Synthesis and Enzymatic Activity of Some New Purine Ring System Analogues of Adenosine 3′,5′-Cyclic Monophosphate

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A series of novel adenosine 3′,5′-cyclic monophosphate (cAMP) analogues, as well as their 6-deamino and 6-nitro derivatives, were synthesized where the purine ring was replaced by indazole, benzotriazole, and benzimidazole. The 3′,5′-cyclic monophosphates of indazole and benzotriazole ribofuranosides, where the sugar–phosphate moiety is attached to the N-2 nitrogen atoms of the heterocycles, were also prepared. The biological efficiency of the analogues was tested by their ability to activate purified cAMP-dependent protein kinase I (PK-I) from rabbit skeletal muscle and cAMP-dependent protein kinase II (PK-II) from bovine heart. Each cyclic nucleotide is capable of activating both PK isozymes in half-maximum concentrations (K_a) ranging from $2.0 \cdot 10^{-8}$ to $4.8 \cdot 10^{-6}$ M. The cyclic phosphate of *N*-1-β-D-ribofuranosylindazole (**13**) proved to be a very poor activator for both PK-I and PK-II, but when indazole binds by N-2 to ribose or when the hydrogen atom at C-4 is substituted by a nitro or amino group, activities of the analogues increase considerably. The activating potencies of benzotriazole derivatives are similar to that of cAMP, irrespective of the C-4 substituents. The K_a' values of cyclic nucleotides containing benzimidazole were found to be higher for PK-II than for PK-I; e.g. the activity of 4-nitro-1-β-D-ribofuranosylbenzimidazole 3′,5′-cyclic monophosphate (**32**) is nearly 20 times as high for PK-II than for PK-I.

Introduction

Cyclic AMP analogues capable of specific binding to one of the cAMP-dependent protein kinase isozymes, type I (PK-I) or type II (PK-II), and differentiating between site 1 and site 2 of the regulatory subunits are of great importance in the analysis of cAMP-mediated effects. These analogues are not only useful in mapping the structural requirements of cAMP binding sites, but they can selectively activate PK isozymes under in vivo conditions, as well.[1,2] A great number of cAMP analogues have been prepared so far by systematically changing the sugar and phosphate moieties and the purine ring, moreover by attaching different substituents to C-2, C-6, or C-8.

The binding affinity of these analogues to PK-I and PK-II as well as their isozyme specificity was thoroughly studied during the last 2 decades.[3] In addition, more than 10 years ago it was recognized that the regulatory subunits of the two isozymes (R_I and R_{II}) contained two different intrachain binding sites, namely, site 1 and site 2. The results indicated that for each isozyme the two distinct binding sites had different cAMP (or cAMP analogue) dissociation rates and different analogue specificity.[4]

It was found that C-2- or C-8-substituted analogues bound preferentially to site 1, while C-6-modified derivatives preferred site 2 of both R_I and R_{II}. Further in vitro studies demonstrated that binding of an analogue selective for either site stimulated binding of an analogue selective for the other site.[5,6] This synergism was utilized in the growth arrest of different human tumor cell lines when certain site 1 and site 2 selective analogues were added together at micromolar concentrations.[2,7] At the same time, these experiments also revealed the involvement of the cellular type II protein kinase in the growth inhibition of these tumors,[2,7] as well as in the lipolysis in intact adipocytes.[6] In addition, single or, even more so, combined application of certain site 1 and site 2 selective analogues could also induce differentiation and modulation of protein kinase gene expression in human leukemic cells.[8,9]

Of the analogues tested, 8-Cl-cAMP, N^6-benzyl-cAMP, and N^6-phenyl-8-[(*p*-chlorophenyl)thio]-cAMP were found to be the most potent inhibitors of tumor proliferation.[2,9]

On the basis of enzymatic studies[3] the site 2 selective N^6-benzyl analogue is somewhat better activator of PK-I

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than of PK-II, but it is less potent for both isozymes than cAMP. The site 1 selective 8-Cl-cAMP as well as the other C-8-thio or -halogen analogues preferentially bind to site 1 of type II rather than of type I protein kinase.[2,5] Both 8-Cl-cAMP and the N^6-phenyl-8-[(*p*-chlorophenyl)thio] derivative are better activators of PK-II than cAMP itself.[10]

Hereupon it seemed to be reasonable to prepare some novel cAMP analogues, in the hope of gaining potent and specific activators of PK-II. It is known that modifications in sugar–phosphate moiety are not tolerated by either PK-I or PK-II,[3,11] whereas the formerly mentioned base derivatives, substituted and modified in the purine ring system, are generally fairly good activators of both isozymes. Our attention turned to the less thoroughly studied 1,3-dideaza analogues since according to earlier systematic studies[12] neither the 6-amino group nor the N-1 and N-3 endocyclic nitrogens play significant role in binding to R_{II}. However, introduction of electron-withdrawing substituents to the C-2, C-6, or C-8 position in nearly all cases provides potent and specific activators for one of the two isozymes;[3] e.g. 2-chloro and 2-(trifluoromethyl)-cAMP are much more potent as activators of PK-I,[13] while 6-chloro-cAMP somewhat prefers PK-II.[3]

Starting from these results we synthesized some 1,3-dideaza analogues of cAMP varying the imidazole ring of the purine skeleton and the location of the sugar–phosphate moiety. In addition, we wished to investigate the enzymatic properties of the analogues bearing a nitro group in place of the cAMP 6-amino group in comparison with those of the corresponding unsubstituted and amino-substituted derivatives.

Results and Discussion

Chemistry. The starting nucleosides bearing no substituent at C-4 of the heterocycles (**5–8**) have been prepared earlier by an acid-catalyzed fusion method using 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (**3**) as glycosylating agent.[14,15] In our coupling reactions standard conditions were applied, but 1-*O*-acetyl-2,3,5-tris-*O*-(*p*-chlorobenzoyl)-β-D-ribofuranose (**4**) was used instead of **3**. Starting from D-ribose, **4** can be prepared readily in high overall yield as white crystals in contrast to the preparation of **3**.[16] Nucleosides with *p*-chlorobenzoyl protecting groups are also known as easily crystallizable derivatives.

Application of **4** in the fusion couplings with indazole (**1**) and benzotriazole (**2**), respectively, under usual conditions[17] enhanced the ratio of N-2 isomers in both cases compared to similar couplings with **3**. Although the overall yields were lower (40% versus 90%[14] and 50% vs 94%,[15] respectively), these reactions revealed that besides the catalyst proportion[17] the nature of the acyl protecting groups could also strongly influence the regioselectivity of fusion couplings. The difference was extremely surprising in the case of the fusion of **4** with **1**, which afforded the thermodynamically less stable *N*-2-β-ribofuranoside as the main product while the other isomers formed only in negligible amount.

Condensation of **2** with **4** under the same conditions resulted in somewhat better overall yield (50% vs 40%) and—in contrast to the former reaction—gave the N-1-β isomer as the main product. The proportion of N-2 isomers (>6%) was only a bit higher compared to the isomer distribution obtained in the fusion of **2** with **3**.[15] The site of ribosylations and anomeric configurations were determined after deacylations by comparison of the physical data of deprotected nucleosides with those reported in the literature[14,15] (Scheme I).

The ribofuranosides of 4-nitroindazole, 4-nitrobenzotriazole, and 4-nitrobenzimidazole (**24–26**) were prepared by condensation of mercury salts of the corresponding heterobases with 2,3,5-tris-*O*-(*p*-chlorobenzoyl)-D-ribofuranosyl chloride (**20**) in boiling xylene. The application of **20** (a new crystalline ribofuranosyl halide) proved to be a good choice, giving relatively high yields for the requisite *N*-1-β-ribofuranosides. Coupling of **20** with the Hg(II) salt of **17** was complete in 1.5 h and provided the unknown **21** in 77% yield and a minor amount (1.5%) of its α anomer. Earlier Revankar et al.[18] synthesized the 4-nitro-2-β-D-ribofuranosylindazole by combination of the silyl method with fusion techniques, but they did not isolate any of the N-1 isomers. Reaction of **20** with the mercury salts of **18** and **19**, respectively furnished the required N-1-β nucleosides in higher yields (35% vs 15%[19] and 34% vs 22%[20]) than similar condensations with different ribosylating agents.[19,20] In addition, the novel *N*-2-β-ribofuranoside of 4-nitrobenzotriazole (**22a**) was also isolated. Structures of the unknown β-ribofuranosides (**24** and **25a**) were identified by UV and ^{1}H NMR spectroscopy. The sites of ribosylations were determined by comparison of their UV spectra with those of the corresponding *N*-methyl derivatives.[18,21]

Since we managed to isolate all N-1-α isomers as well, it made possible the unambiguous determination of anomeric configuration of the N-1-substituted products by comparison of chemical shifts of H-1′ protons of the anomeric pairs.[22] The β-anomeric configuration of **25a**

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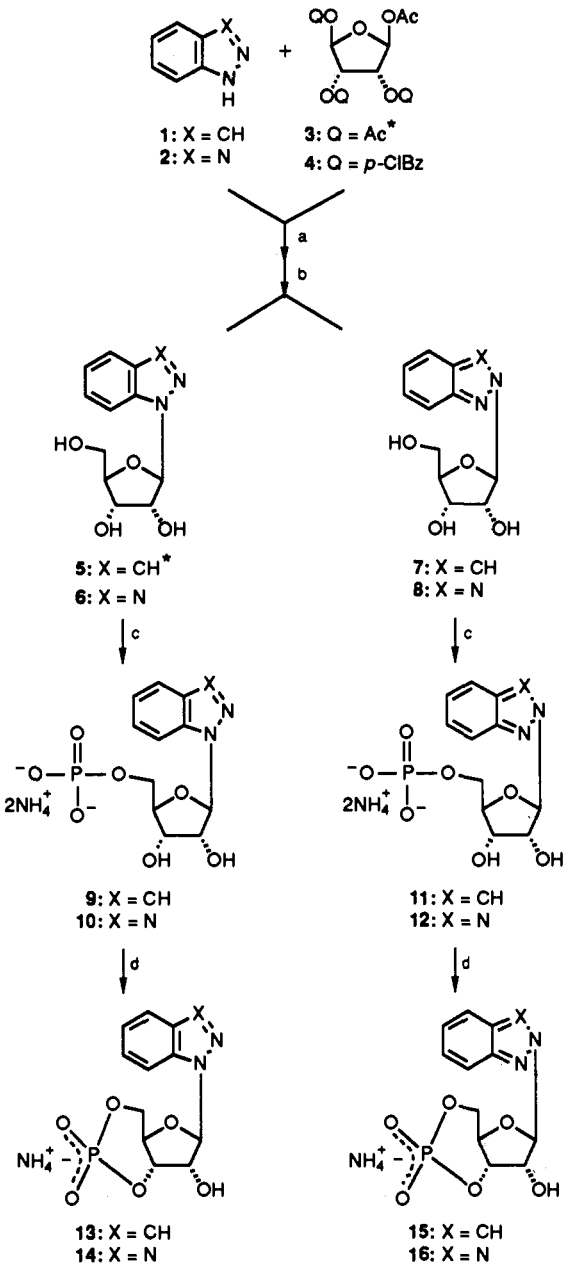
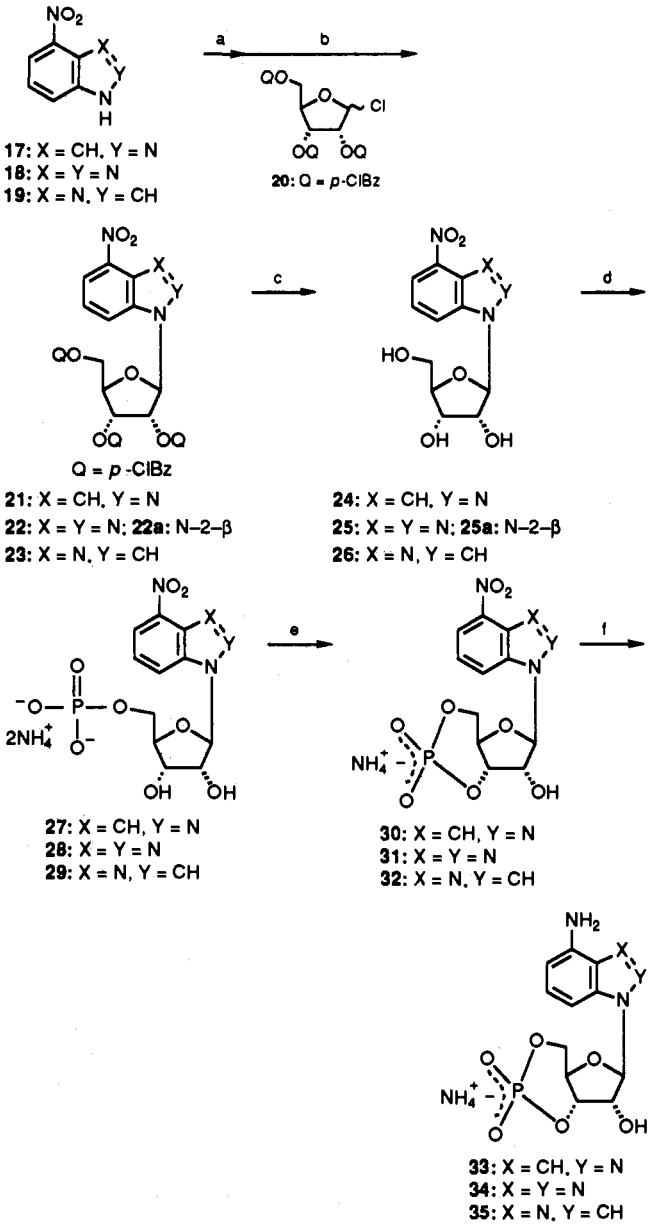
Scheme I [a]



[a] (a) p-TsOH, 160 °C, 20 mmHg; (b) NaOMe–MeOH; (c) (i) $POCl_3$–$(EtO)_3PO$, 0 °C, (ii) ion-exchange chromatography; (d) (i) DCC–pyridine, (ii) ion-exchange chromatography. **5** was prepared by the reaction of **1** with **3**, while **7**, **6**, and **8** were obtained by the fusion of **4** with **1** and **2**, respectively.

Scheme II [a]



[a] (a) (i) NaOH–EtOH–H_2O; (ii) $HgCl_2$; (b) (i) xylene, 1.0–4.5 h reflux, (ii) silica gel chromatography; (c) (i) NaOMe–MeOH; (d) (i) $POCl_3$–$(EtO)_3PO$, 0 °C, (ii) ion-exchange chromatography; (e) (i) DCC–pyridine, (ii) ion-exchange chromatography; (f) H_2–10% Pd/C, EtOH–H_2O.

was supported by high negative optical rotation value,[23] besides the apparently favored formation of β-nucleosides in ribosylations with 2-*O*-acylribofuranosyl halides (Scheme II). The 5′-monophosphates of nucleosides prepared (**9**–**12** and **27**–**29**) were synthesized with minor modification of the Yoshikawa method.[24] We found that reaction time of these phosphorylations was highly influenced by the structure and substituents of the base moiety. The nature of heterocycles was found to be the primary factor. Benzotriazole ribofuranosides can be phosphorylated much more slowly than benzimidazole or indazole analogues. This finding proved to be consequent both in the series of unsubstituted and 4-nitro-substituted derivatives. Phosphorylation of nitro nucleosides required longer time as compared to that of the unsubstituted ribofuranosides. In addition, N-2-substituted analogues reacted somewhat less readily with $POCl_3$ than N-1 ribosides with the same heterobase. Owing to the chemical instability of parent nucleoside, we failed to prepare the 5′-monophosphate of **25a**. TLC monitoring of the reaction showed only a weak spot in the usual region of nucleotides even after 2 days. Higher temperature, however, led to discoloration and gradual decomposition of **25a**. The structures of the 5′-monophosphates were identified by ^{13}C NMR spectroscopy (Table I). Carbon chemical shift and $^3J_{P,C4'}$ and $^2J_{P,C5'}$ coupling constant values characterizing the sugar–phosphate moiety are in agreement with the reported literary

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Table I. Proton-Decoupled ^{13}C NMR Data for 9–12 and 27–29 in D_2O

	heterocycle								ribose						
compd	C2	C3	C4a	C4	C5	C6	C7	C7a	C1′	C2′	C3′	C4′	C5′	$^3J_{P,C4'}$	$^2J_{P,C5'}$
9		138.06	125.44	129.50	123.56	123.03	110.84	141.82	90.74	71.98	74.17	84.45	66.30	8.4	4.8
10			132.03	119.00	127.90	124.29	111.82	145.59	90.96	70.72	72.79	84.60	64.48	7.3	4.0
11		121.16	125.91	121.16	123.03	117.18	121.16	147.99	94.76	70.54	75.39	83.77	64.37	6.0	3.0
12			144.43	118.34	128.67	128.67	118.34	144.43	96.92	71.40	75.30	84.95	65.38	8.0	4.8
27		123.33	114.98	151.00	127.69	127.28	126.63	141.28	96.21	70.72	76.85	85.82	66.35	8.4	4.4
28			138.71	147.79	129.26	129.26	128.58	138.27	99.22	72.80	76.94	86.61	66.86	7.9	4.9
29	139.10		137.52	147.25	125.04	122.20	120.83	136.68	90.97	72.29	76.13	86.38	65.90	8.6	4.3

Table II. Proton-Decoupled ^{13}C NMR Data for 13–16 and 30–32 in D_2O

	heterocycle								ribose								
compd	C2	C3	C4a	C4	C5	C6	C7	C7a	C1′	C2′	C3′	C4′	C5′	$^3J_{P,C'}$	$^2J_{P,C3'}$	$^3J_{P,C4'}$	$^2J_{P,C5'}$
13		136.76	124.65	128.17	122.51	122.03	110.12	140.73	93.98	73.16	78.41	72.56	67.34	6.8	3.6	2.2	4.3
14			132.63	119.28	128.18	124.45	110.36	145.07	92.55	72.80	77.55	72.60	66.15	7.8	4.7	2.4	6.2
15		120.87	121.13	121.55	126.30	123.49	117.45	148.91	97.29	73.42	77.54	72.50	66.62	7.3	3.8	2.0	5.8
16			144.68	118.57	129.04	129.04	118.57	144.68	99.44	73.69	78.38	73.43	68.40	8.7	4.1	4.3	6.7
30		123.28	114.68	151.19	127.74	127.30	126.60	141.10	98.85	74.74	79.12	74.00	69.11	7.9	3.7	3.6	6.6
31			138.27	147.47	128.77	128.48	128.34	137.88	100.81	74.82	79.08	74.66	69.26	8.0	4.2	4.2	7.2
32	139.07		137.16	146.41	125.23	122.27	119.79	136.17	94.73	74.27	79.34	73.79	96.26	8.0	4.2	4.3	6.8

data[25] for other nucleoside 5′-monophosphates. The cyclization of the 5′-nucleotides was accomplished in refluxing dry pyridine containing DCC as the condensing agent according to the method of Drummond et al.[26] The 3′,5′-cyclic monophosphates thus obtained were purified by ion-exchange chromatography, giving the corresponding ammonium salts. Ring closures were also confirmed by ^{13}C NMR spectroscopy (Table II). Our sugar carbon chemical shift assignment follows the revised order reported by Kainosho.[27] Overlapping of the corresponding carbon signals (C-4 with C-7 and C-5 with C-6) in the ^{13}C NMR spectra of 12 and 16 provided an additional proof for the N-2 attachment of the sugar moiety in these compounds.

Catalytic hydrogenation of the nitro derivatives (30–32) resulted in the corresponding cAMP analogues (33–35) which were identified by UV, IR, and ^1H NMR spectroscopy. The signal of H-1′ proton appeared as a singlet in all three ^1H NMR spectra, which is characteristic of the 3′,5′-cyclic monophosphates of β-ribofuranosides.[28]

Activation of Protein Kinase Isozymes by cAMP Analogues. A generally used method for testing the biological efficiency of cAMP analogues is the determination of their half-maximum activating concentration (K_a) and its comparison with that of cAMP. Figure 1 shows the percent activation of PK-1 and PK-II as a function of the concentration of cAMP and some analogues, plotted against a logarythmic scale.

A summary of K_a' values is shown in Table III. Considering that, except 33, all of our cyclic phosphates are better activators of type II than of type I kinase, we gave a reciprocal specificity (the ratio of K_a' for PK-II to K_a' for PK-I) in each case. It can be seen that PK-II is more sensitive to the electron distribution of heterocycle

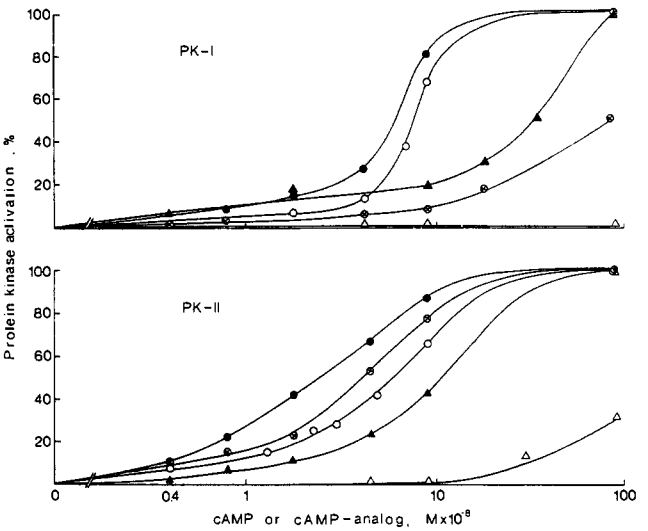

Figure 1. Activation of PK-I and PK-II by some cAMP-analogues. The concentrations of cAMP and cAMP analogues are plotted against a logarythmic scale. Protein kinase activities were determined by the amount of ^{32}P transferred from [γ-^{32}P]-ATP into histone IIA as described in Methods. Symbols: ○, cAMP; △, 13; ●, 30; ⊗, 32; ▲, 35.

than PK-I, although compound 32 and especially compound 13 proved to be very poor activators of PK-I, too.

The latter derivative is unique among the adenine ring system analogues, being very weak activator of both isozymes. In the case of PK-II we can find similarly poor activators (e.g. 3-deaza- or 2-aza-cAMP), but they activate PK-I fairly well.[3] At the same time, it is striking that a change in the position of indazole, relative to the fixed sugar–phosphate moiety, can significantly improve the activating potency of the analogue (compound 15) toward both isozymes, especially for PK-II. The same trend can be observed in the case of the corresponding benzotriazole derivatives, where the nonisosteric N-2 analogue (compound 16) is also a better activator of both isozymes than the isosteric N-1 nucleotide (compound 14). The N-2 analogues are particularly well-tolerated by PK-II; e.g. compound 16 is the most potent activator of this isozyme of all purine ring system analogues, whereas 15 is 10 times more potent as an activator of PK-II than of PK-I. Earlier studies[3,12] indicated that the adenine moiety bond to R_{II}

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Table III. Activation of cAMP-Dependent Protein Kinase Isozymes by cAMP Analogues

compd	heterocycle	attachment of ribose	C-4 substit	K'_a[a] PK-I	K'_a[a] PK-II	specificity: K'_a(PK-II)/K'_a(PK-I)
cAMP	purine	N-9	NH_2	1.0	1.0	1.00
13	indazole	N-1	H	0.014	0.048	3.45
30		N-1	NO_2	1.17	2.50	2.14
33		N-1	NH_2	0.65	0.54	0.83
15		N-2	H	0.14	1.40	10.00
14	benzotriazole	N-1	H	0.64	0.88	1.38
31		N-1	NO_2	0.88	1.00	1.14
34		N-1	NH_2	0.88	0.93	1.06
16		N-2	H	0.82	3.50	4.27
Yagura et al.[12]	benzimidazole	N-1	H	0.25	0.70	2.80
32		N-1	NO_2	0.08	1.56	19.50
35		N-1	NH_2	0.20	0.67	3.35

[a] K'_a values represent the ratio of the apparent K_a for cAMP to the apparent K_a for the derivative, where K_a is the concentration of cAMP or analogue required for half-maximum activation of the protein kinase isozymes. K_a values were determined by the activation curves demonstrated in Figure 1. In the case of cAMP it was found to be 70 nM for both isozymes. Details of measurements are described in Methods.

exhibited significant freedom of motion; therefore steric factors (i.e. different location of the heterocycle) are not likely to play a determining role in the improvement of activating potencies of the N-2 analogues related to those of the corresponding N-1 derivatives.

At the same time, due to the quinoidal structures of heterocycles, the benzene rings of **15** and **16** are electron deficient compared to those of **13** and **14**, respectively. It shows that reduction of electron density in the plane of the benzene ring results in highly increased activating potency toward PK-II. This recognition is further confirmed by the activating properties of nitro derivatives (compounds **30–32**), which—surely owing to the electron-withdrawing effect of the nitro groups—are also potent activators of the type II isozyme. Of them, compound **32** was found to be the far most specific purine ring system analogue, being nearly 20 times more potent an activator of PK-II than of PK-I.

Type I protein kinase is generally known to have stricter steric requirements,[12] but is less sensitive for changes in the electron distribution of the heterocycle.[3] The presence of electron-withdrawing substituents in the C-2 position appeared to be unambiguously beneficial for the activation of PK-I.[13] According to our results the activating potencies of our C-4-nitro-substituted derivatives for PK-I essentially depend on the nature of the heterocycle.

Thus, the C-4-nitro substituent may exert a beneficial (see compound **30**) or detrimental (see compound **32**) effect on the activation of PK-I, but it may be indifferent (see compound **31**), as well. The differences found in the abilities of the three nitro compounds to activate PK-I show that the overall electron distribution of the heterocycle plays an important role in the activation of PK-I, too. On the basis of their enzymatic activity and isozyme specificity, the probably site 2 selective nitro derivatives as well as the N-2 analogues appear to be promising candidates for further biological studies.

Experimental Section

Materials. Indazole and benzotriazole were purchased from Fluka AG, cAMP and histone IIA were from Sigma Chemical Co., and [γ-^{32}P]ATP was from Izinta (Budapest, Hungary). Triethyl phosphate and xylene were vacuum distilled. Phosphoryl chloride and pyridine were freshly distilled from P_2O_5 prior to use. All reagents were of analytical grade.

Methods. Cyclic AMP dependent protein kinase I was purified from rabbit skeletal muscle and cAMP dependent protein kinase II from bovine heart.[29,30] Protein kinases were assayed according to the filter-paper method of Corbin and Reimann.[31] The reaction mixture (50 μL) contained 40 mM MES buffer (pH 6.8), 0.2 mM EDTA, 0.8 mg/mL bovine serum albumin, 12 mM magnesium acetate, 120 μM [γ-^{32}P]ATP adjusted to a specific radioactivity of 5.5 Bq/pmol, and a constant enzyme amount resulting in a linear ^{32}P incorporation during the 5 min reaction time. Activity measurements were performed at 30 °C in the absence and presence of 10^{-8}–10^{-6} M cAMP or its analogues. Reaction was stopped by pipetting a 40-μL aliquot onto a 1.5 × 1.5 cm^2 Whatman 31 ET filter paper and placing it into cold 20% TCA solution. After three washes the incorporated radioactivity was determined by measuring the Cerenkov radiation. It was found that 2 × 10^{-6} M cAMP caused the maximum activity of both PK-I and PK-II. PK preparations without cAMP or with 10^{-3} M 5′-AMP gave a basal phosphate incorporation yielding—in accordance with literary data[32]—10 and 20% of the total activity of PK-I and PK-II, respectively. The differences between basal activity and the activities measured in the presence of various concentrations of cyclic nucleotides tested gave the activating effects, which were expressed as the percent maximum activation elicited by 2 × 10^{-8} M cAMP.

Chromatography. Precoated silica gel (Kieselgel 60 F_{254}, 0.2 mm × 20 cm × 20 cm Merck, Darmstadt, Germany) TLC sheets were used to follow the reactions and check the purity of the products. Solvent systems (v/v) for silica gel TLC were (A) $CHCl_3$/ether = 4:1, (B) EtOAc/MeOH = 19:1, (C) benzene/EtOAc = 4:1, (D) $CHCl_3$, (E) nPrOH/25% ammonium hydroxide/water = 7:2:1. DEAE Sephadex A-25 was purchased from Pharmacia Fine Chemicals. Ion-exchange column chromatographic separations were monitored with the help of a Spectromom 195 spectrophotometer (MOM) equipped with a flow-through cell (Starna Ltd.) and a potentiometric recorder (Type OM814/1, Radelkis).

Spectroscopy. ^{1}H NMR spectra were recorded with a Varian XL-100/15 FT NMR system operating at 100.1 MHz using dioxane (δ 3.70) for internal reference. Carbon-13 NMR spectra were acquired on a Varian XL-100/15 disk-augmented FT NMR system operating at 25.1 MHz. Dioxane (67.71 ppm downfield from Me_4Si) was used as the internal standard. Infrared spectra were recorded in potassium bromide on a Nicolet 7199 FT IR spectrophotometer. UV spectra were recorded with a Zeiss Specord UV–vis spectrophotometer. Spectroscopic-grade MeOH and distilled water were used to dissolve the samples in the case of nucleosides and nucleotides, respectively. Maxima are reported

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in nanometers ($\epsilon \times 10^{-3}$). Optical rotation measurements were taken by a Polamat A (Zeiss) polarimeter. Melting points were determined by a Koefler apparatus and are uncorrected.

1-O-Acetyl-2,3,5-tris-O-(p-chlorobenzoyl)-β-D-ribofuranose (4). Methyl 2,3,5-tris-O-(p-chlorobenzoyl)-D-ribofuranoside[33] (96.00 g, 0.166 mol) was dissolved in the mixture of AcOH (300 mL) and Ac_2O (180 mL) by stirring at 70 °C for 20 min. After cooling to 50 °C, concentrated H_2SO_4 was added dropwise in 1 h. The clear brown solution obtained was stirred at ambient temperature for 2 h further and then was kept in a refrigerator overnight. The white crystals precipitated were filtered off and washed with cold water (3 × 200 mL). The crude product was dissolved in CH_2Cl_2 (600 mL), extracted with warm saturated aqueous $NaHCO_3$ solution (3 × 200 mL), and dried over $CaCl_2$. The CH_2Cl_2 solution was concentrated to a smaller volume (100 mL) and then MeOH was added to induce crystallization. The white precipitate was recrystallized from MeOH, giving chromatographically pure 4. Yield 82.17 g (1.135 mol, 81.3%). Mp: 142 °C. R_f (A): 0.67. Anal. ($C_{28}H_{21}O_9Cl_3$): C, H, Cl.

2,3,5-Tris-O-(p-chlorobenzoyl)-D-ribofuranosyl Chloride (20). Compound 4 (37.45 g, 0.061 mol) was dissolved in dry CH_2Cl_2 (150 mL) and cooled to 0 °C, and then dry HCl was introduced with intensive stirring. After 1 h the solution became opalescent and a fine white precipitate appeared in the mixture. Gas introduction was continued for 2 h further. The white crystalline product was filtered off, washed with dry ether (3 × 30 mL), and dried over KOH to give 30.74 (0.052 mol) of **20** as an anomeric mixture. Yield: 85.4%. R_f (A): 0.45, 0.38 (β and α anomer, respectively). Anal. ($C_{26}H_{18}O_7Cl_4$): C, H, Cl.

Coupling of 2 with 4 by the Acid-Catalyzed Fusion Method. Benzotriazole (2) (1.78 g, 15.0 mmol) and 4 (9.11 g, 15.0 mmol) was melted together at 160 °C, and then p-toluenesulfonic acid (228 mg, 1.2 mol) was added and the melted mixture was stirred for 10 min, at 160 °C in 20 mmHg vacuum. The dark brown mixture obtained was cooled to ambient temperature and dissolved in $CHCl_3$ (100 mL). The chloroformic solution was treated with a small amount of active carbon, filtered, extracted with saturated $NaHCO_3$ solution (2 × 60 mL), and dried over $MgSO_4$. After filtration and evaporation the residue was purified by silica gel chromatography (60 g of Kieselgel 60; $CHCl_3$; flow rate, 2 mL/min). Evaporation of fractions 60–81 afforded a pale yellow syrup, which gave white crystals on treatment with cold ether. Yield: 0.30 g (0.45 mmol), 3.0%. Mp: 152 °C. R_f (D): 0.73. $[\alpha]^{25}_D = -109.6°$ (c = 1.0, $CHCl_3$). Anal. ($C_{32}H_{22}O_7N_3Cl_3$): C, H, N, Cl.

By identification of the deacylated nucleoside on the basis of literary data,[15] this product proved to be 2-(2,3,5-tris-O-(p-chlorobenzoyl)-β-D-ribofuranosyl)benzotriazole.

Fractions 152–230 contained the main product, which was also crystallized with ether, after evaporation of the combined fractions. Yield: 4.45 g (6.67 mmol), 44.5%. Mp: 156 °C. R_f (D): 0.43. $[\alpha]^{25}_D = -77.6°$ (c = 1.0, $CHCl_3$). Anal. ($C_{32}H_{22}O_7N_3Cl_3$): C, H, N, Cl.

By subsequent identification of the deprotected nucleoside, this product was found to be the protected N-1-β isomer.

Fusion of 1 with 4. Coupling of 1 (2.36 g, 20 mmol) with 4 (9.11 g, 15 mmol) in the presence of p-TsOH (228 mg, 1.2 mmol) was effected as described above. The only product isolated after chromatography was found to be 2-(2,3,5-tris-O-(p-chlorobenzoyl)-β-D-ribofuranosyl)indazole on the basis of identification of the deprotected nucleoside. Yield: 3.63 g (5.46 mmol), 36.4%. Mp: 179 °C. R_f (D): 0.65. $[\alpha]^{25}_D = -66.4°$ (c = 1.0, $CHCl_3$). Anal. ($C_{33}H_{23}O_7N_2Cl_3$): C, H, N, Cl.

Reaction of 1 with 3. Compound 1 (10.93 g, 93.10 mmol), **3**[16] (22.17 g, 69.72 mmol), and p-TsOH (928 mg, 4.88 mmol) were fused for 20 min under the usual conditions. Chromatography—using $CHCl_3/Et_2O$ = 4:1 as eluent—afforded the acetylated 1-β-D-ribofuranosylindazole as the main product. Yield: 15.90 g (42.30 mmol), 60.7%. R_f (A): 0.51. ^1H NMR ($CDCl_3$): δ 6.50 (1 H, d, $^3J_{1',2'}$ = 3.1 Hz, H1'). In addition, the corresponding N-1-α isomer was also isolated. Yield: 2.88 g (7.67 mmol), 11.0%. R_f (A): 0.32. ^1H NMR ($CDCl_3$): δ 6.68 (1 H, d, $^3J_{1',2'}$ = 5.0 Hz, H1').

General Method for the Synthesis of Mercury Salts of 4-Nitro Heterobases. Compound 17,[34] 18,[35] or 19,[36] (30.65 mmol) was dissolved in a hot mixture of EtOH (450 mL) and H_2O (50 mL) containing an equimolar molar amount (1.23 g) of NaOH. After the nitro compound had gone into solution, 8.45 g (31.1 mmol) of $HgCl_2$ in 35 mL of hot EtOH was added in one portion. Although the majority of mercury salts precipitated immediately, the mixture was stirred for 30 min further, at 100 °C to drive the reaction to completion. Then the mixture was cooled to ambient temperature and concentrated to ~250 mL in volume. The yellow heavy-metal salts were filtered off, washed with cold water until no more chloride ion could be detected in the filtrate, and dried under an infrared lamp. Yield: 91–96%. On the basis of the N and Cl elemental analyses, the products prepared are mixtures of chloromercury and mercury salts of the corresponding nitro bases.

General Procedure for the Coupling of Heavy-Metal Salts of Nitro Bases with 20. A mercury salt (10.0 mmol) and an equimolar amount of **20** was refluxed in dry xylene, with stirring for 2–5 h. The mixture was then cooled to room temperature and the xylene was removed in vacuo. The solid residue was taken up with $CHCl_3$ (70 mL), the insoluble inorganic salts were removed by filtration, and the filtrate was extracted with 30% aqueous KI solution (2 × 40 mL) and water (50 mL). After separation, the organic phase was dried over $CaCl_2$, filtered, and evaporated to dryness to give a yellow syrup. The crude products were purified on a silica gel column containing 80 g of Kieselgel 60, using $CHCl_3$ as eluent. The ribofuranosides, obtained after evaporation of the appropriate fractions, can be readily crystallized with cold ether when allowed to stand for a short time.

4-Nitro-1-[2,3,5-tris-O-(p-chlorobenzoyl)-β-D-ribofuranosyl]indazole (21). Reaction time: 1.5 h. Yield: 77.2%. mp: 158 °C. R_f (A): 0.70. $[\alpha]^{25}_D = -53.50°$ (c = 1.0, $CHCl_3$). Anal. ($C_{33}H_{22}O_9N_3Cl_3$): C, H, N, Cl.

4-Nitro-1-[2,3,5-tris-O-(p-chlorobenzoyl)-β-D-ribofuranosyl]benzotriazole (22). Reaction time: 2.5 h. Yield 34.8%. mp: 161 °C. R_f (A): 0.68. $[\alpha]^{25}_D = -89.10°$ (c = 1.0, $CHCl_3$). Anal. ($C_{32}H_{21}O_9N_4Cl_3$): C, H, N, Cl. A significant amount of the N-2-β isomer (**22a**) was also isolated. Yield: 22.8%. mp: 171 °C. R_f (A): 0.64. $[\alpha]^{25}_D = -142.7°$ (c = 1.0, $CHCl_3$). Anal.: C, H, N, Cl.

4-Nitro-1-[2,3,5-tris-O-(p-chlorobenzoyl)-β-D-ribofuranosyl]benzimidazole (23). Reaction time: 5 h. Yield: 33.6%. mp: 188 °C. R_f (C): 0.17. $[\alpha]^{25}_D = -130.3°$ (c = 1.0, $CHCl_3$). Anal. ($C_{33}H_{22}O_9N_3Cl_3$): C, H, N, Cl.

General Procedure for Deacylation of Protected Nucleosides. Protected nucleoside (1.0 mmol) was suspended in dry MeOH (15 mL) and then 0.3 M NaOMe/MeOH solution was added dropwise with stirring. On addition of NaOMe solution, the mixture gradually cleared up, indicating the progress of the reaction. The clear solution was left to stand at room temperature overnight, and then H_2O (2.0 mL) was added and pH was adjusted to 7.0 with DOWEX 50 (H^+) resin. After filtration and removal of the MeOH in vacuo, the evaporation was repeated with 3 × 20 mL of water to remove the majority of the methyl p-chlorobenzoate. The solid residue was thoroughly washed with cold ether and dried on air. All the products can be recrystallized from EtOH, if necessary.

Physical constants (mp, $[\alpha]^{20}_D$, UV, and ^1H NMR data) of the nucleosides 5–8 were in good correlation with the reported literary data,[14,15] except the melting point of 8, which was 123 °C, while in the literature[15] it was described as an oil.

4-Nitro-1-β-D-ribofuranosylindazole (24). Yield: 84.5%. mp: 178 °C. R_f (B): 0.46 $[\alpha]^{25}_D = -102.0°$ (c = 1.0, MeOH). UV λ_{max}: nm 230 (10.1) 307 (6.5), 357 (7.9). IR ν_{max}: cm^{-1} 1337 $\nu_s(NO_2)$, 1534 $\nu_{as}(NO_2)$. ^1H NMR (DMSO-d_6): δ 6.16 (1 H, d, $^3J_{1',2'}$ = 3.1 Hz, H1'), 8.96 (1 H, s, H3), 8.10, 8.08 (2 H, m, H5, H7), 7.41 (1 H, m, H6). Anal. ($C_{12}H_{13}N_3O_6$: C, H, N.

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4-Nitro-1-β-D-ribofuranosylbenzotriazole (25). Yield: 81.2%. mp: 159 °C. R_f (B): 0.43. $[\alpha]^{25}_D$ = −83.2° (c = 1.0, MeOH). UV λ_{max}: nm 205 (13.2), 306 (11.7). IR ν_{max}: cm^{-1} 1354 $\nu_s(NO_2)$, 1531 $\nu_{as}(NO_2)$. ^{1}H NMR (DMSO-d_6): δ 6.46 (1 H, d, $^3J_{1',2'}$ = 2.5 Hz, H1'), 7.64 (1 H, m, H6), 8.34–8.37 (2 H, m, H5, H7). Anal. ($C_{11}H_{12}N_4O_6$): C, H, N.

4-Nitro-2-β-D-ribofuranosylbenzotriazole (25a). Yield: 73.1%. mp: 153 °C. R_f (B): 0.42. $[\alpha]^{25}_D$ = −288.1° (c = 1.0, MeOH). UV λ_{max}: nm 212 (11.2), 234 (7.3), 312 (7.1). IR ν_{max}: cm^{-1} 1352, 1532. ^{1}H NMR (DMSO-d_6): δ 6.74 (1 H, d, $^3J_{1',2'}$ = 2.8 Hz, H1'), 7.53 (1 H, m, H6), 8.30, 8.41 (2 H, m, H5, H7). Anal. ($C_{11}H_{12}N_4O_6$): C, H, N.

4-Nitro-1-β-D-ribofuranosylbenzimidazole (26). Yield: 87.6%. mp: 195 °C. R_f (B): 0.18. $[\alpha]^{25}_D$ = −29.4° (c = 1.0, MeOH). UV λ_{max}: nm 210 (10.0), 220 (9.1), 309 (8.1). IR ν_{max}: cm^{-1} 1337, 1525. ^{1}H NMR (DMSO-d_6): δ 6.06 (1 H, d, $^3J_{1',2'}$ = 5.9 Hz, H1'), 8.78 (1 H, s, H2), 7.52 (1 H, m, H6), 8.18, 8.22 (2 H, m, H5, H7). Anal. ($C_{12}H_{13}N_3O_6$): C, H, N.

General Procedure for the Synthesis of Nucleoside 5'-Monophosphate Diammonium Salts (9–12 and 27–29). Nucleoside (5–8 and 24–26) (1.0 mmol) was dissolved in dry triethyl phosphate (4.0 mL) and cooled to 0 °C, and 2.0 mmol (0.18 mL) of $POCl_3$ was added in one portion. The mixture was stirred for several hours at 0 °C and the reaction was followed by TLC. When most of the starting material had reacted, but the formation of 3',5'-diphosphate had not been considerable yet, the mixture was worked up in the following way. It was poured into ice water (10 mL), the pH was adjusted to 3.0 with concentrated ammonia solution. The water was evaporated in vacuo, inorganic ammonium salts were filtered off and washed with $(EtO)_3PO$ (4 mL), and the pH value of the filtrate was made slightly basic by aqueous concentrated ammonia solution. The solution thus obtained was applied to a DEAE Sephadex A-25 (HCO_3^-) column (350 mL). The column was washed with water (300 mL), and then the corresponding monophosphates were eluted with a linear gradient of water (1.5 L) and 1 M NH_4HCO_3 (1.5 L) (flow rate, 2 mL/min). The appropriate fractions were combined and evaporated to dryness. Evaporation was repeated with dry EtOH (2 × 25 mL) and the products were precipitated by addition of dry acetone. After filtration the white hygroscopic diammonium salts were dried in vacuo, over P_2O_5.

1-β-D-Ribofuranosylindazole 5'-Monophosphate Diammonium Salt (9). Reaction time: 15 min. Yield: 90.0%. $[\alpha]^{25}_D$ = −61.90° (c = 1.0, H_2O). R_f (E): 0.25. UV λ_{max}: nm 251 (3.4), 258 (3.5), 287 (3.19), 292 (3.8), 297 (3.3). IR ν_{max}: cm^{-1} 761 ν_s(P–O–C), 1078 ν_{as}(P–O–C), 1120–1240 ν_s(P=O). Anal. ($C_{12}H_{21}O_7N_4P$): C, H, N. P.

1-β-D-Ribofuranosylbenzotriazole 5'-Monophosphate Diammonium Salt (10). Reaction time: 4 h. Yield: 83.3%. $[\alpha]^{25}_D$ = −70.3° (c = 1.0, H_2O). R_f (E): 0.21. UV λ_{max}: nm 254 (6.3), 281 (3.6). IR ν_{max}: cm^{-1} 750, 1060, 1150, 1240. Anal. ($C_{11}H_{20}O_7N_5P$): C, H, N, P.

2-β-D-Ribofuranosylindazole 5'-Monophosphate Diammonium Salt (11). Reaction time: 30 min. Yield: 71.5%. $[\alpha]^{25}_D$ = −50.5° (c = 1.0, H_2O). R_f (E): 0.24. UV λ_{max}: nm 276 (5.9), 292 (5.4). IR ν_{max}: cm^{-1} 760, 1060, 1120–1240. Anal. ($C_{12}H_{21}O_7N_4P$): C, H, N, P.

2-β-D-Ribofuranosylbenzotriazole 5'-Monophosphate Diammonium Salt (12). Reaction time: 4.5 h. Yield: 85.2%. $[\alpha]^{25}_D$ = −81.3° (c = 1.0, H_2O). R_f (E): 0.31. UV λ_{max}: nm 278 (14.5), 281 (15.9), 287 (12.6). IR ν_{max}: cm^{-1} 745, 1067, 1100–1250. Anal. ($C_{11}H_{20}O_7N_5P$): C, H, N, P.

4-Nitro-1-β-D-ribofuranosylindazole 5'-Monophosphate Diammonium Salt (27). Reaction time: 1 h. Yield 86.0%. $[\alpha]^{25}_D$ = −83.90° (c = 1.0, H_2O). R_f (E): 0.19. UV λ_{max}: nm 233 (7.6), 316 (5.9), 362 (7.7). IR ν_{max}: cm^{-1} 740 ν_{as}(P–O–C), 1065 ν_s(P–O–C), 1110–1240 ν_s(P=O), 1349 $\nu_s(NO_2)$, 1523 $\nu_{as}(NO_2)$. Anal. ($C_{12}H_{20}O_9N_5P$): C, H, N, P.

4-Nitro-1-β-D-ribofuranosylbenzotriazole 5'-Monophosphate Diammonium Salt (28). Reaction time: 6 h. Yield: 72.0%. $[\alpha]^{25}_D$ = −24.3° (c = 1.0, H_2O). R_f (E): 0.13. UV λ_{max}: nm 205 (11.4), 230 (4.1), 315 (6.4). IR ν_{max}: cm^{-1} 738, 1068, 1110–1250, 1350, 1527. Anal. ($C_{11}H_{19}O_9N_6P$): C, H, N, P.

4-Nitro-1-β-D-ribofuranosylbenzimidazole 5'-Monophosphate Diammonium Salt (29). Reaction time: 1.5 h. Yield: 80.1%. $[\alpha]^{25}_D$ = −42.1° (c = 1.0, H_2O). R_f (E): 0.11. UV λ_{max}: nm 222 (7.5), 318 (8.1). IR ν_{max}: cm^{-1} 735, 1085, 1110–1240, 1341, 1522. Anal. ($C_{12}H_{20}O_9N_5P$): C, H, N, P.

The ^{13}C NMR data of all nucleoside 5'-monophosphates are listed in Table I.

General Method for the Synthesis of Nucleoside 3',5'-Cyclic Monophosphate Ammonium Salts (13–16 and 30–32). Compounds 9–12 and 27–29 (1.0 mmol) were dissolved in water (2.0 mL) and to this solution was added *N,N'*-dicyclohexyl-4-morpholinecarboxamidine (1.0 mmol) in pyridine (30 mL). The solution was evaporated to dryness and evaporation was repeated with 3 × 15 mL of dry pyridine. The solid residue was dried overnight in vacuo over P_2O_5. The dry organic salt thus obtained was dissolved in dry pyridine (100 mL). This solution was added dropwise slowly to the refluxing solution of DDC (2.0 mmol) in dry pyridine (100 mL) with vigorous stirring. After addition, the stirred reaction mixture was kept boiling for 2 h further and then was cooled to ambient temperature and evaporated to dryness. Evaporation was repeated with toluene (2 × 40 mL) and the residue was taken up with water (20 mL) and ether (20 mL). The insoluble *N,N'*-dicyclohexylurea was filtered off, the filtrate was separated, and the aqueous phase was reextracted with ether (20 mL) followed by concentration to a smaller volume. This aqueous solution was applied to a column (350 mL) made of DEAE Sephadex A-25 (HCO_3^-). Preparation of 3',5'-cyclic monophosphates was carried out on the same way as it was described for the 5'-monophosphates.

1'-β-D-Ribofuranosylindazole 3',5'-Cyclic Monophosphate Ammonium Salt (13). Yield: 74.4%. $[\alpha]^{25}_D$ = −119.8° (c = 1.0, H_2O). R_f (E): 0.34. UV λ_{max}: nm 252 (5.4), 259 (5.6), 288 (6.0), 293 (5.9), 297 (5.3). IR ν_{max}: cm^{-1} 749 ν_s(P–O–C), 1084 ν_{as}(P–O–C), 1237 ν_s(P=O). Anal. ($C_{12}H_{16}O_6N_3P$): C, H, N, P.

1-β-D-Ribofuranosylbenzotriazole 3',5'-Cyclic Monophosphate Ammonium Salt (14). Yield: 74.4%. $[\alpha]^{25}_D$ = −135.2° (c = 1.0, H_2O). R_f (E): 0.45. UV λ_{max}: nm 254 (7.2), 281 (3.9). IR ν_{max}: cm^{-1} 758, 1085, 1234. Anal. ($C_{11}H_{15}O_6N_4P$): C, H, N, P.

2-β-D-Ribofuranosylindazole 3',5'-Cyclic Monophosphate Ammonium Salt (15). Yield: 59.4%. $[\alpha]^{25}_D$ = −82.3° (c = 1.0, H_2O). R_f (E): 0.41. UV λ_{max}: nm 276 (6.4), 292 (5.5). IR ν_{max}: cm^{-1} 749, 1087, 1240. Anal. ($C_{12}H_{16}O_6N_3P$): C, H, N, P.

2-β-D-Ribofuranosylbenzotriazole 3',5'-Cyclic Monophosphate Ammonium Salt (16). Yield: 86.7%. $[\alpha]^{25}_D$ = −95.6° (c = 1.0, H_2O). R_f (E): 0.38. UV λ_{max}: nm 278 (21.8), 281 (22.7), 287 (19.8). IR ν_{max}: cm^{-1} 747, 1085, 1230. Anal. ($C_{11}H_{15}O_6N_4P$): C, H, N, P.

4-Nitro-1-β-D-ribofuranosylindazole 3',5'-Cyclic Monophosphate Ammonium Salt (30). Yield: 87.3% $[\alpha]^{25}_D$ = −102.5° (c = 1.0, H_2O). R_f (E): 0.39. UV λ_{max}: nm 233 (9.8), 317 (9.3), 362 (10.7). IR ν_{max}: cm^{-1} 739 ν_s(P–O–C), 1084 ν_{as}(P–O–C), 1238 ν_s(P=O), 1341 $\nu_s(NO_2)$, 1527 $\nu_{as}(NO_2)$. Anal. ($C_{12}H_{15}O_8N_4P$): C, H, N, P.

4-Nitro-1-β-D-ribofuranosylbenzotriazole 3',5'-Cyclic Monophosphate Ammonium Salt (31). Yield: 66.3%. $[\alpha]^{25}_D$ = −71.1° (c = 1.0, H_2O). R_f (E): 0.34. UV λ_{max}: nm 205 (13.0), 231 (7.1), 314 (9.8). IR ν_{max}: cm^{-1} 738, 1087, 1249, 1351, 1529. Anal. ($C_{11}H_{14}O_8N_5P$): C, H, N, P.

4-Nitro-1-β-D-ribofuranosylbenzimidazole 3',5'-Cyclic Monophosphate Ammonium Salt (32). Yield: 78.2%. $[\alpha]^{25}_D$ = −36.1° (c = 1.0, H_2O). R_f (E): 0.29. UV λ_{max}: nm 221 (9.3), 317 (9.8). IR ν_{max}: cm^{-1} 738, 1087, 1256, 1343, 1525. Anal. ($C_{12}H_{15}O_8N_4P$): C, H, N, P.

The ^{13}C NMR data of all nucleoside 3',5'-cyclic monophosphates are listed in Table II.

General Method for the Preparation of cAMP Analogues (33–35). A nitro compound (30–32) (0.2 mmol) was dissolved in water (2.0 mL) and ethanol (16 mL). This solution was shaken in a H_2 atmosphere in the presence of 10% Pd–C catalyst (20 mg) until H_2 consumption was observed. After approximately the theoretical volume of H_2 had been consumed the catalyst was filtered off through an asbestos pad and washed with EtOH (10 mL), and the filtrate was evaporated to dryness. This was repeated with several portions of EtOH. The pale yellow hygroscopic solid thus obtained was dried in vacuo over P_2O_5.

4-Amino-1-β-D-ribofuranosylindazole 3',5'-Cyclic Monophosphate Ammonium Salt (33) (1,3,7-Trideaza-8-aza-cAMP·NH$_3$). Reaction time: 75 min. Yield: 92.7%. R_f (E):

0.35. ^{1}H NMR (DMSO-d_6): δ 6.20 (1 H, s, $^3J_{1',2'} < 0.7$ Hz, H1'), 6.90–7.50 (3 H, m, H5, H6, H7), 8.44 (1 H, s, H3). UV λ_{max}: nm 224 (12.7), 268 (4.9), 316 (3.7). IR ν_{max}: cm^{-1} 750 ν_s(P–O–C), 1087 ν_{as}(P–O–C), 1246 ν_s(P=O). Anal. ($C_{12}H_{17}O_6N_4P$): C, H, N, P.

4-Amino-1-β-D-ribofuranosylbenzotriazole 3',5'-Cyclic Monophosphate Ammonium Salt (34) (1,3-Dideaza-8-aza-cAMP·NH$_3$). Reaction time: 45 min. Yield 94.1%. R_f (E): 0.35. ^{1}H NMR (DMSO-d_6): δ 6.46 (1 H, s, $^3J_{1',2'} < 0.7$ Hz, H1'), 7.10–7.70 (3 H, m, H5, H6, H7). UV λ_{max}: nm 230 (18.3), 271 (6.0), 315 (3.9). IR ν_{max}: cm^{-1} 748, 1082, 1244. Anal. ($C_{11}H_{16}O_6N_5P$): C, H, N, P.

4-Amino-1-β-D-ribofuranosylbenzimidazole 3',5'-Cyclic Monophosphate Ammonium Salt (35) (1,3-Dideaza-cAMP·NH$_3$). Reaction time: 120 min. Yield: 91.7%. R_f (E): 0.23. ^{1}H NMR (DMSO-d_6): δ 6.18 (1 H, s, $^3J_{1',2'} < 0.7$ Hz, H1'), 6.80–7.50 (3 H, m, H5, H6, H7), 8.45 (1 H, s, H2). UV λ_{max}: nm 216 (15.7), 266 (5.8), 288 (5.1). Anal. ($C_{12}H_{17}O_6N_4P$): C, H, N, P.