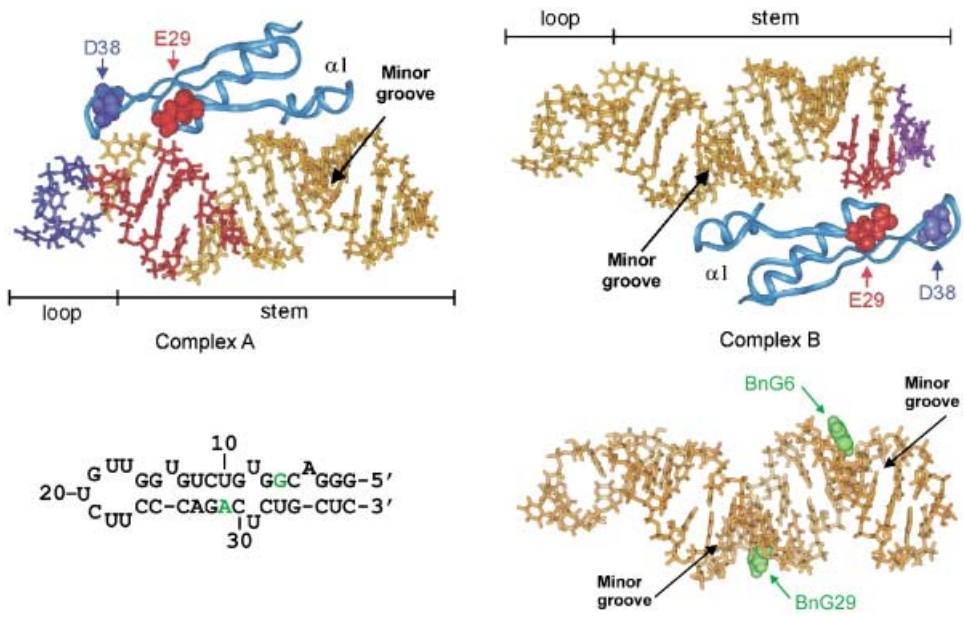
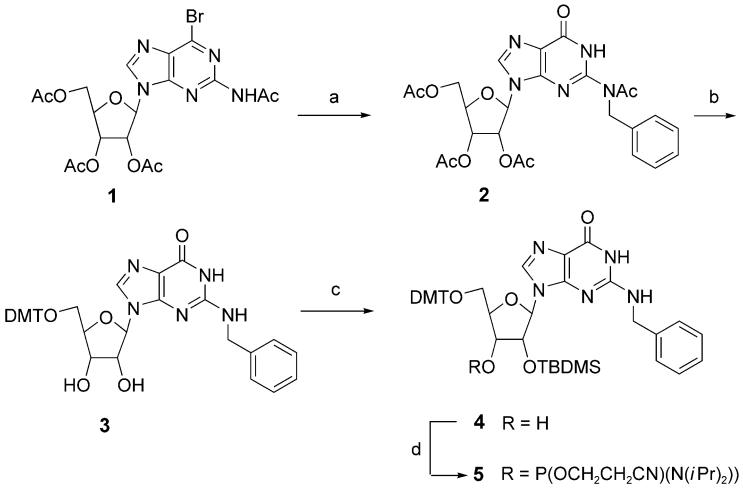


Figure 1. *(Top) Two different PKR dsRMBI-mEBER1 complexes (**A** and **B**) invoked to explain the cleavage patterns observed.[20] Colored nucleotides are cleaved by proteins modified by EDTA·Fe at the amino acid position with the corresponding color (E29 = red, D38 = blue). (Bottom, left) Sequence of mEBER RNA ligand of PKR with G6 and A29 indicated in green. (Bottom right) Model for mEBER containing* N^2*-benzylguanosine at position 6 (BnG6) and 29 (BnG29).*

ically disrupt complex **B** and should not affect complex **A**. Indeed, when E29C-EDTA·Fe-modified PKR dsRBD was used to analyze binding on this RNA, a significant decrease in cleavage efficiency corresponding to complex **B** was observed relative to that observed for the complex **A** site (Figure 3 A). Comparing the effects of modification of mEBER at the two different sites (G6 and A29) clearly demonstrates that site-specific benzylation can be used to control the extent to which PKR binds to form either complex **A** or complex **B** (Figure 3 B).

PKR has been shown by our laboratory and others to bind RNA ligands selectively.[16, 17, 20, 24] The basis for this binding selectivity is not fully understood. We hypothesize that in addition to seeking stretches of A-form duplex of a certain length, PKR's dsRBMs have preferred sequences to which they bind due to the unique arrangement of functional groups in the minor grooves at those sites. Interestingly, the



Scheme 1. *a) i) benzyl bromide, LiOH, DMF, RT, 8 h. ii) LiOAc·2* H_2O*, DMF, 55 °C, 24 h, 80 % (two steps). b) i)* NH_3*/MeOH, 55 °C, 8 h. ii) DMTCl, pyr, 54 % (two steps). c) TBDMSCl,* $AgNO_3$*, THF, 37 %. d) 2-cyanoethyl (N,N-diisopropylamino)chlorophosphite, Hünig's base, THF, 70 %.*

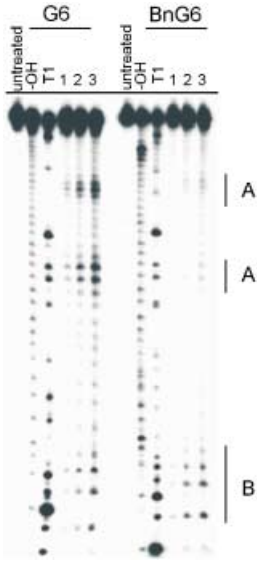


Figure 2. *Site-specific benzylation of mEBER RNA at G6 eliminates PKR complex **A**. G6: unmodified mEBER RNA. BnG6: mEBER RNA benzylated at* N^2 *of G6. Untreated: RNA alone in the absence of cleavage reagents. –OH: alkaline hydrolysis lane. T1: ribonuclease T1 (G lane). Lanes 1: Cleavage by PKR RBD E29C-EDTA-Fe at 8 μM. Lanes 2: Cleavage by PKR RBD E29C-EDTA-Fe at 16 μM. Lanes 3: Cleavage by PKR RBD E29C-EDTA-Fe at 32 μM. Cleavage bands arising from complexes **A** and **B** are labeled. The 3′-end cleavage products from complex **B** are not resolved on this gel.*

model. The fact that binding occurs at the complex **B** site confirms that the benzyl modification does not cause a large change in the overall structure of mEBER and that the effect is specific to complex **A**. Importantly, this result indicates that the two PKR binding sites on mEBER are independent and do not arise from cooperative binding of monomers in a PKR dimer.

As an additional test of this approach to controlling PKR binding, we prepared a mEBER with N^2-benzylguanosine at nucleotide position 29. This substitution is predicted to specif-

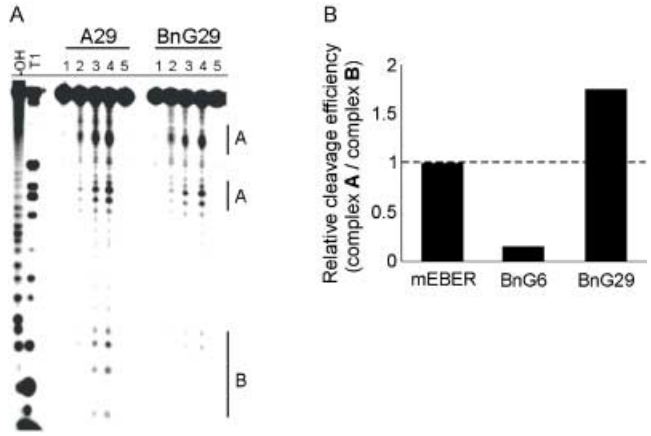


Figure 3. *A) Site-specific benzylation of mEBER RNA at position 29 eliminates complex* ***B****. A29: unmodified mEBER RNA. BnG29: mEBER RNA with* N^2*-benzylguanosine incorporated at position 29. −OH: alkaline hydrolysis lane. T1: ribonuclease T1 (G lane). Lanes 1: RNA in the absence of cleavage reagents. Lanes 2: Cleavage by PKR RBD E29C-EDTA-Fe at 4* μ*M. Lanes 3: Cleavage by PKR RBD E29C-EDTA-Fe at 8* μ*M. Lanes 4: Cleavage by PKR RBD E29C-EDTA-Fe at 16* μ*M. Lanes 5: Cleavage by PKR RBD E29C-EDTA-Fe in absence of* H_2O_2 *and ascorbic acid. Cleavage bands arising from complexes* ***A*** *and* ***B*** *are labeled. The 3′-end cleavage products from complex* ***B*** *are not resolved on this gel. B) Effect of benzyl modification on the ratio of cleavage observed for complex* ***A*** *compared to complex* ***B****.*

sequence 5′-UCU-3′ exists near each of the binding sites for α1 of PKR's dsRBMI on mEBER. Further studies are required to determine the importance of this sequence to PKR binding and the role of the other EBER1 nucleotides in dictating binding-site selectivity. A full comprehension of the basis for selective binding by PKR's dsRBMs is necessary to predict how PKR's dsRBD will assemble on various RNA ligands. In addition, it is significant to define modifications of duplex RNA that prevent PKR from binding, as these will probably be necessary in the development of RNA interference reagents (siRNAs) that will not interact with PKR or other double-stranded RNA-binding proteins.[25]

In conclusion, we have developed a method by which the binding of PKR, and likely other members of the dsRBM family, can be controlled on RNA ligands through site-specific steric occlusion of the minor groove. This was accomplished by chemical synthesis of the RNA by using a phosphoramidite of N^2-benzylguanosine. An efficient new procedure to prepare guanosine derivatives modified at N^2 was used for the generation of this compound. RNA prepared with the N^2-benzylguanosine phosphoramidite allowed us to define further the interaction between PKR and EBER1, a kinase-inhibiting RNA generated by Epstein – Barr virus that has two PKR binding sites.

Experimental Section

PKR dsRBD E29C-EDTA·Fe protein was prepared as previously described.[17] All RNA synthesis was carried out by using a Perkin – Elmer-ABI 392 DNA/RNA synthesizer with 5′-dimethoxytrityl-2′-*tert*-butyldimethylsilyl-protected adenosine, guanosine, cytidine, and uridine phosphoramidites (Glen Research, Sterling, VA (USA)). For the coupling of phosphoramidite (**5**), the standard 1.0 μmol RNA synthesis cycle was modified to have an extended coupling time of 20 min. RNAs were deprotected and purified as previously reported.[17] G6-benzyl mEBER was analyzed by MALDI-TOF mass spectrometry on a PerSeptive Biosystems Voyager-DE SDR spectrometer. MALDI-TOF analysis: calcd: 11 884.03, found: 11 877.08. The standard error related to the MALDI-TOF analysis is ±0.1 % or ±12 mass units for this RNA. Affinity cleavage reactions were carried out with 5′-end-labeled mEBER or benzylated mEBER RNAs, as previously described.[20] Comparisons of the relative cleavage efficiencies presented in Figure 3 B were determined by quantifying the cleavage band intensities for nucleotides 15 and 16 (complex **A**) and nucleotides 2 and 3 (complex **B**). The ratio of these intensities was assigned a value of 1.0 for mEBER.

***N^2,$O^{2'}$,$O^{3'}$,$O^{5'}$*-Tetraacetyl *N^2*-benzylguanosine (2)**: LiOH (87.5 mg, 2.08 mmol) and benzyl bromide (356.7 mg, 2.08 mmol) were added to a solution of N^2,$O^{2'}$,$O^{3'}$,$O^{5'}$-tetraacetyl-6-bromopurine ribonucleoside (**1**; 715 mg, 1.39 mmol) in DMF (25 mL), and the reaction mixture was stirred at room temperature overnight. Then, the mixture was diluted with EtOAc/hexanes (100 mL, 4:1) and successively washed with water (2 × 20 mL) and brine (1 × 20 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The syrup obtained was dissolved in DMF (15 mL) and $LiOAc \cdot 2H_2O$ (851 mg, 8.34 mmol) was added to the solution. After the mixture had been stirred at 55 °C for 24 h, the solution was concentrated under reduced pressure. Purification by flash column chromatography (3 % MeOH/$CHCl_3$) afforded **2** as a light yellow foam (602.3 mg, 80 % for two steps). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.77 (s, 1 H), 7.36 – 7.28 (m, 3 H), 7.16 – 7.11 (m, 2 H), 5.80 – 5.75 (m, 2 H, H1′, H2′), 5.60 (d, J = 17.7 Hz, 1 H), 5.19 (d, J = 17.7 Hz, 1 H), 5.09 (dd, J = 6.3, 5.4 Hz, 1 H), 4.25 – 4.20 (m, 1 H), 3.98 (dd, J = 12.3, 3.3 Hz, 1 H), 3.70 (dd, J = 12.3, 6 Hz, 1 H), 2.32 (s, 3 H), 2.05 (s, 3 H), 2.02 (s, 3 H), 1.97 ppm (s, 3 H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 175.6, 170.1, 169.4, 169.1, 155.5, 149.6, 147.1, 138.1, 135.5, 129.0, 127.7, 125.4, 122.2, 87.1, 78.9, 72.7, 69.8, 62.9, 49.9, 25.3, 20.6, 20.3, 20.2 ppm; HR FAB MS calcd for $C_{25}H_{28}N_5O_9$: 542.1887 $[M+H]^+$; found: 542.1912.

5′-*O*-(4,4′-Dimethoxytrityl)*N^2*-benzylguanosine (3): Compound **2** (78 mg, 0.14 mmol) was dissolved in methanolic ammonia (100 mL), and the tube was capped. The solution was heated at 55 °C for 12 h, then the mixture was concentrated to obtain crude N^2-benzylguanosine. Complete conversion was monitored by thin-layer chromatography. This compound was then vacuum dried and rendered anhydrous by coevaporation with dry pyridine. The residue was dissolved in dry pyridine (3 mL), and dimethoxy trityl chloride (DMTCl; 59 mg, 0.17 mmol) was added. After being stirred under inert argon atmosphere at room temperature for 12 h, the mixture was diluted with ethyl acetate and washed three times with aqueous saturated $NaHCO_3$. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash column chromatography on a column of silica gel eluted with CH_2Cl_2/MeOH (19:1) to give **3** as a white powdered solid (53 mg, 54 %). ^{1}H NMR ([D_6]DMSO, 300 MHz): δ = 7.84 (s, 1 H), 7.36 – 7.19 (m, 14 H), 6.84 – 6.78 (m, 5 H), 5.49 (d, J = 6 Hz, 1 H), 5.18 (d, J = 6 Hz, 1 H), 4.52 (q, J = 5.4 Hz, 1 H), 4.43 – 4.29 (m, 2 H), 4.20 (q, J = 5.4 Hz, 1 H), 3.71 (s, 6 H), 3.34 (s, 2 H), 3.23 – 3.10 ppm (m, 2 H); ^{13}C NMR ([D_6]DMSO, 75 MHz): δ = 158.0, 156.6, 152.4, 150.5, 144.9, 138.75 136.4, 135.6, 135.5, 129.7, 128.4, 127.8, 127.7, 127.6, 127.1, 126.6, 117.3, 113.1, 87.8, 85.4, 82.9, 73.0, 70.4, 64.1, 55.0, 54.9, 44.1 ppm; HR FAB MS calcd for $C_{38}H_{37}N_5O_7$: 676.2688 $[M+H]^+$; found: 676.2695.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)*N^2*-benzylguanosine (4): A mixture of compound **3** (675.27 mg, 0.83 mmol) and imidazole (105 mg, 1.54 mmol) was dissolved in freshly distilled THF (10 mL); then $AgNO_3$ (281.6 mg, 1.65 mmol) was added to the solution. After this mixture had been stirred for 1 min, *tert*-

butyldimethylsilyl chloride (230 mg, 1.53 mmol) was added. After being stirred at room temperature for 12 h under argon atmosphere, the mixture was diluted with ethyl acetate and washed twice with saturated $NaHCO_3$. The organic phase was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by flash column chromatography on a column of silica gel eluted with CH_2Cl_2/MeOH (98:2–97.5:2.5) to give **4** as a white powder (244 mg, 37%). ^{1}H NMR (CD_2Cl_2, 300 MHz): $\delta = 9.22$ (s, 1H), 7.42 (d, $J = 5.9$ Hz, 2H), 7.31–7.15 (m, 13H), 6.87 (s, 1H), 6.76 (d, $J = 9$ Hz, 4H), 5.67 (d, $J = 4.5$ Hz, 1H), 4.64 (t, $J = 4.8$ Hz, 1H), 4.44 (dd, $J = 15$, 5.4 Hz, 1H), 4.32 (dd, $J = 15$, 5.4 Hz, 1H), 4.14–4.07 (m, 2H), 3.71 (s), 3.41 (dd, $J = 10.2$, 5.4 Hz, 1H), 3.30 (dd, $J = 10.2$, 3.3 Hz, 1H), 2.6 (d, $J = 3.2$ Hz, 1H), 0.84 (s, 9H), −0.08 (s, 3H), −0.19 ppm (s, 3H); ^{13}C NMR (CD_2Cl_2, 75 MHz): $\delta = 159.9$, 159.2, 153.6, 151.7, 145.4, 139.8, 136.3, 136.1, 130.6, 130.5, 128.9, 128.5, 128.4, 128.2, 127.4, 117.7, 113.6, 95.4, 89.1, 86.9, 84.2, 75.4, 71.8, 64.8, 55.7, 45.3, 30.2, 26.1, 26.0, 25.9, 18.3, −4.6, −4.9 ppm; HR FAB MS calcd for $C_{44}H_{51}N_5O_7Si$: 790.35 $[M+H]^+$; found: 790.40.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)-(*N*,*N*-diisopropylamino)phosphino]-2′-*O*-(*tert*-butyldimethylsilyl)N^2-benzylguanosine (5): Compound **4** (79 mg, 0.10 mmol) was dissolved in freshly distilled THF (0.60 mL), then *N*,*N*-diisopropylethylamine (104 μL, 0.60 mmol) and 2-cyanoethyl (*N*,*N*-diisopropylamino)chlorophosphite (85 μL, 0.38 mmol) were added to the solution. This solution was allowed to stir for 8 h at room temperature, and was subsequently quenched with methanol and then diluted with ethyl acetate. The reaction was followed by a TLC system of acetone/methanol/hexane (34:6:60–34:8:58). The resulting solution was washed twice with saturated $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on a column of silica gel (pretreated with 0.1% triethylamine in CH_2Cl_2) eluted with CH_2Cl_2/MeOH (98:2) to give compound **5** as a white powdered solid (69 mg, 70%). ^{31}P NMR (CD_2Cl_2, 121 M+Na]$^+$; found: 1012.3.

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