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Derivatives of 1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate that mimic the actions of adenosine 3′,5′-phosphate (cAMP) and guanosine 3′,5′-phosphate (cGMP) *

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ABSTRACT

A series of new analogues of 1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (cBIMP) has been designed according to the properties predicted by the MNDO method, and synthesised from substituted benzimidazoles. Dipole vectors and HOMO and LUMO energies for each benzimidazole base were calculated by the MNDO method and the lipophilicities of the cBIMP derivatives were determined. In general, the cBIMP derivatives activate cAMP-dependent protein kinases I and II and preferentially bind to site B, especially for the type II kinase, with 2-trifluoromethyl-cBIMP and 5,6-difluoro-cBIMP exhibiting the highest site selectivity. Each cBIMP derivative can stimulate cGMP-stimulated cyclic phosphodiesterase (cGS-PDE), with 5,6-dimethyl-cBIMP being as potent as cGMP, and also inhibit cGMP-inhibited phosphodiesterase (cGI-PDE). Only the 2-trifluoromethyl-cBIMP and the Rp-phosphorothioates (cBIMPS) (equatorial P=S) were resistant to hydrolysis by cPDE. The Sp-phosphorothioates were hydrolysed slowly, if at all. In addition to exhibiting a high lipophilicity, the most active compounds for the induction of apoptosis and inhibition of proliferation were also resistant to cPDE (Sp-5,6-dichloro-cBIMPS) and/or were potent activators of cAMP-dependent protein kinase (5,6-dichloro-cBIMP).

INTRODUCTION

A ubiquitous mechanism for relaying a hormonal signal inside cells is by the action of second messengers. Regulation by adenosine 3′,5′-phosphate (cAMP) is

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widespread throughout all organisms and affects diverse physiological and biochemical responses[1]. In addition to mediating the effects of hormones in cells, cAMP is involved in the control of cell proliferation and differentiation[2–4], in the onset of programmed cell death (apoptosis)[5], and in acting as a first messenger in such organisms as *Dictyostelium discoideum*[6].

Regulation by guanosine 3′,5′-phosphate (cGMP) is less widespread than the action of cAMP, and cGMP-controlled metabolic processes occur in specialised cell types[1].

The cAMP signal is perceived by various receptor proteins, e.g., catabolite gene-activator protein (CAP and CRP, respectively) in bacteria[7], cell-surface receptors (CSR) in *Dictyostelium discoideum*, and two general types of cAMP-dependent protein kinases each with two subtypes, as well as specific ion channels[8] in eukaryotes. cGMP effects are mediated by three subtypes of cGMP-dependent protein kinases (cGKs), cGMP-regulated ion channels (e.g., in the retina)[8], and cGMP-controlled phosphodiesterases. For many tissues, extensive interaction of processes regulated by cAMP, cGMP, calcium ions, and inositol phosphate has been characterised[9,10]. Both cAMP and cGMP are hydrolysed rapidly in cells by various cyclophosphodiesterases (cPDEs)[9].

Because of the involvement of cAMP in multiple cellular-regulation processes, including cellular proliferation and growth control[11,12], cAMP analogues are a prime target of pharmacological research[13–17], as illustrated by the recent development of a modified cAMP as a new antitumour agent[12,18].

Rationales for the design of cyclic nucleotide analogues for process-selective actions in cells.—Since the development of cAMP 2′,6-dibutyrate[19], several hundred cAMP analogues have been synthesised with the aim of obtaining a selective, highly active, metabolically stable, and lipophilic cAMP agonist for pharmacological and therapeutical use. Substitution of equatorial P=O by P=S led to the first cyclic nucleotide antagonists, Rp-cAMPS for cAMP-dependent protein kinase[20] and Rp-cGMPS for cGMP-dependent protein kinase[21]. The most promising target enzymes in mammals are the isozymes of cyclic nucleotide-dependent protein kinases and the various cPDEs. Fourteen different binding sites on cAKs and cGKs have been characterised in mammalian systems (two kinetically different sites on cAK-Iα,β, cAK-IIα,β[22], cGK Iα,β, and cGK II[10,23,24]). In addition, six different cyclic nucleotide-binding sites on cPDEs have been identified (two on cPDEs specific for either cGMP or cAMP, two on unspecific cPDEs, and one catalytic and one allosteric binding site on the cGMP-stimulated cPDE)[9].

The design of new cyclic nucleotide analogues selective for a specific biological process has to take into account the numerous enzymes involved in the metabolism of cyclic nucleotides.

The following are requirements for a process-specific cyclic nucleotide analogue: (*a*) binding constants to cAKs must be several orders of magnitude lower than to cGKs and other cyclic nucleotide-regulated proteins; (*b*) the derivative must be enzyme subtype-specific or at least highly selective; (*c*) only full agonists

or antagonists of a specific enzyme are useful; (*d*) a low concentration of the analogue should be sufficient to trigger the biological effect; (*e*) analogues should be highly lipophilic in order to ensure passage through cell membranes; (*f*) cAMP/cGMP antagonists should not inhibit cPDEs significantly in order to avoid an increase in endogenous cAMP and/or cGMP; (*g*) cAMP agonists should not activate the cGMP-stimulated cPDE; and (*h*) cyclic nucleotide analogues should be resistant to hydrolysis in order to avoid side effects by metabolism.

With current knowledge and biochemical characterisation of the enzymes involved, the above criteria can be met at least partially.

Two approaches have been used to characterise the binding sites of cyclic nucleotides by means of analogues. The test-kit concept uses a limited number of analogues in order to define and compare molecular interactions essential to the binding of cyclic nucleotides[22,25,26]. Determination of site and isozyme selectivity has been studied systematically for more than 100 cyclic nucleotide analogues in order to characterise the molecular properties that define the binding to sites A and B of cAK I and II[2,27–29]. The cGKs[28,30] and cPDEs[31,32] have been characterised less extensively by analogue mapping.

The chemical groups able to increase membrane permeability and hydrophobicity of the base moiety are known[26,33]. Mapping studies for the cAKs and cGKs have also defined clearly the structural elements of the cyclic nucleotides that must not be altered. These elements are somewhat less well established for the cPDEs. The substitution of equatorial P=O by P=S promotes antagonism for cAKs[20] and cGKs[21], and resistance to hydrolysis[34].

A significant finding is the difference in hydrogen bonding to the base moiety of cyclic nucleotides in cAKs and cGKs. Whereas, for cGKs, a specific hydrogen bond is formed to N-2 of the purine moiety[23], binding of the adenine moiety to cAKs does not involve hydrogen bonding but presumably hydrophobic and/or charge transfer interactions[22,35–37].

The significance of theoretically determined dipole moments and HOMO and LUMO energies of the bases in cyclic nucleotide derivatives has been demonstrated[38] for the hydrolysis of these derivatives by cPDE. The influence of the dipole vector for the recognition and binding of cyclic nucleotide derivatives by cGK has been shown by Butt[30].

For type II cAK, site-A selectivity can be achieved with 6-substituted cAMP analogues, whereas 8-substituted derivatives preferentially bind to site B. The situation for the type I cAK is not yet clearly defined.

Based on these rationales and results, a series of benzimidazole derivatives has been synthesised in order to test ideas for the design of new cyclic nucleotide analogues with specific biological activities.

Benzimidazole derivatives as a model for the rational design of cyclic nucleotide analogues.—1-β-D-Ribofuranosylbenzimidazole 3′,5′-phosphate (**1**) (cBIMP) was synthesised first within a screening programe[39] and was subsequently investigated in several biological systems[29,35,36,39,40] as part of the cAMP/cGMP test-kits[25].

cBIMP activates cAK I and II, for which the site selectivity has been determined, and is a good activator of the hydrolysis of cAMP by cGMP-stimulated cPDE[41,42].

We have used cBIMP as a basic structure in order to check the validity of the rationales discussed above, and now report on the effects of systematic modification at the 5-, 6-, and 5,6-positions with fluorine, chlorine, bromine, nitro, and methyl substituents. Table I shows the derivatives synthesised. Phosphorothioate analogues, with sulfur axial (Sp) or equatorial (Rp), were synthesised of those phosphate derivatives with interesting biological activity.

According to MNDO calculations (Table I), the dipole moment of the benzimidazole base can be adjusted in magnitude and direction through regioselective mono-nitration at positions 4–6 (Fig. 1). The largest change in the direction of the dipole can be achieved by introduction of a trifluoromethyl group at position 2 (**8**).

The benzimidazole derivatives are more lipophilic than purine derivatives and the log k_w values[33] are given in Table I. Using MNDO, the HOMO and LUMO energy levels of the bases were calculated in order to assess the charge-transfer interaction potentials and the results are shown in Table I.

Synthesis of benzimidazole derivatives.—The analogues **1–19** were synthesised conventionally from the appropriate benzimidazole derivatives by the sequence (*a*) trimethylsilylation, (*b*) trimethylsilyltriflate-catalysed reaction with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose[43], (*c*) *O*-deacylation (MeOH–NH_3), (*d*) 3′,5′-phosphorylation[44,45] ($POCl_3$ or $PSCl_3$ in triethyl phosphate), (*e*) saponification (MeCN–H_2O–KOH). The phosphorothioates Rp- and Sp-cBIMPS were synthesised according to Genieser et al.[46,47], each as a mixture of diastereomers that was resolved by chromatography.

The phosphorylation method described initially follows the well-established procedure of Yoshikawa et al.[48] and presumably results in the formation of a 5′-phosphorodichloridate. In contrast to the normally applied hydrolysis, which yields mainly the 5′-phosphates or 5′-thiophosphates[49], the phosphorylation mixture is poured into a large volume of a basic medium (MeCN–H_2O–KOH) in order to promote cyclisation[44,46]. The incorporation of MeCN to the minimum necessary for the solubilisation of potassium hydroxide decreased hydrolysis to the phosphates.

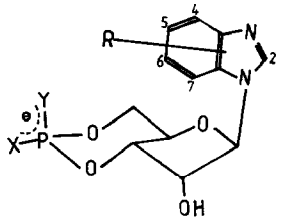
The general cyclophosphorylation procedure described (see Experimental) is the result of systematic variation of the reaction parameters, including solvents, bases, or dilution factors, and is considered to be about the optimum obtainable. The moisture content seems not to be a critical parameter since the highly water-sensitive phosphoryl chloride generally reacted more quickly and gave higher yields than the less sensitive thiophosphoryl chloride. Intensive drying of nucleosides had little or no effect on the phosphorylation procedure and, in contrast to previous results, there was no improvement on adding small quantities of water[50]. The 5,6-dihalogenated benzimidazole nucleosides were difficult to thiophosphorylate and the yields on cyclisation were low. However, there was no striking correlation between structural or steric properties of a nucleoside and its be-

TABLE I

1-β-D-Ribofuranosylbenzimidazole 3′,5′-phosphate and 3′,5′-phosphorothioate derivatives: lipophilicity (log k_w[33]), dipole vectors and directions (deviation relative to that of adenine), and HOMO/LUMO-energies of the bases (calculated by MNDO)

	R	Log k_w	HOMO (eV)	LUMO (eV)	Dipole (D)	Angle (°)
cAMP		1.10	−8.48	−0.14	2.03	0
cGMP		0.68	−8.56	−0.07	5.73	131
1 1-β-D-Ribofuranosylbenzimidazole 3′,5′-phosphate (cBIMP)	H	1.55	−8.86	−0.02	3.12	93
2 4-Nitro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (4-N-cBIMP)	4-NO_2	1.52	−9.63	−1.27	5.69	96
3 4-Amino-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (4-A-cBIMP)	4-NH_2	1.14	−7.88	+0.29	1.29	76
4 5-Nitro-1-β-D-ribofuranosylbenzimidazole-3′,5′-phosphate (5-N-cBIMP)	5-NO_2	1.71	−9.69	−1.10	8.09	60
5 6-Nitro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (6-N-cBIMP)	6-NO_2	1.69	−9.73	−1.34	6.00	21
6 5-Methyl-1-β-D-ribofuranosylbenzimidazole 3′,5′phosphate (5-Me-cBIMP)	5-Me	1.91	−8.86	−0.02	3.13	92
7 6-Methyl-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (6-Me-cBIMP)	6-Me	1.90	−8.80	−0.09	3.07	93
8 1-β-D-Ribofuranosyl-2-trifluoromethylbenzimidazole 3′,5′-phosphate (2-CF_3-cBIMP)	2-CF_3	2.17	−9.38	−0.67	4.00	147
9 5,6-Difluoro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (5,6-F_2-cBIMP)	5,6-F_2	2.12	−9.20	−0.79	5.33	51
10 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (5,6-Cl_2-cBIMP)	5,6-Cl_2	2.81	−9.37	−0.71	5.00	54
11 Rp-5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphorothioate (Rp-5,6-Cl_2-cBIMPS)	5,6-Cl_2	n.d. [a]	−9.37	−0.71	5.00	54
12 Sp-5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphorothioate (Sp-5,6-Cl_2-cBIMPS)	5,6-Cl_2	2.93	−9.37	−0.71	5.00	54
13 5,6-Dibromo-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (5,6-Br_2-cBIMP)	5,6-Br_2	3.05	−9.27	−0.60	4.38	60
14 Rp-5,6-Dibromo-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphorothioate (Rp-5,6-Br_2-cBIMPS)	5,6-Br_2	3.10	−9.27	−0.60	4.38	60
15 Sp-5,6-Dibromo-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphorothioate (Sp-5,6-Br_2-cBIMPS)	5,6-Br_2	3.34	−9.27	−0.60	4.38	60
16 5,6-Dinitro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (5,6-N_2-cBIMP)	5,6-$(NO_2)_2$	2.00	−10.41	−1.66	10.02	34
17 Rp-5,6-Dinitro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphorothioate (Rp-5,6-N_2-cBIMPS)	5,6-$(NO_2)_2$	n.d. [a]	−10.41	−1.66	10.02	34
18 Sp-5,6-Dinitro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphorothioate (Sp-5,6-N_2-cBIMPS)	5,6-$(NO_2)_2$	n.d. [a]	−10.41	−1.66	10.02	34
19 5,6-Dimethyl-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (5,6-Me_2-cBIMP)	5,6-$(Me)_2$	2.28	−8.79	−0.06	3.08	95

[a] Not determined.



1–19 (see Table I for R)

1–10, 13, 16, 19	X = Y = O
11, 14, 17	X = S, Y = O
12, 15, 18	X = O, Y = S

haviour on phosphorylation and cyclisation.

The phosphorylation procedure and the subsequent cyclisation yielded numerous byproducts, including monophosphates, diphosphates, 2′,3′-phosphates, 5′-chloro-5′-deoxynucleosides, and remnants of the starting riboside and the nucleobase. Some of these byproducts are potential metabolites of the cyclic phosphates and therefore have value as reference compounds.

With trimethyl phosphate as the solvent in the phosphorylation, the yields were generally improved but, due to the carcinogenicity of this solvent, the corresponding ethyl ester was used.

The 3′,5′-phosphates were purified by reversed-phase flash-column chromatography, but this process was sometimes difficult and resulted in relatively low yields. The synthesis procedures were not optimised with respect to yield, because the biological activities could be determined on small amounts of analogues.

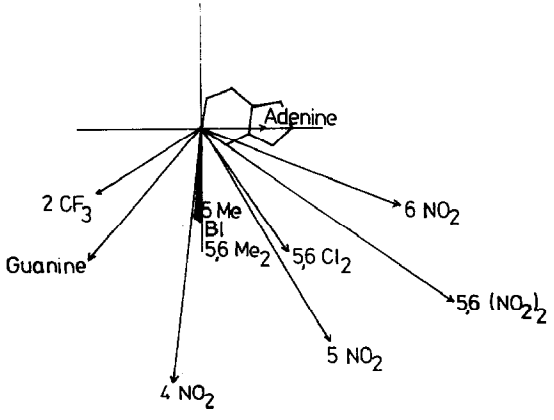


Fig. 1. Representation of the dipole moments of some substituted benzimidazoles. The direction of the dipole moment is standardised as a clockwise angle relative to the dipole vector of cAMP, with the origin of the co-ordinate system set to position 2 of adenine; BI = unsubstituted benzimidazole.

TABLE II

Activation constants for cAK I and II, and relative affinities and selectivities for site A and B [a]

	cAK I				cAK II			
	K_a(nM)	K_i'AI	K_i'BI	$\frac{K_i'BI}{K_i'AI}$	K_a(nM)	K_i'AII	K_i'BII	$\frac{K_i'BII}{K_i'AII}$
cAMP	50	1.0	1.0	1.0	80	1.0	1.0	1.00
cGMP	10200 *	0.005	0.012	2.5	60000	0.004	0.0009	1/4.5
cBIMP (1)	340 *	0.058	0.38	6.5	500	0.19	0.86	4.5
4-N-cBIMP (2)	2300 *	0.008	0.06	7.5	630	0.014	0.40	29
5-N-cBIMP (4)	3300 *	0.018	0.013	1/1.5	44	0.04	3.3	83
6-N-cBIMP (5)	2100 *	0.0073	0.08	11	680	0.007	1.03	150
2-CF_3-cBIMP (8)	280 *	0.15	0.22	1.5	830	0.0004	6.4	16000
5,6-F_2-cBIMP (9)	550 *	0.016	0.51	32	500	< 0.00001	0.033	3300
5,6-Cl_2-cBIMP (10)	67 *	0.11	5.1	46	9	0.38	42	110
R_p-5,6-Cl_2-cBIMPS (11)	n.d. [b]	0.0004	0.02	50	n.d. [b]	0.0017	0.46	270
S_p-5,6-Cl_2-cBIMPS (12)	930 *	0.02	0.13	6.5	250	0.034	14	412
R_p-5,6-Br_2-cBIMPS (14)	20000	0.00025	0.03	120	2500	0.0006	0.45	750
S_p-5,6-Br_2-cBIMPS (15)	1100 *	0.015	0.15	10	550	0.031	8.3	270
5,6-N_2-cBIMP (16)	8300	0.005	0.007	1.5	600	0.0004	0.15	375
5,6-Me_2-cBIMP (19)	610	0.017	0.40	24	30	0.28	6.23	22

[a] The affinity of cAMP analogues for sites A and B of the RI (purified from rabbit skeletal muscle) and RII subunit (purified from bovine heart) was deduced from competition[57] with ^{3}H-cAMP. The relative affinity for a binding site is expressed as K_i'(analogue) = K_i(cAMP)/K_i(analogue), K_i'AI and K_i'AII are the relative affinities of an analogue for site A of RI and RII, respectively, and K_i'BI and K_i'BII are the relative affinities for site B of RI and RII, respectively. The apparent K_a value is the concentration of a nucleotide to induce 50% activation. The K_a values (denoted by *) for cAK I were calculated according to the method of Ogreid[65]. [b] Not determined.

Biochemical studies with cAK I and cAK II.—Activation constants (K_a) for cAK I and II were either determined experimentally or calculated according to Øgreid et al.[29] (Table II). Each of the 3′,5′-phosphates and the 3′,5′-phosphorothioates with an axial sulfur (Sp) could activate each kinase. The activity varied within a 100-fold range, depending on the type and position of the benzimidazole substituents. Replacement of P=O by P=S led to a decrease in activity. Some derivatives with an equatorial sulfur (Rp) did not activate or inhibit the cAKs. A detailed investigation of the Rp-phosphorothioate derivatives is in progress.

There was no significant correlation between the data calculated by MNDO (Table I) and the activation constants. The presence of hydrophobic interactions at the cAMP binding sites on each kinase[35] was corroborated by a general increase of activity with higher lipophilicity of the nucleobase (Table I).

The reduced biological activity of the Sp-phosphorothioate derivatives **12** and **15** accords with data for Sp-cAMPS and its derivatives[36,51]. A mechanistic interpretation of this effect has been proposed[51,52]. None of the derivatives showed specificity for cAK I or II. Most of the compounds had similar K_a values.

The high preference (100-fold) of 5-N-cBIMP (**4**) for cAK type II and the reasonably high preferences for 5,6-Me_2-cBIMP (**19**, 20-fold) and 5,6-N_2-cBIMP

(**16**, 10-fold) make these derivatives starting points for the development of more specific cBIMP analogues.

Site-selective binding studies for cAK I and cAK II were performed in order to determine the affinities for sites A and B according to the method of Øgreid et al.[29]. The K_i' values shown in Table II were standardised against the affinities of cAMP for the same sites [K_i' (analogue) = K_i(cAMP)/K_i(analogue)]. In addition, values for the site selectivity on cAK I and cAK II (K_i'A/K_i'B) are included in Table II. The K_i' values for some of the analogues have been published[51].

Each of the benzimidazole derivatives was bound preferentially to site B in each enzyme, but exhibited higher site selectivity in cAK II than in cAK I. Regardless of the type of benzimidazole substituent, substitution increased the site selectivity for cAK II site B (Table II). The most profound effect was observed with the 2-trifluoromethyl group (**8**). Another compound highly discriminative for site B was 5,6-F_2-cBIMP (**9**).

It has been postulated that, in the cAMP molecule, 6-substitution (corresponding to 4-subtitution in benzimidazoles) leads to site-A selectivity in each kinase. For cAMP, 2- and 8-substitution (corresponding to the 6- and 2-substitution in benzimidazoles) should produce site B-selective compounds[27]. Our results indicate that no such clear-cut prediction about the site selectivity of 3′,5′-phosphates can be made and other factors must be involved.

Since 2-CF_3-cBIMP (**8**) mimics an 8-modified cAMP derivative, it should be highly site-B selective for each kinase. Although site-B selectivity was observed for cAK II, no site selectivity was found for cAK I. On the other hand, 4-N-cBIMP (**2**), which resembles a 6-modified cAMP and should be site-A selective according to the theory[27], was site-B selective in each cAK.

Affinity for a distinct site, as compared to that of cAMP, varied from 40-fold higher (**10**) for site B of cAK II to five orders of magnitude lower (**9**) for site A of cAK II. Reduction in the affinity for AI and AII compared to cAMP was found for all of the derivatives **1**–**19** (Table II).

No simple correlations were found between the affinity or site selectivity for each enzyme with respect to log k_w and the theoretical parameters determined.

cPDE studies.—Twelve of the benzimidazole derivatives were studied as activators of the cyclic GMP-stimulated cPDE, as inhibitors of cGMP-inhibited cPDE, and for hydrolysis by cGS-PDE, cGI-PDE, and the Ca calmodulin-stimulated cPDE. The results are given in Table III. Each of the benzimidazole derivatives studied except 2-CF_3-cBIMP (**8**) could activate cGS-PDE, and 5,6-Me_2-cBIMP (**19**) was as active as cGMP. A 5–30-fold reduction in activity was observed for 5- (**4**) and 6-N-cBIMP (**5**), 5- (**6**) and 6-Me-cBIMP (**7**), and 5,6-Cl_2-cBIMP (**10**). Substitution of P=O by P=S lowered the affinity further (**11**, **12**, **14**, and **15**). A slight regioselectivity in favour of the Rp-derivative (2-fold difference) was observed.

Each of the derivatives, except 5-Me-cBIMP (**6**), was an inhibitor of cGI-PDE, but none was as good as cGMP. The best inhibitor was 5,6-Cl_2-cBIMP (**10**) with

TABLE III

Specificity of phosphodiesterases [a]

	Activation	Inhibition		Hydrolysis		
	cGS-PDE K_a (μM)	cGI-PDE K_i (μM)	K_a/K_i	cGS-PDE V'_{max}	cGI-PDE V'_{max}	CaM-PD V'_{max}
cAMP				1	1	1
cGMP	0.35	0.14	2.5	1	0.3	0.7
4-N-cBIMP (**2**)	n.d. [b]	30	n.d. [b]	0.56	4.2	n.d. [b]
5-N-cBIMP (**4**)	1.5	130	1/87	0.13	3.2	n.d. [b]
6-N-cBIMP (**5**)	2.5	16	1/6	0.8	2.8	n.d. [b]
5,6-F_2-cBIMP (**9**)	4	28	1/7	0.25	2.6	0.5
5,6-Cl_2-cBIMP (**10**)	12	1.5	8	0.9	2.4	0.56
R_p-5,6-Cl_2-cBIMPS (**11**)	40	100	1/2.5	None	None	None
S_p-5,6-Cl_2-cBIMPS (**12**)	100	30	3	< 0.01	None	None
R_p-5,6-Br_2-cBIMPS (**14**)	18	9	2	None	None	None
S_p-5,6-Br_2-cBIMPS (**15**)	40	5.5	7	< 0.01	None	None
5-Me-cBIMP (**6**)	4	> 1000	1/250	0.08	n.d. [b]	n.d. [b]
6-Me-cBIMP (**7**)	6	100	1/17	0.06	0.80	n.d. [b]
5,6-Me_2-cBIMP (**19**)	0.3	180	1/600	1	2.5	None
2-CF_3-cBIMP (**8**)	None	140	selective	None	None	None

[a] The cyclic nucleotides shown were subjected to hydrolysis by cGS-PDE, cGI-PDE, or CaM-PDE, and assessed for activation of cGS-PDE and inhibition of cGI-PDE. K_a is the concentration of cyclic nucleotide required for 50% activation of the hydrolysis of cAMP and K_i the concentration required for 50% inhibition. K_a/K_i represents the discrimination between the activation site on cGS-PDE and the inhibition site on cGI-PDE; $V'_{max} = V'_{max}$(analogue)$/V'_{max}$ cAMP. The data for Sp-5,6-Cl_2-cBIMPS (**12**) have been reported[55]. [b] Not determined.

only a 10-fold reduced affinity compared to that of cGMP. Sulfur substitution again led to a reduction in affinity, but with the opposite regioselectivity.

5,6-Me_2-cBIMP (**19**) exhibited the highest discrimination between the activation site of cGS-PDE and the inhibition site of cGI-PDE. 5-Substitution with methyl or nitro also led to rather high discrimination, whereas, for the corresponding 6-substituted derivative, a difference of only one order of magnitude was observed.

cGS-PDE has been mapped by using cAMP and cGMP analogues, and a model for the essential interactions of cGMP with the activation site has been proposed[41]. The K_a values obtained for the benzimidazole derivatives accord with the proposal that N-7 of the guanine base (corresponding to N-3 of benzimidazoles) is involved in recognition and binding. The benzimidazole derivative with the highest electron density at N-3 (**19**, 5,6-Me_2-cBIMP) also exhibited the highest affinity and it is proposed that hydrophobic forces and charge-transfer interactions may also be involved in binding. The inactivity of 2-CF_3-cBIMP (**8**), which adopts the *syn*-conformation, towards cGS-PDE accords with the model that proposes an *anti*-conformation for the guanine base.

The cGI-PDE-inhibitory binding site has not been mapped. Benzimidazole derivatives alone did not allow the essential interactions involved to be proposed. However, because of profound differences in affinity observed for some of the derivatives **1**–**19**, this site must be different from the activation site of cGS-PDE.

TABLE IV

Effects of cBIMP analogues on the viability of BNS leukemia cells and on the synthesis of DNA by C6 glioma cells [a]

		Cytolysis	Proliferation
		BNS cells $K_{0.5}$ (mM)	C6 cells IC_{50} (mM)
4-N-cBIMP	(2)	> 1	> 1
5-N-cBIMP	(4)	> 1	> 1
6-N-cBIMP	(5)	> 1	n.d. [b]
2-CF_3-cBIMP	(8)	0.5	0.6
5,6-F_2-cBIMP	(9)	> 1	0.8
5,6-Cl_2-cBIMP	(10)	0.2	0.4
Rp-5,6-Cl_2-cBIMPS	(11)	n.d. [b]	> 1
Sp-5,6-Cl_2-cBIMPS	(12)	0.08	0.2
Rp-5,6-Br_2-cBIMPS	(14)	> 0.5	> 0.5
Sp-5,6-Br_2-cBIMPS	(15)	0.15	0.4
5,6-Me_2-cBIMP	(19)	0.2	0.4

[a] $K_{0.5}$ is the concentration required for a 50% decrease in the viability of the cells; IC_{50} is the concentration that causes a 50% decrease in the synthesis of DNA. [b] Not determined.

The hydrolysis studies (Table III) showed that only Rp-5,6-Cl_2-cBIMPS (**11**), Rp-5,6-Br_2-cBIMPS (**14**), and 2-CF_3-cBIMP (**8**) were resistant to each cPDE. The Sp-derivatives (**12** and **15**) were hydrolysed slowly, if at all. These results accord with earlier observations that an equatorial P=S confers resistance to hydrolysis by mammalian cPDE, an axial P=S reduces the rates of hydrolysis drastically[34,52], and an 8-substituent reduces the rate of hydrolysis[14].

cGI-PDE hydrolysed the derivatives at a rate higher than those for cGMP and cAMP, wheras cGS-PDE hydrolysed the corresponding derivatives at a lower rate.

5,6-Me_2-cBIMP (**19**) was resistant to hydrolysis by CaM-PDE as was 2-CF_3-cBIMP (**8**), whereas 5,6-F_2-cBIMP (**9**) and 5,6-Cl_2-cBIMP (**10**) were hydrolysed nearly as rapidly as cGMP.

Cell culture studies.—The cBIMP derivatives were tested against the human leukemia cell line BNS and the rat C6 glioma.

The BNS cells provide an excellent test system for cyclic nucleotide analogues as inducers of apoptosis[5], which ultimately leads to cytolysis. The viability of BNS cells in the presence of benzimidazole derivatives was determined by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide colorimetric assay (MTT) for mitochondrial dehydrogenase enzymic activity[5] and the concentrations ($K_{0.5}$) leading to cytolysis of 50% of the cells are given in Table IV. The proliferation of C6 glioma cells was used as a model to study inhibition and differentiation by the cyclic nucleotide analogues. Proliferation of the C6 cells was determined on the basis of the increase of DNA after incubation for 4 days with each cBIMP derivative over the concentration range 5–1000 μM. The concentrations leading to 50% inhibition are given as IC_{50} values in Table IV.

Nitro substitution of the benzimidazole moiety rendered the cBIMP analogues inactive in each bioassay, as did the introduction of equatorial P=S (Rp derivatives). The corresponding axial P=S compounds were active. Sp-5,6-Cl_2-cBIMPS (**12**) was the most active compound and was more active than 5,6-Cl_2-cBIMP (**10**). 2-CF_3-cBIMP (**8**) and 5,6-Me_2-cBIMP (**19**) were also good activators of cytolysis and inhibitors of proliferation, whereas 5,6-F_2-cBIMP (**9**) had low (glioma) or no activity (leukemia). Ranking the biological activity in each system led to an identical sequence, which suggests similar mechanisms of action. The most active compounds were rather lipophilic, indicating a possible influence of cellular uptake. The cBIMP analogues were either resistant to the cPDEs investigated, or slowly hydrolysed, or at least resistant to one cPDE (**19**, 5,6-Me_2-BIMP), indicating that metabolic stability is a prerequisite for activity.

None of the derivatives reached the inhibitory potential of 8-Cl-cAMP in glioma (25 μM) which is dependent on a synergistic effect of the cyclic nucleotide and its metabolite 8-chloroadenosine[53,54].

In order to exclude the possible involvement of a cyclic GMP-dependent protein kinase as a target for cBIMP derivatives, K_a values were determined for the three most active compounds (data not shown). Sp-5,6-Cl_2-cBIMP (**12**) activated cGK only at concentrations 100-fold higher than for cAK I and II. 2-CF_3-cBIMP (**8**) and 5,6-Me_2-cBIMP (**19**) also exhibited a much lower affinity for cGK than for the cAKs. The cAK selectivity of Sp-5,6-Cl_2-cBIMPS (**12**) has been characterised in cell extracts and intact platelets[55].

CONCLUSIONS

Previous studies indicated that substitution of benzimidazole for adenine in cAMP gave a derivative with only 10-fold reduced activity towards cAK I and II, and a cGMP-like affinity for cGS-PDE. This is not suprising since models of the binding of cAMP to cAKs postulate dipole–dipole and/or charge-transfer interactions of the adenine base and one or two aromatic residues of the cAK[22,35,37,51] in addition to hydrophobic forces, and benzimidazole provides these properties. The relatively simple structure of benzimidazole limits the number of interactions with the target receptor proteins, but allows much scope for chemical modifications.

Although some of the analogues **1–19** have promising biological properties, correlation with dipole moments or HOMO/LUMO values of the nucleobases could not be confirmed. The nitro derivatives 5- (**4**) and 6-N-cBIMP (**5**) and 5,6-N_2-cBIMP (**16**), which have low LUMO energies, had low activities in the test systems which showed that designing on the basis of a single physicochemical parameter is not sufficient to produce specific biological results.

Presumably, the large dipole moments of these derivatives cancel out electronic effects that may enhance binding to cAKs. cGMP, which activated the cAKs only at a very high concentration, also has a large dipole moment (Fig. 1).

High hydrophobicity of the base seems to be a valid parameter for enhanced binding to cAKs.

Stability towards cPDEs could be achieved by introducing sulfur into the phosphate moiety. Thus, the Rp diastereomers (**11**, **14**, and **17**) were stable and the corresponding Sp isomers (**12**, **15**, and **18**) were metabolised only slowly.

The study reported here produced several derivatives (**8**, **9**, **12**, and **19**) with biological activity that warrants more detailed investigation or are in use as biochemical tools because of their specific activity profile[55] (effective differentiation between cAK and cGK, or between cPDE isozymes).

Some compounds (**8**, **9**, and **14**) also showed a high selectivity for site B of both type I and II cAK. Sp-5,6-Cl_2-cBIMPS (**12**), for example, combines high selectivity and activation potency for the cAKs, is not hydrolysed by cPDE, has a high lipophilicity, and will not interfere strongly with cGS-PDE- and cGI-PDE-regulated pathways, so that it meets several of the requirements (*a*)–(*h*) noted above for an ideal cyclic nucleotide.

2-CF_3-cBIMP (**8**) and 5,6-F_2-cBIMP (**9**) are excellent candidates for studies of the synergism between A- and B-selective compounds[5] because of their high selectivity for site B in cAK II.

5,6-Me_2-cBIMP (**19**) is a good discriminator for cGMP activation of cGS-PDE and cGMP inhibition of cGI-PDE.

The results show that the generalisation regarding 6-substituted cAMP derivatives as site-A selective, and the 2- and 8-substituted derivatives as site-B selective for each cAK, is an oversimplification. The behaviour of 4-N-cBIMP (**2**), which mimics a 6-substituted cAMP, and 2-CF_3-cBIMP (**8**), which resembles an 8-substituted cAMP, does not fit with this simple rule for determining site selectivity.

EXPERIMENTAL

General methods.—Flash-column chromatography was performed on LiChrosorb RP18 (20–40 μm, Merck) and analytical HPLC on LiChrosorb RP18 (10 μm, Merck). For the purification of the mixtures of 5- and 6-nitrobenzimidazole and 5- and 6-methylbenzimidazole derivatives, a Cyclobond-1 column (Astec) was used. Yields were determined by weight for ribosides and by UV spectroscopy for the 3′,5′-phosphates and 3′,5′-phosphorothioates. The ^1H and ^{31}P NMR spectra (internal Me_4Si and external H_3PO_4, respectively) were recorded with a Bruker WH-360 spectrometer for solutions in MeOD. Only those ^1H NMR data characteristic for the structure are given. UV spectra were recorded for solutions in MeOH, using a Perkin–Elmer Model 554 spectrometer. Mass spectra were obtained with a Finnigan MAT spectrometer (Model 8222).

5-Nitrobenzimidazole was purchased from Aldrich (Steinheim), 5,6-dimethylbenzimidazole from Sigma (Deisenhofen), ^3H-cAMP from ICN (Eschwege), [^{32}P]ATP from Amersham Buchler (Braunschweig), the peptide Kemptide from Peninsula Chemicals (UK), and snake venom (*Crotalus adamanteus*) from Sigma

(Deisenhofen). All tissue culture media, solutions, and foetal calf serum were purchased from Gibco Ltd. (Eggenstein).

The log K_w values were determined according to the procedure of Braumann and Jastorff[33]. Dipole vectors and LUMO and HOMO energies were calculated by the MNDO (Modified Neglect of Diatomic Overlap) method using the program based on the SCF-LCAO method (Linear Combination of Atomic Orbitals)[56].

Protein kinase assay.—The type II cAMP-dependent protein kinase and the soluble type I cGMP-dependent protein kinase were purified from bovine heart as described[40]. The activity of each purified kinase was measured by the phosphocellulose method (FAD) using Kemptide as a substrate. Briefly, the activity was assayed at 30° in a total volume of 100 μL containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.01% of bovine serum albumin, 10 μg of Kemptide (130 μM), 50 ng of protein kinase, and various concentrations of cyclic nucleotides. Each reaction was started by the addition of 50 μM ^{32}P-ATP (100 cpm/pmol) and terminated after 5 min by the addition of 0.1 mM EDTA. Activity is expressed as the amount of phosphate transferred per min per mg of protein kinase. The assay for inhibition of ^{3}H-cAMP binding and site selectivity followed the procedure of Døskeland and Øgreid[57]. The activation experiments with cAK II were carried out in the laboratory of Dr. U. Walter (Universität Würzburg).

cPDE assays.—Calmodulin-dependent cPDE (CaM-PDE), cGMP-stimulated cPDE (cGS-PDE), and cGMP-inhibited cPDE (cGI-PDE) were purified using specific antibodies[58]. Phosphodiesterase activity was determined by incubating at 30° in a total volume of 250 μL containing 40 mM 3-morpholinepropanesulfonic acid (MOPS) (pH 7.5), 0.8 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), 15 mM Mg acetate, 0.2 mg/mL of BSA, 1 μM ^{3}H-cAMP, and various concentrations of each cyclic nucleotide. Each reaction was stopped after 10 min by boiling the sample for 1 min, the mixture was cooled, 10 μL of the 2.5 mg/mL snake venom was added, and the sample was incubated for 5 min at 30°. cAMP was removed by an anion-exchange resin (Sigma A-25) by elution with 2 mL of 20 mM Tris (pH 7.5). Radioactivity was determined with a scintillation counter (Hewlett–Packard Tricarb) after adding 3 mL of scintillation fluid (Ultima Gold).

Phosphodiesterase hydrolysis was measured using the phosphate release assay[59]. Briefly, cPDE activity was assayed in a total volume of 100 μL containing 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, and 0.5 mg/mL of BSA. The microtitre plate was warmed up to 30°, snake venom (50 μg) and cyclic nucleotide derivative were added, and the sample was incubated for 1–30 min. The cPDE reaction was stopped at $< 10\%$ hydrolysis by adding 50 μL of 6% SDS. The colorimetric reagent (0.1 M H_2SO_4, 0.1% ammonium molybdate, and 0.2% of ascorbic acid) was added, and the wells were read with a microtitre plate reader (Dynatech Laboratories) using an 800-nm filter. The rates of hydrolysis of cAMP were 0.75 nmol/min for cGS-PDE, 0.25 nmol/min for cGI-PDE, and 0.3 nmol/min for CaM-PDE. The phosphodiesterase experiments were carried out in the laboratory of Dr. J.A. Beavo (University of Washington, Seattle, USA).

Cell culture studies.—C6 rat glioma cells were grown routinely in Dulbeccos modified Eagles medium (DMEM) containing 10% of foetal calf serum (FCS), penicillin–streptomycin solution, and extra glutamine (2 mM) at 37° in a humidified 10%-CO_2 atmosphere. For growth experiments, 3×10^4 cells/dish were seeded in a stock culture medium. After 24 h, the medium was changed to DMEM containing 0.5% of heat-inactivated FCS (30 min at 56°), and the cBIMP derivative was added in various concentrations between 5 and 1000 μM. The culture medium and the cBIMP derivative were changed after 48 h. After a further 48 h, the medium was replaced and the cells were washed twice with phosphate-buffered saline solution (PBS) before lysis with an acidic DNA reagent (5 g of diphenylamine in 490 mL of acetic acid, 10 mL of H_2SO_4, and 10 μL/mL of aq 1% acetaldehyde). The DNA content was determined colorimetrically by the method of Burton[60]. The analogue concentration resulting in a 50% decrease in the DNA content (IC_{50}) was obtained by comparing the DNA content of treated cells to that of untreated control cells. The $K_{0.5}$ values for cytolysis of BNS cells were determined as described by Lanotte et al.[5].

Cyclophosphorylation (cyclothiophosphorylation)[44,46].—A solution of nucleoside (1 mmol) dried over P_2O_5 in freshly distilled trimethyl phosphate or triethyl phosphate (5 mL) was cooled to room temperature, and freshly distilled $POCl_3$ (2 mmol, 186 μL) or $PSCl_3$ (200 μL) was added. The mixture was stirred at 0° (room temperature for $PSCl_3$) and the reaction was monitored by HPLC on RP-18 with MeOH–100 mM triethylammonium formate buffer (TEAF). When the nucleoside had reacted completely, the mixture was added to a stirred mixture of 0.08 M KOH in H_2O–MeCN (4:6, 120 mL) at 0°. The mixture was neutralised immediately with HCl, concentrated to 5 mL, extracted with dry *tert*-butyl methyl ether (2×100 mL), diluted with MeOH (100 mL), filtered, and concentrated. Flash-column (25×3 cm) chromatography of the residue on RP-18, with 0.1 M TEAF buffer in MeOH or MeCN and lyophilisation of the appropriate fractions, gave the cyclic nucleotide.

The following benzimidazole derivatives were amorphous and not amenable to elemental analysis.

4-Nitro-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphate (**2**, *4-N-cBIMP*).—Prepared from 4-nitro-1-β-D-ribofuranosylbenzimidazole[43] and purified by flash-column chromatography (RP-18, 12% MeCN–10 mM TEAF), **2** (9.6%) had T 2.87 (HPLC, RP-18, 22% MeCN–100 mM TEAF); λ_{max} 314 nm (ϵ_{mM} 7500). NMR data: ^{1}H, δ 8.62 (s, 1 H, H-2), 8.20 (d, 1 H, $J_{5,6}$ 7.8 Hz, H-5), 7.55 (t, 1 H, H-6), 8.05 (d, 1 H, $J_{6,7}$ 7.8 Hz, H-7), 6.20 (s, H 1, H-1'), 4.56–4.28 (m, 4 H, H-2'/5'); ^{31}P, δ −0.40. FAB-mass spectrum: m/z 162 [(B − H)$^-$, 25%], 356 [(M − H)$^-$, 21%], 358 [(M + H)$^+$, 2%].

4-Amino-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphate (**3**, *4-A-cBIMP*).—Hydrogen was bubbled through a stirred solution of 4-nitro-1-β-D-ribofuranosylbenzimidazole in acetic acid containing 10% Pd/C for 2 h at room temperature. The mixture was then filtered and lyophilised to give 4-amino-1-β-D-ribofurano-

sylbenzimidazole which was 3′,5′-phosphorylated. Flash-column chromatography (RP-18, 10% MeCN–10 mM TEAF) of the product gave **3** (7.1%), *T* 3.33 (HPLC, RP-18, 16% MeCN–100 mM TEAF); λ_{max} 265 nm (ϵ_{mM} 4100). NMR data: ^{1}H, δ 8.18 (s, 1 H, H-2), 6.59 (d, 1 H, $J_{5,6}$ 7.2 Hz, H-5), 7.10 (dd, 1 H, H-6), 6.86 (d, 1 H, $J_{6,7}$ 7.2 Hz, H-7), 6.00 (s, 1 H, H-1′), 4.23–4.56 (m, 4 H, H-2′/5′); ^{31}P, δ −0.55. FAB-mass spectrum: *m/z* 132 [(B − H)$^-$, 2%], 326 [(M − H)$^-$, 43%], 134 [(B + H)$^+$, 23%], 328 [(M + H)$^+$, 62%].

*5-Nitro- (**4**, 5-N-cBIMP) and 6-nitro-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (**5**, 6-N-cBIMP).*—Using a slightly modified version of the method of Vorbrüggen et al.[43] (1.4 mmol of chlorotrimethylsilane/mmol of base for 6 h; 0.62 mmol of triflate/mmol of base for 2 h, reflux), 5-nitrobenzimidazole was converted into a mixture of 5- and 6-nitro-1-β-D-ribofuranosylbenzimidazole which was 3′,5′-phosphorylated. Flash-column chromatography (RP-18, 15% MeCN–10 mM TEAF) of the products followed by HPLC on Cyclobond-I (50% MeOH–10 mM TEAF) gave the pure isomers that were differentiated by ^{1}H NMR spectroscopy[61]. Compound **4** (2.3%) had *T* 5.78 (HPLC, Cyclobond-I, 50% MeOH–10 mM TEAF); λ_{max} 236 nm (ϵ_{mM} 18300). NMR data: ^{1}H, δ 8.68 (s, 1 H, H-2), 8.63 (d, 1 H, $J_{4,6}$ 1.6 Hz, H-4), 8.27 (dd, 1 H, H-6), 7.85 (d, 1 H, $J_{6,7}$ 7.9 Hz, H-7), 6.24 (s, 1 H, H-1′); ^{31}P, δ −0.40. FAB-mass spectrum: *m/z* 162 [(B − H)$^-$, 33%], 356 [(M − H)$^-$, 100%], 358 [(M + H)$^+$, 57%]. Compound **5** (4.3%) had *T* 7.13 (HPLC, Cyclobond-I, 50% MeOH–10 mM TEAF); λ_{max} 236 nm (ϵ_{mM} 18300). NMR data: ^{1}H, δ 8.68 (s, 1 H, H-2), 7.81 (d, 1 H, $J_{4,5}$ 7.9 Hz, H-4), 8.3 (dd, 1 H, H-5), 8.61 (d, 1 H, $J_{5,7}$ 1.6 Hz, H-7), 6.18 (s, 1 H, H-1′); ^{31}P, −0.40. FAB-mass spectrum: *m/z* 162 [(B − H)$^-$, 40%], 356 [(M − H)$^-$, 100%], 358 [(M + H)$^+$, 2%].

*5-Methyl- (**6**, 5-Me-cBIMP) and 6-methyl-1-β-D-ribofuranosylbenzimidazole 3′,5′-phosphate (**7**, 6-Me-cBIMP).*—5-Methylbenzimidazole was converted into **6** and **7** by the procedure of Vorbrüggen et al.[43] (1.4 mmol of chlorotrimethylsilane/mmol of base, 0.5-h reflux; 6.2 mmol of triflate/mmol of base) and the mixture of isomers was 3′,5′-phosphorylated. Flash-column chromatography (RP-18, 16% MeCN–100 mM TEAF) followed by HPLC on Cyclobond-I (40% MeOH–100 mM TEAF) then gave the pure isomers.

Compound **6** (2.8%) had *T* 2.10 (HPLC, RP-18, 25% MeCN–100 mM TEAF), *T* 18.33 (HPLC, Cyclobond-I, 40% MeOH–100 mM TEAF); λ_{max} 247 nm (ϵ_{mM} 4400). NMR data: ^{1}H, δ 8.32 (s, 1 H, H-2), 7.51 (s, 1 H, H-4), 7.22 (d, 1 H, $J_{6,7}$ 7.9 Hz, H-6), 7.49 (d, 1 H, H-7), 2.50 (s, 3 H, CH_3), 6.08 (s, 1 H, H-1′), 4.25–4.60 (m, 4 H, H-2′/5′); ^{31}P, δ −0.5. FAB-mass spectrum (mixture of **6** and **7**): *m/z* 325 [(M − H)$^-$, 100%], 651 [(2 M − H)$^-$, 5%], 265 [(B + H)$^+$, 5%], 327 [(M + H)$^+$, 13%].

Compound **7** (1.8%) had *T* 3.15 (HPLC, RP-18, 25% MeCN–100 mM TEAF), *T* 21.43 (HPLC, Cyclobond-I, 40% MeOH–100 mM TEAF); λ_{max} 247 nm (ϵ_{mM} 4400). NMR data: ^{1}H, δ 8.30 (s, 1 H, H-2), 7.58 (d, 1 H, $J_{4,5}$ 7.9 Hz, H-4), 7.18 (d, 1 H, H-5), 7.42 (s, 1 H, H-7), 2.54 (s, 3 H, CH_3), 6.08 (s, 1 H, H-1′), 4.29–4.59 (m, 4 H, H-2′/5′); ^{31}P, δ −0.5.

1-β-D-Ribofuranosyl-2-trifluoromethylbenzimidazole 3',5'-phosphate (**8**, *2-CF_3-cBIMP*).—Using a slight modification (10 mmol) of base and 10 mmol of chlorotrimethylsilane) of the method of Vorbrüggen et al.[43], 2-trifluoromethylbenzimidazole[62] was converted into the nucleoside, then 3',5'-phosphorylated. Flash-column chromatography (RP-18, 10% MeCN–100 mM TEAF) of the product gave **8** (37%), *T* 8.56 (HPLC, RP-18, 18% MeCN–100 mM TEAF); λ_{max} 274 nm (ϵ_{mM} 6200). NMR data: ^{1}H, δ 7.62 (d, 1 H, H-4), 7.53 (dt, 1 H, H-5), 7.46 (dt, 1 H, H-6), 7.89 (d, 1 H, H-7), 5.99 (s, 1 H, H-1'), 4.36 (d, 1 H, H-2'), 4.49 (ddd, 1 H, H-3'), 4.02 (ddd, 1 H, H-4'), 4.27 (ddd, 1 H, H-5'*ax*), 4.21 (ddd, 1 H, H-5'*eq*); ^{31}P, δ −0.27. FAB-mass spectrum: *m/z* 379 [(M − H)$^-$, 84%], 185 [(B − H)$^-$, 95%], 381 [(M + H)$^+$, 20%], 187 [(B + H)$^+$, 40%].

5,6-Difluoro-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphate (**9**, *5,6-F_2-cBIMP*). —5,6-Difluorobenzimidazole[63] was converted[43] into the nucleoside (hexamethyldisilazane, 1 h, reflux; 1 mmol of triflate/mmol of base, 3.5 h, reflux) and then 3',5'-phosphorylated. Flash-column chromatography (RP-18, 30% MeOH–100 mM TEAF) of the product gave **9** (5%), *T* 7.22 (HPLC, RP-18, 35% MeOH–100 mM TEAF); λ_{max} 277 nm (ϵ_{mM} 6300). NMR data: ^{31}P, δ −0.74. FAB-mass spectrum: *m/z* 347 [(M − H)$^-$, 100%], 153 [(B − H)$^-$, 20], 349 [(M + H)$^+$, 14], 155 [(B + H)$^+$, 17].

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphate (**10**, *5,6-Cl_2-cBIMP*).—3',5'-Phosphorylation of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and flash-column chromatography (RP-18, 16% MeCN–100 mM TEAF) of the product gave **10** (25%), *T* 3.95 (HPLC, RP-18, 50% MeOH–100 mM TEAF); λ_{max} 252.5 nm (ϵ_{mM} 6400). NMR data: ^{31}P, δ −0.6. FAB-mass spectrum: *m/z* 381 [(M + H)$^+$], 187 [(B + H)$^+$].

Rp-5,6-Dichloro- (**11**, *Rp-5,6-Cl_2-cBIMPS*) *and Sp-5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphorothioate* (**12**, *Sp-5,6-Cl_2-cBIMPS*).—3',5'-Thiophosphorylation of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and flash-column chromatography (RP-18, 20% MeCN–100 mM TEAF) of the product gave **11** (3%) and **12** (2%).

Compound **11** had *T* 8.45 (HPLC, RP-18, 25% MeCN–100 mM TEAF); λ_{max} 252.5 nm (ϵ_{mM} 6400). NMR data: ^{31}P, δ 56.5. FAB-mass spectrum: *m/z* 395 [(M − H)$^-$, 55%].

Compound **12** had *T* 10.47 (HPLC, Rp-18, 25% MeCN–100 mM TEAF); λ_{max} 252.5 nm (ϵ_{mM} 6400). NMR data: ^{31}P, δ 55.0. FAB-mass spectrum: *m/z* 395 [(M − H)$^-$, 4%].

5,6-Dibromo-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphate (**13**, *5,6-Br_2-cBIMP*).—3',5'-Phosphorylation of 5,6-dibromo-1-β-D-ribofuranosylbenzimidazole[63] and flash-column chromatography (RP-18, 40% MeOH–100 mM TEAF) of the product gave **13** (15%), *T* 5.10 (HPLC, RP-18, 28% MeCN–100 mM TEAF); λ_{max} 255 nm (ϵ_{mM} 5900). NMR data: ^{31}P, δ −0.6 (D_2O). FAB-mass spectrum: *m/z* 469 [(M − H)$^-$, 40%].

Rp-5,6-Dibromo- (**14**, *Rp-5,6-Br_2-cBIMPS*) *and Sp-5,6-Dibromo-1-β-D-ribo-*

furanosylbenzimidazole 3',5'-phosphorothioate (**15**, *Sp-5,6-Br_2-cBIMPS*).—3',5'-Thiophosphorylation of 5,6-dibromo-1-β-D-ribofuranosylbenzimidazole and flash-column chromatography (RP-18, 42% MeOH–100 mM TEAF) of the product gave **14** (3%) and **15** (2%).

Compound **14** had *T* 6.53 (HPLC, RP-18, 28% MeCN–100 mM TEAF); λ_{max} 255 nm (ϵ_{mM} 5900). NMR data: ^{31}P, δ 56.4. FAB-mass spectrum: *m/z* 485 [(M – H)$^-$, 14%].

Compound **15** had *T* 8.00 (HPLC, RP-18, 8% 2MeCN–100 mM TEAF); λ_{max} 255 nm (ϵ_{mM} 5900). NMR data: ^{31}P, δ 55.1. FAB-mass spectrum: *m/z* 485 [(M – H)$^-$, 15%].

5,6-Dinitro-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphate (**16**, *5,6-N_2-cBIMP*).—5,6-Dinitrobenzimidazole[64] was converted[43] into the nucleoside, then 3',5'-phosphorylated. Flash-column chromatography (RP-18, 17% MeCN–10 mM TEAF) of the product gave **16** (44%), *T* 3.8 (HPLC, RP-18, 30% MeCN–100 mM TEAF); λ_{max} 240 nm (ϵ_{mM} 12500). NMR data; ^1H, δ 8.81 (s, 1 H, H-2), 8.46 (s, 1 H, H-4), 8.39 (s, 1 H, H-7), 6.25 (s, 1 H, H-1'); ^{31}P, δ −0.68. FAB-mass spectrum: *m/z* 207 [(B – H)$^-$, 95%], 401 [(M – H)$^-$, 23%].

Rp-5,6-Dinitro- (**17**, *Rp-5,6-N_2-cBIMPS*) *and Sp-5,6-Dinitro-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphorothioate* (**18**, *Sp-5,6-N_2-cBIMPS*),—3',5'-Thiophosphorylation of 5,6-dinitro-1-β-D-ribofuranosylbenzimidazole and flash-column chromatography (RP-18, 16% MeCN–100 mM TEAF) of the products gave **17** (7%) and **18** (5%).

Compound **17** had *T* 7.45 (HPLC, RP-18, 22% MeCN–100 mM TEAF); λ_{max} 240 nm (ϵ_{mM} 12500). NMR data: ^{31}P, δ 56. FAB-mass spectrum: *m/z* 417 [(M – H)$^-$, 10%], 207 [(B – H)$^-$, 100%].

Compound **18** had *T* 9.55 (HPLC, RP-18, 22% MeCN–100 mM TEAF); λ_{max} 240 nm (ϵ_{mM} 12500). NMR data: ^{31}P, δ 54. FAB-mass spectrum: *m/z* 417 [(M – H)$^-$, 11%], 207 [(B – H)$^-$, 100%].

5,6-Dimethyl-1-β-D-ribofuranosylbenzimidazole 3',5'-phosphate (**19**, *5,6-Me_2-cBIMP*).—5,6-Dimethyl-1-β-D-ribofuranosylbenzimidazole was synthesised by a slight modification of the method of Vorbrüggen et al.[43] (1.4 mmol of chlorotrimethylsilane/mmol of base, 12 h; 0.62 mmol of triflate/mmol of base, 77 h, reflux) and then 3',5'-phosphorylated. Flash-column chromatography (RP-18, 17% MeCN–10 mM TEAF, pH 6.6) of the product gave **19** (10%), *T* 4.75 (HPLC, RP-18, 25% MeCN–10 mM TEAF); λ_{max} 279 nm (ϵ_{mM} 7500). NMR data: ^1H, δ 8.42 (s, 1 H, H-2), 7.48 (s, 1 H, H-4), 7.41 (s, 1 H, H-7), 2.52 (s, 3 H, CH_3-5), 2.49 (s, 3 H, CH_3-6), 6.07 (s, 1 H, H-1); ^{31}P, δ −0.52. FAB-mass spectrum: *m/z* 145 [(B – H)$^-$, 19%], 339 [(M – H)$^-$, 51%], 147 [(B + H)$^+$, 57%], 341 [(M + H)$^+$, 33%].

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REFERENCES

1 J.A. Nathanson and J.W. Kebabian (Eds.), *Handbook of Experimental Pharmacology*, Springer, Berlin, 1982.
2 W.L. Ryan and M.L. Heidrick, *Adv. Cyclic Nucleotide Res.*, 4 (1974) 81–115.
3 J.R. Sheppard, in W. Braun, L.M. Lichtenstein, and C.W. Parker (Eds.), *Cyclic AMP Cell Growth Immune Response*, Proc. Symp., Springer, Berlin, 1974, pp 290–301.
4 A.L. Boynton and J.F. Whitfield, *Adv. Cyclic Nucleotide Res.*, 15 (1983) 193–294.
5 M. Lanotte, J.B. Riviere, S. Hermouet, G. Houge, O.K. Vintermyr, B.T. Gjersten, and S.O. Døskeland, *J. Cell. Physiol.*, 146 (1991) 73–88.
6 T.M. Konijn, *Adv. Cyclic Nucleotide Res.*, 1 (1972) 17–31.
7 P. Peterkowsky, *Adv. Cyclic Nucleotide Res.* 7 (1976) 1–48.
8 U.B. Kaupp, *Trends Neurol. Sci.*, 14 (1991) 150–157.
9 J. Beavo and M.D. Housley (Eds.), *Cyclic Nucleotide Phosphodiesterases: Structures, Regulation and Drug Action*, Wiley, London, 1990.
10 U. Walter, *Rev. Physiol. Biochem. Pharmacol.*, 113 (1989) 41–88.
11 D.L. Friedman, ref. 1, pp 151–188.
12 Y.S. Cho-Chung, T. Clair, P. Tagliaferry, S. Ally, D. Katsaros, G. Tortora, L. Neckers, T.L. Avery, G.W. Crabtree, and R.K. Robins, *Cancer Invest.*, 7 (1989) 161–177.
13 L. Szaduykis-Szadurski, G. Weimann, and F. Berti, *Pharmacol. Pharm. Res. Commun.*, 4 (1974) 63–69.
14 G. Michal, K. Mühlegger, M. Nelboeck, C. Thiessen, and G. Weimann, *Pharm. Res. Commun.*, 6 (1974) 203–219.
15 J.P. Miller and R.K. Robins, *Annu. Rep. Med. Chem.*, 11 (1976) 291–300.
16 G.R. Revankar and R.K. Robins, ref. 1, pp 17–151.
17 L.H. Parker-Botelho, J.D. Rothermel, R.V. Coombs, and B. Jastorff, *Methods Enzymol.*, 159 (1988) 159–172.
18 R.K. Robins, G.R. Revankar, and Y. Chang, Eur. Pat. PCT/US88/0217 (1989); *Chem. Abstr.*, 112 (1990) P 210975d.
19 T. Posternack, E.W. Sutherland, and W.F. Henion, *Biochim. Biophys. Acta*, 65 (1962) 558–560.
20 J.D. Rothermel, W.J. Stec, J. Baraniak, B. Jastorff, and L.H. Parker-Botelho, *J. Biol. Chem.*, 258 (1983) 12125–12128.
21 E. Butt, M.X.P. van Bemmelen, L. Fischer, and B. Jastorff, *FEBS Lett.*, 263 (1990) 47–50.
22 B. Jastorff, E. Maronde, M.X.P. van Bemmelen, M. Zorn, and R. Stoermann, in D. Shugar, W. Rode, and E. Borowski (Eds.), *Molecular Aspects of Chemotherapy*, 3rd. Int. Symp., Polish Scientific Publishers, Warszawa; and Springer, Berlin; 1991.
23 L. Wolfe, J.D. Corbin, and S.H. Francis, *J. Biol. Chem.*, 264 (1989) 7734–7741.
24 H.R. de Jonge, *Adv. Cyclic Nucleotide Res.*, 14 (1981) 315–333.
25 B. Jastorff, E.G. Abbad, G. Petridis, W. Tegge, R.J.W. De Wit, C. Erneux, W.J. Stec, and M. Morr, *Nucleic Acids Res. Symp. Ser.*, 9 (1981) 219–223.
26 N. Nass, C. Colling, M. Cramer, H.G. Genieser, E. Butt, E. Winkler, L. Jaenicke, and B. Jastorff, *Biochem. J.*, 285 (1992) 129–136.
27 S.J. Beebe and J.D. Corbin, in E.G. Krebs and P.D. Boyer (Eds.), *The Enzymes*, Vol. 17A, Academic Press, Orlando, FL, 1986, pp 43–111.
28 J.D. Corbin, D. Øgreid, J.P. Miller, R.H. Suwa, B. Jastorff, and S.O. Døskeland, *J. Biol. Chem.*, 261 (1986) 1208–1214.
29 D. Øgreid, R. Ekanger, R.H. Suva, J.P. Miller, and S.O. Døskeland, *Eur. J. Biochem.*, 181 (1989) 19–31.

30 E. Butt, Thesis, Universität Bremen, 1989.
31 C. Erneux, D. Couchie, J.E. Dumont, and B. Jastorff, *Adv. Cyclic Nucleotide Prot. Phos. Res.*, 16 (1984) 107–118.
32 M.M. Van Lookeren-Campagne, F. Villalba Diaz, B. Jastorff, E. Winkler, H.G. Genieser, and R.H. Kessin, *J. Biol. Chem.*, 265 (1990) 5847–5854.
33 T. Braumann and B. Jastorff, *J. Chromatogr.*, 350 (1985) 105–118.
34 P.M.J. Burgers, F. Eckstein, D.H. Hunnemann, J. Baraniak, R.W. Kinas, K.W. Lesiak, and W.J. Stec, *J. Biol. Chem.*, 254 (1979) 9959–9961.
35 B. Jastorff, J. Hoppe, and M. Morr, *Eur. J. Biochem.*, 101 (1979) 555–561.
36 R.J.W. De Wit, D. Hekstra, B. Jastorff, W.J. Stec, J. Baraniak, R. Van Driel, and P.J.M. Van Haastert, *Eur. J. Biochem.*, 142 (1984) 255–260.
37 I.T. Weber, T.A. Steitz, J. Bubis, and S.S. Taylor, *Biochemistry*, 26 (1987) 343–351.
38 T. Braumann, C. Erneux, G. Petridis, W.D. Stohrer, and B. Jastorff, *Biochim. Biophys. Acta*, 871 (1986) 199–206.
39 T.S. Yagura, Z. Kazimierczuk, D. Shugar, and J.D. Miller, *Biochem. Biophys. Res. Commun.*, 97 (1980) 737–743.
40 M. Van Ments-Cohen, H.G. Genieser, B. Jastorff, P.J.M. Van Haastert, and P. Schaab, *Microbiol. Lett.*, 82 (1991) 9–14.
41 C. Erneux, D. Couchie, J.E. Dumont, J. Baraniak, W.J. Stec, E.D. Abbad, G. Petridis, and B. Jastorff, *Eur. J. Biochem.*, 115 (1981) 503–510.
42 D. Couchie, G. Petridis, B. Jastorff, and C. Erneux, *Eur. J. Biochem.*, 136 (1983) 571–575.
43 H. Vorbrüggen, K. Krolikiewicz, and B. Bennua, *Chem. Ber.*, 114 (1981) 1234–1255.
44 H.G. Genieser, E. Butt, U. Bottin, W. Dostmann, and B. Jastorff, *Synthesis*, (1989) 53–54.
45 H.G. Genieser, W. Dostmann, U. Bottin-Weber, and B. Jastorff, Ger. Pat. DE 3802 866 A1 (1989); *Chem. Abstr.*, 112 (1990) P 56588.
46 H.G. Genieser, W. Dostmann, U. Bottin, E. Butt, and B. Jastorff, *Tetrahedron Lett.*, 29 (1988) 2803–2804.
47 H.G. Genieser, W. Dostmann, U. Bottin-Weber, and B. Jastorff, Ger. Pat. DE 3802 865 A1 (1989); *Chem. Abstr.*, 112 (1990) P 56587.
48 M. Yoshikawa, T. Kato, and T. Takenishi, *Tetrahedron Lett.*, 50 (1967) 5065–5068.
49 A.W. Murray and M.R. Atkinson, *Biochemistry*, 11 (1968) 4023–4029.
50 W.H. Dawson, R.L. Cargill, and R.B. Dunlap, *J. Carbohydr. Nucleosides, Nucleotides*, 4 (1977) 363–375.
51 W.R.G. Dostmann, S.S. Taylor, H.G. Genieser, B. Jastorff, S.O. Døskeland, and D. Øgreid, *J. Biol. Chem.*, 265 (1990) 10484–10491.
52 P.J.M. Van Haastert, P.A.M. Dijkgraaf, T.M. Konijn, E. Garcia-Abbad, G. Petridis, and B. Jastorff, *Eur. J. Biochem.*, 131 (1983) 659–666.
53 M.M. Van Lookeren Campagen, F. Villalba Diaz, B. Jastorff, and R.H. Kessin, *Cancer Res.*, 51 (1991) 1600–1605.
54 R.H. Kessin, F. Villalba-Diaz, M.M. Van Lookeren Campagne, and B. Jastorff, *Cancer Res.*, 51 (1991) 6206–6208.
55 M. Sandberg, E. Butt, C. Nolte, L. Fischer, M. Halbrügge, J. Beltman, T. Jahnsen, H.G. Genieser, B. Jastorff, and U. Walter, *Biochem. J.*, 279 (1991) 521–527.
56 M.J.S. Dewar and W. Thiel, *J. Am. Chem. Soc.*, 99 (1977) 4899–4906.
57 S.D. Døskeland and D. Øgreid, *J. Biol. Chem.*, 259 (1984) 2291–2301.
58 S.A. Harrison, D.H. Reifsnyder, B. Gallis, G.G. Cadd, and J.A. Beavo, *Mol. Pharmacol.*, 25 (1986) 506–514.
59 P.G. Gillespie and J.A. Beavo, *Mol. Pharmacol.*, 36 (1989) 773–781.
60 K. Burton, *Biochemistry*, 62 (1956) 315–323.
61 A. Burkhard, A. Dworsky, R. Jeck, M. Pfeiffer, S. Pundak, and C. Woeckhaus, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 1079–1090.
62 M.A. Phillips, *J. Chem. Soc.*, (1928) 2395–2397.
63 Z. Kazimierczuk, L. Dudycz, R. Stolarski, and D. Shugar, *Z. Naturforsch, Teil C*, 35 (1980) 30–35.
64 Z. Kazimierczuk and D. Shugar, *Nucleosides Nucleotides*, 8 (1989) 1379–1385.
65 D. Øgreid, S.O. Døskeland, and J.P. Miller, *J. Biol. Chem.*, 258 (1983) 1041–1049.